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Relationship among growth, steroid production and immunolocalization of transforming growth factor- β 1 in the normally developing mouse follicles cultured *in vitro*

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Abstract

This study examined the relationship among growth, steroid production and transforming growth factor- β 1 (TGF- β 1) immunolocalization in the mouse follicles cultured *in vitro* to evaluate the hypothesis that normally developing follicles should express TGF- β 1 in the granulosa cells around the time of antrum formation. Preantral follicles with 151-175 μ m (large category) and 125-150 μ m (small category) of initial diameters were used as models for normal and retarded follicles, respectively. Growth rate and timing of antrum formation in both categories were comparable to those of *in-vivo* grown follicles. At the time of antrum formation, follicular diameters were similar between the two follicle categories; however, antral follicles from the large category showed larger number of granulosa cells, higher estradiol production and proportion of follicles with TGF- β 1 positive granulosa cells. Two days after antrum formation, there were no differences in the number of granulosa cells and the proportions of follicles with TGF- β 1 positive granulosa or theca cells between the two categories. Temporal association in large follicles between the increase in estradiol production and proportion of follicles with TGF- β 1 positive granulosa cells at the time of antrum formation supports our hypothesis. Furthermore, this study demonstrated the usefulness of the follicle culture system in the investigations of follicular physiology.

Key words : estradiol, follicle culture, granulosa cells, mouse.

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Introduction

The practical goal of follicle culture is to produce fertile oocytes from the primordial follicles because they are most abundant. Besides the potential for oocyte production, intact follicle culture systems can provide valuable information about ovarian function and follicular development. Previous studies using individual/spherical follicle culture have focused on follicles with initial diameters of 170-250 μm ^{5,10,12,14,18,20,21}. Our previous study³ indicates that smaller preantral follicles cultured *in vitro* with initial diameters of 151-175 μm can grow at a similar rate and produce estradiol with similar pattern to *in vivo*. Furthermore, follicles of this size developed to the preovulatory stage during a 6-day-culture period, and their oocytes had capacity to develop to the blastocyst stage³. However, follicles with 125-150 μm of initial diameter seem less potent in their growth, steroidogenesis and production of fertile oocytes^{3,15,22}. These observations indicate that the growth phase of follicles with diameters of 125-150 μm may be critical to follicles and their oocytes for acquisition of normal developmental competence. The present culture system could not sustain a thorough development of small follicles at the critical phase. Thus, the follicles with 151-175 μm (large category) and 125-150 μm (small category) of initial diameter would serve as models for normal and retarded follicles, respectively.

In an attempt to improve development of small follicles *in vitro*, we have tried to understand the regulatory mechanism of follicular development. The differential expression of factors at key points during follicular development modulates the action of gonadotropins on follicular cells⁶. Transforming growth factor- β 1 (TGF- β 1) is one of such factors and has been shown to have roles in differentia-

tion of isolated granulosa and theca-interstitial cells^{7,9,13}. *In-vitro* studies using isolated granulosa and theca cells are of limited value, since processes of differentiation such as steroidogenesis depend on cross-talk between these two cell-types in an intact follicle. Our previous studies of TGF- β 1 expression in mouse ovarian follicles *in vivo* suggested that normally developing follicles would express this factor in the granulosa cells around the time of antrum formation⁴. It was, therefore, hypothesized that *in-vitro* grown follicles originated from the large category, but not small category, would express TGF- β 1 in the granulosa cells around the time of antrum formation with normal growth and steroid production. To examine this hypothesis, this study evaluated the relationship among growth, steroid production and immunolocalization of TGF- β 1 in *in-vitro* cultured preantral follicles of small and large categories.

Materials and Methods

Animals : Four- to seven-week-old female ICR mice purchased from Japan SLC Inc.

(Hamamatsu, Shizuoka, Japan) were housed in a temperature (23°C) and light controlled room with a 14 hr light : 10 hr dark photoperiod and provided with feed and water ad libitum. The handling of animals was done according to guidelines laid down by the university.

Preantral follicle isolation : Mice were killed by cervical dislocation. Ovaries were removed to Leibovitz L-15 medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 3 mg/ml bovine serum albumin (fraction V, Sigma, St Louis, MO, USA), 75 $\mu\text{g/ml}$ penicillin and 50 $\mu\text{g/ml}$ streptomycin in a 60-mm petri dish (Falcon 1007, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 37°C. Preantral follicles were isolated by me-

chanical microdissection using 25G needle attached to 1 ml syringe. Follicles with diameters of 125-175 μm without irregularities (*i.e.*, non-spherical or non-centrally located oocytes with dark granulosa layers) were selected. Follicle diameter excluding the theca stroma was estimated by measuring two perpendicular diameters (length and width) at $\times 200$ magnification with a pre-calibrated ocular micrometer under an inverted microscope (TMS, Nikon, Tokyo, Japan). Follicles were allocated to one of the two size categories. According to the classification of Pedersen and Peters¹⁷⁾, majority of follicles in the small and large categories were judged as type 4 and type 5a, respectively.

Preantral follicle culture : Preantral follicles were cultured individually in 25 μl drops of α -minimal essential medium (Gibco) supplemented with 5% heat-inactivated fetal calf serum (Gibco), 0.1 IU/ml porcine follicle stimulating hormone (FSH, Antrin, Denka Pharmaceutical Co. Ltd., Kawasaki, Kanagawa, Japan), 75 $\mu\text{g}/\text{ml}$ penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 37°C. Follicles were transferred to fresh drops of culture medium every 24 hr.

Assessment of steroid hormone : The spent medium from follicle cultures was assayed for estradiol-17 β , progesterone and testosterone by enzyme immunoassay. Two hundred microliters of pooled sample was extracted with 1 ml of diethylether and reconstituted with assay buffer. The reconstitutes were diluted 1 : 10 for the estradiol-17 β assay in samples from antral stage follicles. Rabbit antiserum raised against estradiol-6-CMO-BSA (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan), progesterone-3-CMO-BSA and testosterone-3-CMO-BSA (Biogenesis Ltd., Poole, UK) were used in the assays. The assay sensitivities were 17.2 pg/well for estradiol-17

β , 4.3 pg/well for progesterone and 1.1 pg/well for testosterone. The intra- and inter-assay coefficients of variation were 4.9 and 6.8% for estradiol-17 β , 3.9 and 6.5% for progesterone, and 7.1 and 8.9% for testosterone, respectively.

Immunohistochemistry : Follicles were fixed in Bouin's solution for 12 hr and were embedded in 3% agar before being processed through alcohol series. The agar blocks were then embedded in paraffin and serially sectioned at 5 μm . The immunostaining was done according to instructions supplied with the staining kit (Histofine, Nichirei Corporation, Tokyo, Japan), consisting of normal goat serum, goat anti-rabbit IgG conjugated with biotin (secondary antibody) and streptavidin-peroxidase complex. Briefly, after deparaffinization and rinsing in Dulbecco's phosphate-buffered saline (PBS), follicle sections were incubated with 3% hydrogen peroxide in methanol for 10 min at room temperature. Subsequent steps were carried out at 4°C, and the sections were rinsed 3 times for 5 min in PBS between steps. Sections were incubated with normal goat serum for 10 min followed by a rabbit-raised polyclonal anti-human TGF- β 1 antibody (SC-146, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution rate of 1 : 10 for 24 hr. Immunoreactivity of the antibody against mouse TGF- β 1 had been tested by the supplier using immunohistochemistry. They were then incubated with secondary antibody and streptavidin-peroxidase complex for 10 and 5 min, respectively. Sections were incubated with 0.03% diaminobenzidine-tetrahydrochloride (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 0.01% hydrogen peroxide dissolved in 0.05 M Tris-HCl buffer (pH 7.6) for 20 min. The follicle sections that served as negative controls were incubated without anti-TGF- β 1 antibody. For positive control, the sections

of mouse adrenal glands were used. Immunoreactivity for TGF- β 1 was judged as positive when the signal was clearly distinguishable from background and as negative when the signal was absent or indistinguishable from background. TGF- β 1 immunoreactivity was assessed on the largest cross-section. The total number of granulosa cells was also counted on the same section.

Experiment 1 : Preantral follicles were cultured for 10 days to determine the growth profile and steroid production of the large and small follicles which showed antra formation on days 4 and 5 of culture, respectively. For hormonal assays, 15 μ l of spent medium was collected daily from each culture droplet after transferring the follicles to fresh medium. At the time of antrum formation and thereafter, spent medium was pooled from the culture droplets in which follicles had shown antrum formation on the same day of culture. The samples were frozen at -80°C until hormone assay.

Experiment 2 : Follicles were fixed to determine granulosa cell number and immunohistochemical localization of TGF- β 1 at the start of culture, on the day of or 2 days after antrum formation if they showed antrum formation on day 4 (large) and 5 (small) of culture. Initial and final follicle diameters were measured before fixation.

Results

The data on frequency and timing of an-

trum formation pooled from experiments 1 and 2 were shown in Table 1. Most antrum formation occurred over a 2-day-period in both categories: on days 5 and 6 in small and on days 4 and 5 in large follicles.

As shown in Fig. 1, mean diameter of large follicles which formed antrum on day 4 was larger than that of small follicles which formed antrum on day 5 on each of the first 6 days of culture ($p < 0.05$). The diameters of large and small follicles became similar from day 7. Estradiol production of large follicles peaked 2 days after antrum formation and was higher than that of small follicles after antrum formation. In both size categories, progesterone and testosterone levels were low throughout the culture.

As shown in Table 2, just after isolation, diameters and granulosa cell numbers in the largest cross sections of large follicles were significantly larger than those of small follicles ($p < 0.05$). The granulosa and theca cells of both small and large follicles were negatively stained for TGF- β 1. On the day of antrum formation, large follicles had a greater number of granulosa cells than small follicles though the diameters were not significantly different ($p < 0.05$). The proportion of large follicles with TGF- β 1 positive granulosa cells was higher than that of small follicles ($p < 0.05$). Two days after antrum formation, there were no significant differences in follicle diameter, number of granulosa cells and proportion of follicles with TGF-

Table 1. Frequency and timing of antrum formation in preantral follicles during *in-vitro* culture

Follicle category	No. of follicles	% of follicles forming antra on each day of culture						Total % of antral follicles
		1	2	3	4	5	6	
Small	190	0	0	0	3.7	31.0	24.2	58.9 ^a
Large	151	0	0	13.2	32.4	25.2	4.6	76.8 ^b

Data were pooled from experiments 1 and 2.

^{a, b} Values with different superscripts differ significantly ($p < 0.05$, chi-square test).

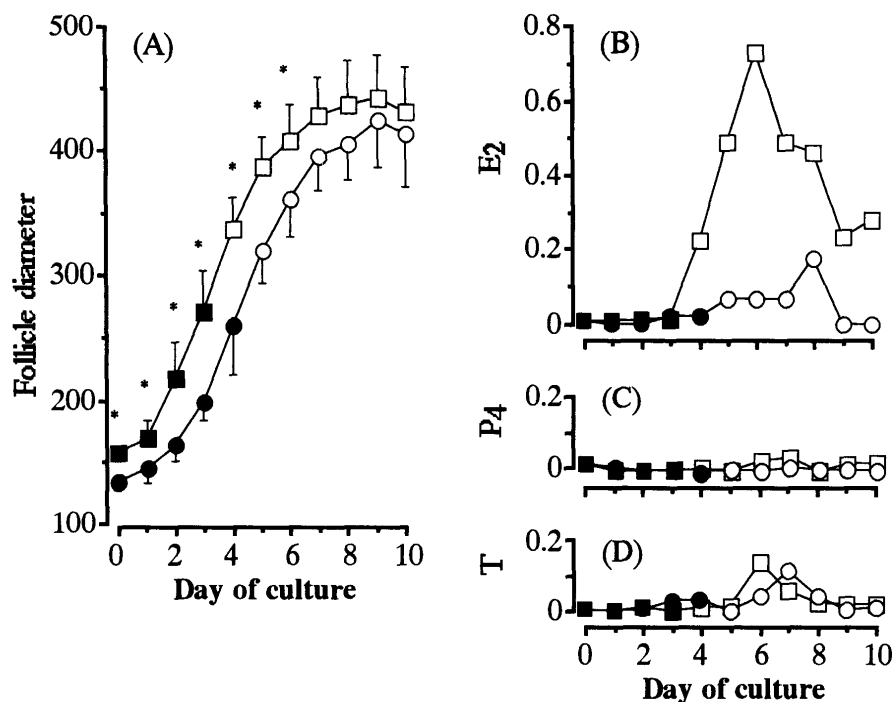


Fig. 1. Growth and steroid production of preantral mouse follicles during 10 days of *in-vitro* culture. Circles and squares represent the follicles derived from small (125-150 µm) and large (151-175 µm) categories which formed antra on day 5 and 4, respectively. Closed and open symbols represent preantral and antral stages, respectively. (A) Growth curves of small (n=8) and large (n=26) follicles. Data represent diameters (mean ± SD µm) and asterisks indicate significant difference on each day of culture ($p < 0.05$). (B)-(D) Steroid production by small (n=8) and large (n=26) follicles. Data before antrum formation were obtained from pooled spent media (small category, n=54; large category, n=69). E₂: estradiol-17β (ng/ml). P₄: progesterone (ng/ml). T: testosterone (ng/ml).

Table 2. Characteristics of follicles before and after culture *in vitro* and TGF-β1 immunolocalization

Parameter	Time of examination	Small follicles with antrum formation on day 5 (n)	Large follicles with antrum formation on day 4 (n)
Follicle diameter (mean ± SD µm)	Before culture	137 ± 7* (23)	161 ± 5 (19)
	At antrum formation	313 ± 22 (15)	333 ± 22 (9)
	2 days after antrum formation	420 ± 35 (27)	408 ± 19 (14)
No. of granulosa cells (mean ± SD)	Before culture	111 ± 34* (6)	143 ± 27 (12)
	At antrum formation	662 ± 101* (9)	862 ± 41 (7)
	2 days after antrum formation	976 ± 304 (7)	1198 ± 202 (14)
% of follicles with TGF-β1 positive granulosa cells	Before culture	0 (6)	0 (12)
	At antrum formation	11* (9)	71 (7)
	2 days after antrum formation	71 (7)	64 (14)
% of follicles with TGF-β1 positive theca cells	Before culture	0 (6)	0 (12)
	At antrum formation	33 (9)	57 (7)
	2 days after antrum formation	100 (7)	86 (14)

Asterisks within rows indicate significant difference between small and large follicles ($p < 0.05$, Student's t-test or Fischer's exact test).

β 1 positive granulosa and/or theca cells. Majority (89%, n=55) of oocytes were positive for TGF- β 1 immunostaining, and the proportion of follicles with TGF- β 1 positive oocytes did not vary with follicle size or timing of sampling.

Discussion

Both the timing and frequency of antrum formation in the present study confirm our previous observation in F1 mice³. The lower antrum formation rate in small follicles was due to higher incidence of premature oocyte extrusion, outgrowth and degeneration compared to large follicles.

The follicles in type 4, which were categorized as small in the present study, require about 2 days before they enter the rapid growth phase (type 5b to type 8), while those in type 5a, large category, require 1 day¹⁶. Under the present culture conditions, the follicles of small and large categories entered a rapid growth phase after 2 and 1 day of culture, respectively. Moreover, after antrum formation, follicles of both categories were at the type 7 stage as judged by the number of granulosa cells (>600 cells)¹⁷ and reached preovulatory size ($\geq 380 \mu\text{m}$)⁵ 2 days after antrum formation. These follicle growth patterns in both categories under the present culture conditions were, therefore, similar to *in vivo* growth patterns¹⁶.

At the time of antrum formation, follicle diameters were similar between the two follicle categories; however, follicles in the large category showed a significant increase in estradiol production and a greater number of granulosa cells compared to those in the small category. These observations confirmed that large and small follicles can serve as models for normal and retarded follicles. Expression of TGF- β 1 mRNA¹⁹ and protein⁴ was reported to become prominent after antrum formation

in vivo where most of follicles undergo atresia. Thus, the timing of TGF- β 1 expression in granulosa cells of normally developing follicles had not been clarified. Around the time of antrum formation, the majority of follicles in the large category showed the expression of TGF- β 1 in the granulosa cells. While the majority of the follicles in the small category did not express TGF- β 1 in the granulosa cells. These results indicate that normally developing follicles express TGF- β 1 in the granulosa cells around the time of antrum formation. The present findings are compatible with the proposed functions of TGF- β 1 to promote proliferation^{2, 8} and estradiol production^{1, 23} in the granulosa cells cultured *in vitro*.

Estradiol production in the large category decreased rapidly after the peak. *In vivo*, when rat follicles reach the preovulatory stage, they die unless being exposed to above baseline levels of FSH¹¹. These observations suggest that even in the follicles in the large category, retardation might initiate 2-3 days after antrum formation under the present culture conditions. Although the roles of TGF- β 1 in the theca cells are not clear, temporal association between this event and the increase in the proportion of follicles with TGF- β 1 positive theca cells would support our previous *in-vivo* finding that the proportion of follicles with TGF- β 1 positive theca cells increased in retarded type 7 and unovulated type 8⁴.

In conclusion, the present study indicates that normally developing follicles express TGF- β 1 in the granulosa cells around the time of antrum formation. Furthermore, our present results demonstrate the usefulness of the present follicle culture system in the investigations of follicular physiology, which could not be determined *in vivo* and using isolated cell culture systems.

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