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The Roles of THY1 and Integrin Beta3 in Cell Adhesion During Theca Cell Layer Formation and the Effect of Follicle-Stimulating Hormone on THY1 and Integrin Beta3 Localization in Mouse Ovarian Follicles

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ABSTRACT

The mechanism of theca cell layer formation in mammalian ovaries has not been elucidated. In the present study, we examined the roles of THY1 and integrin beta3 in theca cell layer formation during mouse folliculogenesis. The localization pattern of THY1 and integrin beta3 in adult mouse ovary was investigated immunohistochemically. The strongest THY1 signal was observed in theca cell layers from secondary to preantral follicles, at which time theca cells have begun to participate in follicle formation. Integrin beta3 also localized to the theca cell layer of secondary to preantral follicles and showed a localization pattern similar to that of THY1. Moreover, the role of THY1 in theca cell layer formation was examined using a follicle culture system. When anti-THY1 antibody was added to this culture, no theca cell layers were formed, and the granulosa cells were distanced from each other. Because a THY1 signal was not observed in ovaries at stages earlier than prepuberty, THY1 localization also appeared to be affected by mouse development. This possibility was examined by determining the effect of administering follicle-stimulating hormone, luteinizing hormone, and 17beta-estradiol to 7-day-old mice on THY1 localization in the ovary 3 days later. Only follicle-stimulating hormone induced a THY1 signal in 10-day-old mouse ovaries. No THY1 signal was observed in untreated 10-day-old ovaries. In conclusion, THY1 might play a role in cell adhesion via binding to integrin beta3 in mouse ovaries. The present results suggest that THY1 localization may be affected by follicle-stimulating hormone in mouse ovaries.

follicle, follicle-stimulating hormone (FSH/FSH receptor), granulosa cells, ovary, theca cells

INTRODUCTION

Follicles in mammalian ovaries constitute a single oocyte, granulosa cells, and theca cells. Although a theca cell layer is not recognizable in primordial follicles, it is recognizable from the stage of secondary follicles onward. Theca cells play important roles in folliculogenesis: They physically support follicle structure, and they produce steroid hormones and other factors for folliculogenesis. The origin of theca cells has not been fully elucidated, but it is believed that ovarian interstitial cells gather around follicles and differentiate into theca cells [1, 2]. Cell adhesion mechanisms would be required for interstitial cells to flatten and spread around follicles to form theca cell layers, and extracellular matrices might be important for theca cell layer formation [3–8]. The presence of extracellular matrices, such as fibronectin [3–5], collagen [3, 5–7], and laminin [3, 5, 8], in ovaries has been reported, and these matrices are localized between interstitial cells as well as between theca cells. However, the morphology of theca cells is different from that of the surrounding interstitial cells. Therefore, adhesion molecules specific for theca cells are required for theca cell layers to form.

THY1 is a 25- to 37-kDa cell surface glycoprotein that belongs to the immunoglobulin (Ig) superfamily [9–12]. THY1 is a glycosylphosphatidylinositol-anchored protein; thus, it has neither a transmembrane domain nor a cytoplasmic domain [12–15]. THY1 is expressed in various types of cells [9], such as thymus [16–18], T cells and B cells [11, 12], neurons [19], fibroblasts [20], ovarian cells [21, 22], endothelial cells [12], and kidney [23, 24], in various animals, including mice [10, 11, 13, 15–19, 25], rats [9, 10, 20, 22–24], and humans [10, 21]. THY1 has been reported to be involved in various functions, such as proliferation and apoptosis [17, 23, 24, 26] as well as adhesion [12, 16, 19, 20, 27, 28]. Of these suggested functions, cell adhesion is one that has gained general acceptance [12]. Some reports also have concerned THY1 expression in ovaries [21, 22]. For instance, THY1 has been shown to be expressed in rat ovaries [22]. In addition, THY1 function might involve the cell cycle-related retinoblastoma protein, and THY1 may induce cell differentiation in human follicles [21]. However, the mechanism by which THY1 mediates cell adhesion, especially the identity of counterparts that interact with THY1 during the process of adhesion, has not been clarified. The most promising candidate counterpart of THY1 is integrin beta3. The effect of integrin beta3 on cell adhesion is mediated by integrin beta3 recognition of the RLD motif within adhesion proteins [29], and this RLD motif is also found in the THY1 sequence [27]. An interaction between integrin beta3 and THY1 has been reported to play a role in cell adhesion [27, 28]. The mRNA expression of various integrins has been reported in mouse ovaries, and integrin beta3 was found to be expressed only in the theca cell layers in follicles [30]. However, a relationship between THY1 and integrin beta3 function has not been shown in ovaries.

These previous data suggest that THY1 and integrin beta3 likely are involved in cell adhesion when theca cell layers are formed. In the present study, to determine if THY1 and integrin beta3 interact to mediate theca cell layer formation, we first compared the localization of these molecules in adult mouse ovary. Then, we investigated the effects of an anti-THY1 antibody on theca cell layer formation in a follicle culture system. Finally, factors that control THY1 localization were

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examined by observing the patterns of THY1 localization during mouse development. THY1 localization in hormone-treated ovaries was also observed.

MATERIALS AND METHODS

Animals

Female ICR mice (Japan SLC, Inc.) were maintained under controlled light conditions (14L:10D) and were given food and water ad libitum. The day of birth was designated as Day 0. On Day 1, each mother was left with eight pups to equalize the growth of pups between litters. Our investigations were conducted in accordance with the Animal Care Committee of Nara Women's University.

Chemicals and Antibodies

Human follicle-stimulating hormone (FSH; Acris Antibodies GmbH), rat luteinizing hormone (LH; Biogenesis Ltd.), and 17beta-estradiol (E_2; Sigma-Aldrich Corp.) were used. Primary antibodies used in the present study were rat anti-mouse THY1 antibody (Biomeda), hamster anti-mouse integrin beta3 antibody (BioLegend), and rabbit anti-human von Willebrand factor (a marker of endothelial cells; DakoCytomation). Secondary antibodies used were goat anti-rat IgG labeled with Alexa Fluor 488 and goat anti-hamster IgG labeled with Alexa Fluor 594 (both from Molecular Probes/Invitrogen Corp.).

Cell culture chemicals used in the present study were Dulbecco modified Eagle medium (DMEM; Nissui Pharmaceutical Co. Ltd.), fetal bovine serum (FBS; Gibco BRL/Invitrogen Corp.), penicillin and streptomycin (Nacalai Tesque, Inc.), collagenase (Wako Pure Chemical Industries Ltd.), DNase (Roche Diagnostics Corp.), and type I collagen (Cellmatrix Type I-A; Nitta Gelatin, Inc.). Rat IgG used as a negative control was rat plasma IgG_{2b} (BioPur AG).

Immunohistochemistry

Isolated ovaries were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15–120 min at 4°C. The time of fixation was adjusted for ovarian size. The fixed ovaries were cryoprotected by immersion in PBS containing increasing concentrations of sucrose (0%, 10%, 20%, and 30%) and then embedded in OCT compound (Sakura Finetek Japan Co., Ltd.) and frozen in liquid nitrogen. Frozen sections (thickness, 7 μm) were prepared using a cryostat (Bright Instrument Co. Ltd.) at −25°C. The following procedures were carried out at room temperature: The sections were washed with PBS and incubated with 10% normal goat serum in PBS for 1 h to reduce nonspecific binding. The sections were then incubated overnight with primary antibody against THY1 or integrin beta3 (diluted 1:1000 [THY1] or 1:2000 [integrin beta3] in 0.5% bovine serum albumin [BSA]/PBS). After washing with PBS, the sections were incubated for 3 h with the appropriate secondary antibody (diluted 1:300 in PBS) and rinsed with PBS. Finally, cell nuclei were stained using 4′,6′-diamidino-2-phenylindole (DAPI; Nacalai Tesque, Inc.). These samples were examined using an Olympus BX51 fluorescent light microscope (Olympus Corp.). Negative controls were treated with 0.5% BSA/PBS instead of primary antibody to assess nonspecific staining. Because we observed autofluorescence of the ovaries, a WIB long-pass filter cube (Olympus Corp.) was used for observation of fluorescent samples. The use of this cube allowed us to distinguish significant signals (green THY1 signals) from autofluorescent signals (yellow signals from red blood cells).

Hormone Treatment

FSH and LH were dissolved in PBS, and E_2 was dissolved in peanut oil. Seven-day-old female mice received a single subcutaneous injection of FSH (0.8 μg/animal), LH (0.25 μg/animal), or E_2 (0.1 μg/animal). Three days after injection, their ovaries were removed and used for immunohistochemical investigation. In addition, 3-wk-old female mice received a single subcutaneous injection of FSH (2 μg/animal). Two days after injection, their ovaries were removed and were used for immunohistochemical investigations. Control animals were treated with vehicle only (PBS or peanut oil).

Three-Dimensional Follicle Culture System

Theca-interstitial cells were isolated from the ovaries of 3-wk-old mice. The ovaries of mice at this age are the largest ovaries that can be obtained without a corpus luteum. The ovaries were collected in culture medium (DMEM with 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 100 ng/ml of FSH) and were freed from connective tissues under a stereomicroscope. The follicles were punctured by fine tweezers to remove granulosa cells and oocytes. In culture medium containing 0.2% collagenase and 0.1% DNase, these ovaries were then cut into 1-mm^3 fragments using scissors and pipetted to facilitate cell dispersion. The suspension of ovarian fragments was incubated at 37°C for 20 min and pipetted after the first 10 min. Ovarian fragments, follicles, and oocytes were removed by filtration through a series of nylon meshes (with pore sizes in the order of 155, 82, 40, 20, and 10 μm). The resulting cell suspension was centrifuged at $250 \times g$ for 5 min, after which the supernatant was removed. To reduce the effects of collagenase to a negligible level, the washing procedure described above was repeated four times. Finally, the number of viable cells was counted.

Preantral follicles (diameter, ~140 μm) were obtained from 10- to 14-day-old female mice. The ovaries were collected in culture medium, and the follicles were mechanically isolated from the ovaries under a stereomicroscope using a 27-gauge needle fitted to a 1-ml syringe barrel. Follicles were selected using a mouth-operated, glass fine pipette and then transferred to a culture dish.

The isolated follicles grew in three dimensions in this culture system. A collagen gel was prepared according to the manufacturer's instructions. Type I collagen and DMEM were mixed, and pH was adjusted to 7.4 with reconstitution buffer. A collagen gel was supplemented with 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 100 ng/ml of FSH. Collagen gel solution (50 μl) was poured into each well of a 96-well culture plate (Microplate 96 Well with Lid; AGC Techno Glass Co. Ltd.) and allowed to gel at 37°C in 5% CO_2 in air and 100% humidity. Theca-interstitial cells were inoculated onto the gel at 6×10^4 cells/well and incubated overnight at 37°C in 5% CO_2 in air. After the medium was removed, the follicles were placed on the theca-interstitial cell layer, and additional collagen gel solution (50 μl) was poured onto the cells to encapsulate the follicles. After the collagen solution had gelled, 50 μl of the culture medium were added. The anti-THY1 antibody (0.1, 1, or 10 μg/ml) was added at initiation of culture to analyze the effect of THY1 on follicular growth. Follicles were cultured for 6 days at 37°C in 5% CO_2 in air and 100% humidity. During the culture period, the culture medium was changed every 2 days, and photographs of the follicles were taken every day to check for cell survival and to measure follicle diameters.

To evaluate the morphology of follicles in collagen gel, the vertical and horizontal diameters were measured by histological methods. Follicles were fixed by 4% paraformaldehyde or Bouin solution at the end of culture, embedded horizontally in paraffin, and serially sectioned (thickness, 7 μm). The horizontal diameters of follicles correspond to the maximum diameters in their serial sections. On the other hand, the vertical diameters of follicles were estimated by the number of follicle sections.

Statistical Analysis

The difference between follicle diameter on Day 0 and that on every following day in the same experimental group was compared using one-factor ANOVA (Fisher protected least significant difference). The effect of anti-THY1 antibody on follicle diameter was assessed by a combination of repeated-measures ANOVA and a paired *t*-test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

THY1 Localization During Folliculogenesis in Adult Mouse Ovaries

Changes in THY1 localization during folliculogenesis of adult mouse ovaries were determined by immunohistochemical analysis. No THY1 signal was detected following negative-control staining (Fig. 1a). A THY1 signal was also not detected in primordial follicles (Fig. 1, c and d, arrows), and only a weak signal was detected in the outer layer of the primary follicles (Fig. 1, c and d, arrowheads). This signal probably existed at the basement membrane. At these stages, the follicles are composed only of oocytes and granulosa cells, and theca cells were not observed. Theca cells participate in follicles as the follicles develop into secondary follicles. Indeed, in secondary and preantral follicles, intense THY1 signals were localized at the surface of theca cells (Fig. 1f, arrow). However, THY1 signals in the theca cell layers were not very strong in antral follicles (Fig. 1h, arrow). In adult ovaries, the most intense THY1 signal was observed in theca cells from secondary to preantral follicles. The THY1 signals of granulosa cells were more variable than those of theca cells. We

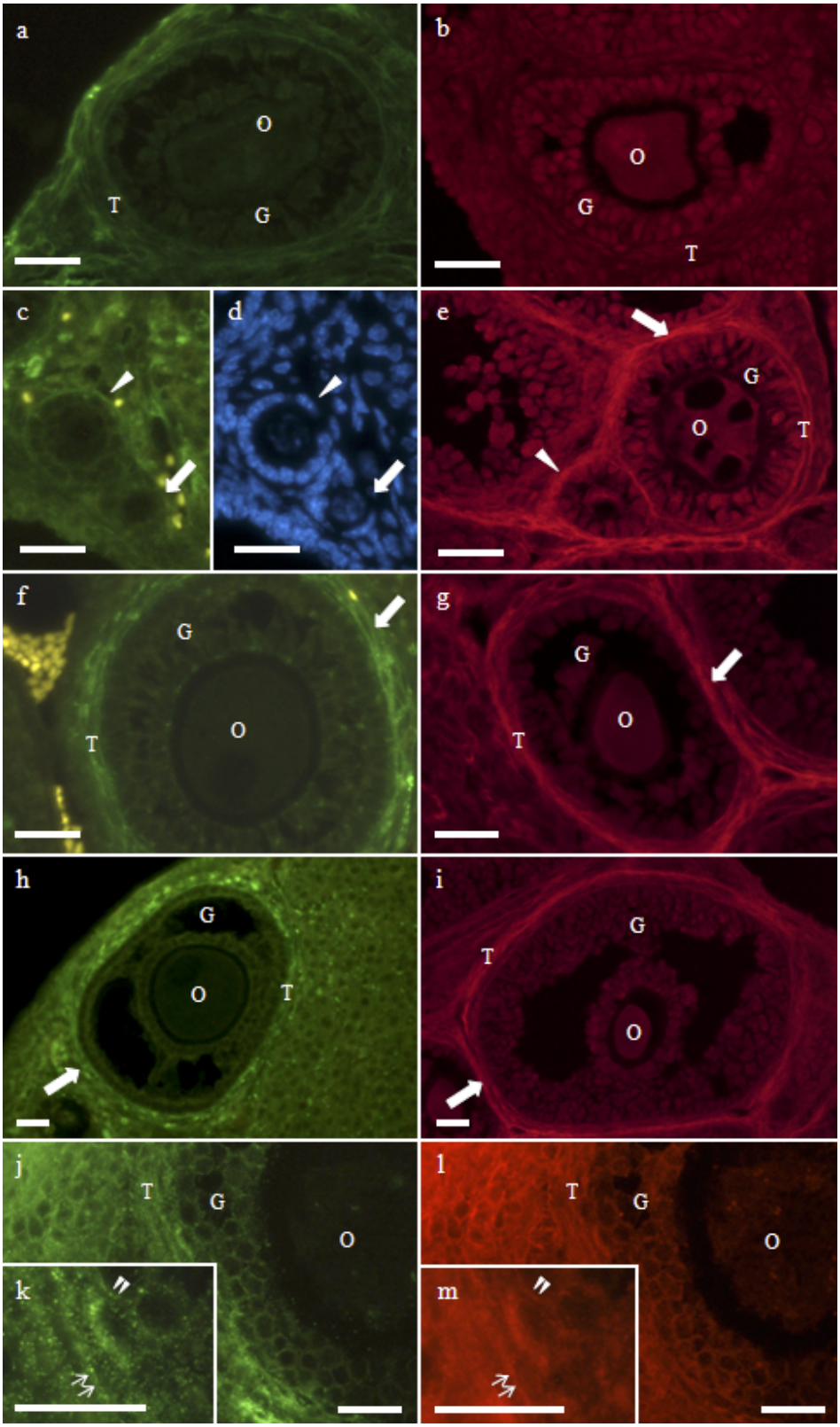

FIG. 1. Immunohistochemical localization of THY1 and integrin beta3 in adult mouse ovary during folliculogenesis. Negative-control staining for THY1 (**a**) and integrin beta3 (**b**) used 0.5% BSA/PBS instead of each antibody. Fixed sections of adult mouse ovaries were stained individually with anti-THY1 antibody (green; **c**, **f**, and **h**) or anti-integrin beta3 antibody (red; **e**, **g**, and **i**) or were double-stained with anti-THY1 and anti-integrin beta3 antibodies (**j**–**m**), followed by labeling with Alexa Fluor 488 and Alexa Fluor 594 secondary antibody, respectively. A section (**c**) was also costained using DAPI (**d**). Fields shown in **c** and **d**, **j** and **l**, and **k** and **m** are identical; **k** and **m** show a higher magnification of **j** and **l**, respectively. The yellow signal in each of the images results from autofluorescence of contaminating red blood cells. THY1 staining of a primordial follicle (arrow) and a primary follicle (arrowhead) is indicated (**c**). Integrin beta3 staining of a primary follicle (arrowhead) and a secondary follicle (arrow) is shown (**e**). StrongTHY1 (**f**) and integrin beta3 (**g**) signals are observed in theca cells (arrows) in the preantral follicle. THY1 (**h**) and integrin beta3 (**i**) signals are observed in theca cells (arrows) in the antral follicle. THY1 (**j**) and integrin beta3 (**l**) signals are also observed throughout ovary. THY1 (**k**) and integrin beta3 (**m**) signals colocalize in theca cells (double arrows) and in granulosa cells (double arrowheads). G, granulosa cell; O, oocyte; T, theca cell. Bar = 25 μm.

occasionally observed a punctate THY1 signal in granulosa cells or an intense THY1 signal throughout the ovaries (Fig. 1, j and k).

THY1 immunoreactivity also localized at capillaries in the ovaries. The capillaries extend to the theca cell layers and supply nutrition for folliculogenesis from about the secondary follicle stage. By staining THY1 together with von Willebrand factor, a marker of endothelial cells, it was confirmed that THY1 signals localized both on the theca cells and on capillaries (data not shown).

Integrin Beta3 Localization During Folliculogenesis in Mouse Ovaries

To investigate the relationship between THY1 and integrin beta3 in follicles, changes in integrin beta3 localization during folliculogenesis in adult ovaries were analyzed using immunohistochemical analysis. No signal was detected following negative-control staining (Fig. 1b). Immunoreactive integrin beta3 was not detected in primordial or small primary follicles. Integrin beta3 signal was observed in the outer layer of large primary follicle (Fig. 1e, arrowhead), and a strong integrin beta3 signal was detected in the theca cell layer of secondary and preantral follicles (Fig. 1, e and g, arrows). In the antral follicles, an integrin beta3 signal was observed in theca cells (Fig. 1i, arrow). Moreover, a strong integrin beta3 signal was occasionally observed throughout the ovaries at cell surfaces of granulosa cells as well as those of theca cells in every follicle (Fig. 1, l and m). This pattern of integrin beta3 localization throughout the ovaries was similar to that of THY1 (Fig. 1, j and k).

Relationship of THY1 and Integrin Beta3 Localization in Mouse Ovaries

Because the localization of THY1 and integrin beta3 in ovaries was similar, we used double immunohistochemical staining of THY1 and integrin beta3 in a normal adult ovary to further analyze their potential colocalization (Fig. 1, j–m). In this ovary, both THY1 and integrin beta3 signals were observed throughout the ovary. Both signals colocalized at the surface of theca cells (Fig. 1, k and m, double arrows) and granulosa cells (Fig. 1, k and m, double arrowheads).

Effects of Anti-THY1 Antibody Treatment on Follicular Development in Culture

To further analyze folliculogenesis, especially the mechanism of theca cell layer formation, an appropriate follicle culture system was needed. We therefore devised a follicle culture system in which a collagen gel was used to generate and maintain a three-dimensional culture. Although follicles were able to grow and maintain a three-dimensional conformation within this collagen gel, theca cell layers were not formed (Fig. 2a). To form theca cell layers in this culture system, we cocultured these follicles in collagen with theca-interstitial cells. In this coculture, the follicles grew very well, and the theca-interstitial cells (Fig. 2b, arrowheads) constructed cell layers around the follicles from Day 3 of the coculture (Fig. 2b, arrows). In addition, follicle diameters significantly increased from Day 2 of the coculture, and at Day 5, the follicle diameter was 145% ± 9.1% of the diameter on Day 0 (Fig. 3). To analyze the three-dimensional morphology of follicles in collagen gel, the vertical and horizontal diameters of follicles were evaluated by histological methods. At the end of culture, the ratio of vertical diameter to horizontal diameter was 1:0.97 ± 0.16 (n = 16), suggesting that in our culture, follicles could grow while keeping a spherical shape.

When the THY1 antibody was added at initiation of this culture system, folliculogenesis exhibited a different appearance from that of the control group, which was treated with rat IgG (10 μg/ml). Control IgG treatment did not affect either follicular growth or the construction of theca cells around the follicles and subsequent theca cell layer formation (Fig. 2c, arrows). Follicle diameters also significantly increased in size from Day 2, and by Day 5, these diameters were 145% ± 8.9% of those diameters on Day 0 (Fig. 3). Although formation of

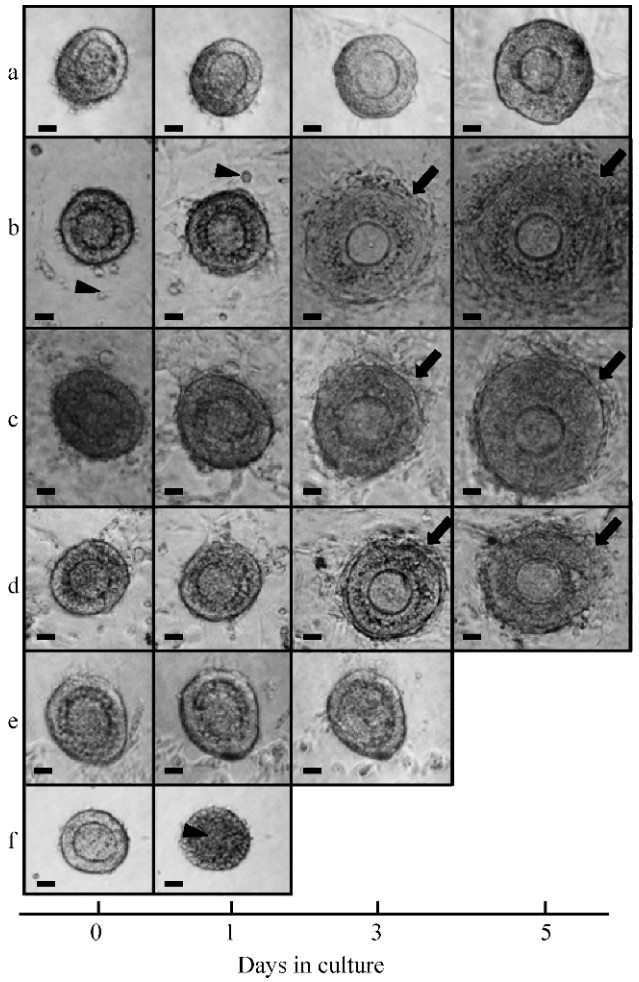

FIG. 2. Effects of anti-THY1 antibody on folliculogenesis in three-dimensional follicle culture. Follicles isolated from mouse ovaries were cultured within a collagen gel without (**a**) or with (**b**–**f**) theca-interstitial cells. The cells were untreated (**b**), treated with 10 μg/ml of rat IgG (**c**), or treated with 0.1, 1, or 10 μg/ml (**d**, **e**, and **f**, respectively) of anti-THY1 antibody. The cells were then observed using phase-contrast microscopy. Cell layers (arrows) were formed in the cultures shown in **b**–**d** but not in those shown in **e** and **f**. Arrowheads indicate theca-interstitial cells. Bar = 25 μm.

cell layers around the follicles was observed (Fig. 2d, arrow) in the anti-THY1 antibody-treated cultures (0.1 μg/ml), these cell layers were thinner than those of the control group. Furthermore, although follicle diameters in the THY1 antibody-treated cultures significantly increased in size from Day 4 of culture and, by Day 5, were 126% ± 8.7% of the diameters on Day 0, the follicle diameters on each day of culture from Day 2 onward were significantly lower than those of the control (Fig. 3). In cultures treated with 1 or 10 μg/ml of anti-THY1 antibody, no cell layers were observed around the follicles in either group (Fig. 2, e and f). At these high concentrations of anti-THY1 antibody (1 and 10 μg/ml), the morphology of the theca-interstitial cells was rounded, not extended (Fig. 2f, arrowhead). In addition, following treatment with 1 or 10 μg/ml of antibody, the oocytes collapsed on Day 3 (Fig. 2e) and on Day 1 (Fig. 2f), respectively. Moreover, granulosa cells did not appear to adhere either to oocytes or to each other in these cultures. Subsequently, follicular growth was arrested (Fig. 3).

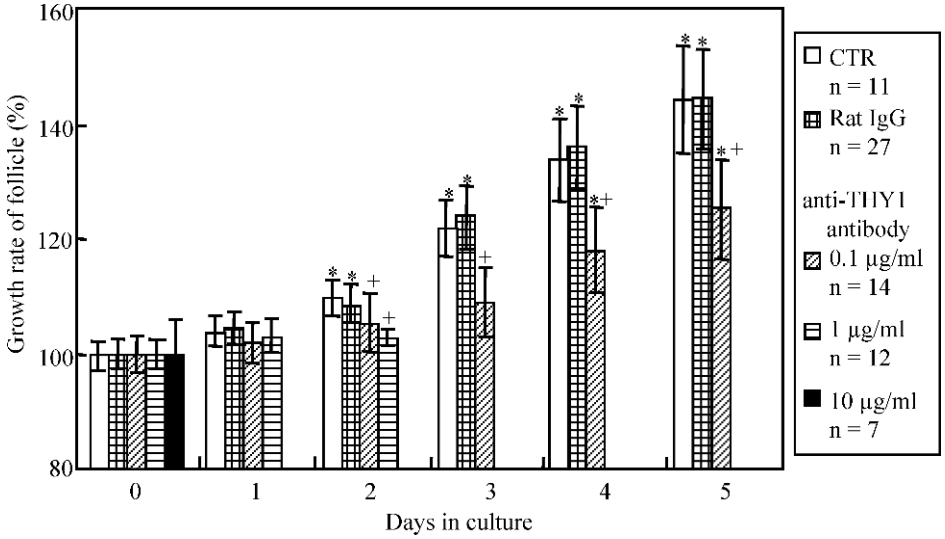

FIG. 3. The effect of anti-THY1 antibody addition on follicular growth in three-dimensional follicle culture. The size of the follicles grown in three-dimensional culture in the presence or absence of anti-THY1 or control (CTR) antibody, as shown in Figure 2, was measured on the indicated days after antibody treatment. Follicle diameters are expressed as a percentage of the follicle diameter on Day 0. $^{*}P < 0.05$ vs. Day 0, $^{+}P < 0.05$ vs. control.

Changes in THY1 Localization During Mouse Development

To determine if the THY1 localization pattern in immature ovary was the same as that in adult ovary, we examined immunohistochemically THY1 localization in the ovary during mouse development (Fig. 4a). The ovaries of 10-day-old mice contained primordial, primary, secondary, and preantral follicles. No THY1 signal was detected in these ovaries (Fig. 4a). THY1 staining in secondary follicles of a 10-day-old ovary is shown in Figure 5a. No THY1 signal was detected in the follicles. The ovaries of 3-wk-old mice contained follicles at all developmental stages, but ovulation had not yet occurred. In 3-wk-old mice, 50% of the ovaries had THY1-positive theca cells, and 7.7% of the ovaries had THY1-positive granulosa cells (Fig. 4a). Secondary follicles that had THY1-positive theca cells in 3-wk-old ovaries are shown in Figure 5e. The ovaries of adult mice contained follicles at all developmental stages, and ovulation had occurred. In the adult mice, 100% of the ovaries had THY1-positive theca cells, and 21.4% of the ovaries had THY1-positive granulosa cells (Fig. 4a).

Several interesting points regarding THY1 localization in ovaries were noted during the examination of THY1 localization in mouse development. First, THY1 signals were not observed in any follicles until the mice reached a certain developmental stage, typically at 2 wk of age. During these developmental stages, THY1 signals simultaneously appeared in follicles throughout an ovary, and the resultant localization pattern of THY1 was identical to that in adult ovaries. Second, THY1 was detected preferentially in theca cell layers rather than in granulosa cell layers. For instance, in an ovary exhibiting a THY1 signal in theca cells, the signal was observed in theca cells of all follicles that contained theca cells. In an ovary exhibiting a THY1 signal in granulosa cells, the signal was observed in granulosa cells of all follicles. Whenever the THY1 signal was observed in granulosa cells, a THY1 signal was also observed in theca cells. In no follicle was the THY1 signal observed only in granulosa cells and not in theca cells.

Effects of Hormonal Treatments on THY1 and Integrin Beta3 Localization in Mouse Ovaries

THY1 signal appeared simultaneously at different regions throughout the ovaries. In addition, the percentage of immuno-positive THY1 ovaries increased as the mice approached puberty. These observations suggested that THY1 localization in ovaries might be regulated by factors, such as gonadotropin, that are involved in the regulation of ovarian function. We therefore assayed the effect of factors known to modulate ovarian function on THY1 localization.

The ovaries of 7-day-old mice contained primordial, primary, secondary, and preantral follicles. No THY1 signal was detected in these ovaries (data not shown). These ovaries were then injected with FSH, LH, E_2, or a control solution, and the localization of THY1 was assayed after 3 days. No THY1 signal was detected in control ovaries 3 days later, either in theca or in granulosa cell layers (Fig. 4b). THY1 staining in secondary follicles of a PBS-treated, 10-day-old ovary is shown in Figure 5b. No THY1 signal was detected in the follicles. In contrast, in FSH-treated ovaries, THY1 immuno-reactivity was detected in 78% of the ovaries (Fig. 4b). Strong THY1 signals were observed in granulosa cell layers as well as in theca cell layers (Fig. 5c). In these granulosa cell layers, the THY1 signals were localized around the granulosa cell surface, and this THY1 localization pattern was similar to that occasionally observed in some of the adult ovaries without treatment (Fig. 1, j and k). In contrast to the effect of FSH, THY1 localization was not affected by injection with LH (Fig. 4b) or E_2 (data not shown). THY1 staining in secondary follicles of an LH-treated, 10-day-old ovary is shown in Figure 5d.

The effect of FSH injection on THY1 localization was also assayed in 3-wk-old mice. In the untreated control mice, 73% of the ovaries had THY1-positive theca cells, and 18% of the ovaries had THY1-positive granulosa cells (Fig. 4b). FSH injection increased the percentage of ovaries with THY1-positive theca cells to 100%. FSH injection also increased the percentage of granulosa cells that were THY1 positive to 88% (Fig. 4b). An antral follicle with THY1-positive theca cells and granulosa cells in an FSH-treated, 3-wk-old ovary is shown in Figure 5f. The pattern of THY1 localization in granulosa cell layers was similar to that of FSH-injected, 7-day-old ovaries (Fig. 5c) and of some adult ovaries without treatment (Fig. 1, j and k). We further confirmed the effect of FSH on integrin beta3 localization in the ovaries of 3-wk-old mice. In control ovaries without treatment, an integrin beta3 signal was observed in theca cells (Fig. 5g). However, in FSH-treated ovaries, integrin beta3 signals were detected not only in theca cells but also in granulosa cells (Fig. 5h). The FSH-treated ovary shown in Figure 5, f and h, is the same ovary. Thus, FSH

stimulates an integrin beta3 signal, especially in granulosa cells, and the pattern of FSH-induced integrin beta3 localization is similar to that of FSH-induced THY1 localization.

DISCUSSION

Roles of THY1 and Integrin Beta3 on Cell Adhesion

In mammalian ovaries, theca cells participate in folliculogenesis from the time of formation of the secondary follicle. It is believed that ovarian interstitial cells gather around follicles and differentiate into theca cells [1, 2]. On the other hand, theca cells may be present at the very outset of follicular growth [31]. The origin of theca cells is still obscure, and many questions about the mechanism of theca cell layer formation remain to be answered. In the present study, we examined the roles of THY1 and integrin beta3 in theca cell layer formation in the mouse ovary.

In adult mouse ovaries, a THY1 signal was observed in the theca cell layer, particularly from the secondary to the preantral follicles, which is the time when theca cell layers begin to form (Fig. 1f). In addition, THY1 antibody treatment of follicle cultures showed that when THY1 was blocked by antibody, theca cell layer formation failed, and interstitial cells became rounded (Fig. 2, e and f). Theca cells spread around the follicle and form a multilayer for which some mechanism of cell adhesion should be required. Our results suggest that the cell adhesion via THY1 might be involved in the theca cell layer formation. It has been reported that THY1 is involved in cell adhesion in various tissues [12, 16, 19, 20, 27]. Thus, pulmonary fibroblasts that express THY1 display elongated focal adhesion structures, form large bundled actin stress fibers, and spread rather than migrate, whereas fibroblasts lacking THY1 exhibit less elongated focal adhesion structures, form fewer stress fibers, and actively migrate [20]. It has been reported that THY1 might play a role in cell adhesion between thymocytes and epithelial cells in the thymus [16]. In neurons, axons that have finished growing express THY1, whereas axons that are still growing do not. It was therefore suggested that THY1 might influence the stability of the synapse through an effect on cell adhesion [19]. Based on these previous data, we considered the possibility that THY1 might mediate theca cell layer formation through modulation of cell adhesion. However, although a THY1 signal was not observed in 10-day-old ovaries (Figs. 4a and 5a), these ovaries contained secondary and preantral follicles with theca cells, suggesting that theca cell adhesion can occur even in the absence of THY1. It is possible that the property of theca cells in neonatal and prepubertal ovaries differ from those of cycling adult ovaries. In these ovaries, the morphology of theca cells is plump and differs from adult ovaries [32]. Therefore, cell adhesion of prepubertal follicles might be weaker than that of adult follicles. It is possible that THY1 expressed during mouse development might reinforce the adhesion of theca cells.

Some reports have appeared regarding the THY1-deficient mouse. Although these mice did not differ from their littermates in breeding, behavior, and health, the regional inhibition of long-term potentiation was observed in the dentate gyrus of the hippocampus [25]. It was also found that the loss of THY1 inhibits alveolar development in the newborn mouse lung [33]. Although these mice appear to be fertile [25], information regarding folliculogenesis in their ovaries has not been mentioned. What shape is the theca cell in the THY1-deficient ovary? How many theca cell layers enclose a follicle in a THY1-deficient ovary? How many follicles reach ovulation in one estrous cycle in the THY1-deficient mouse?

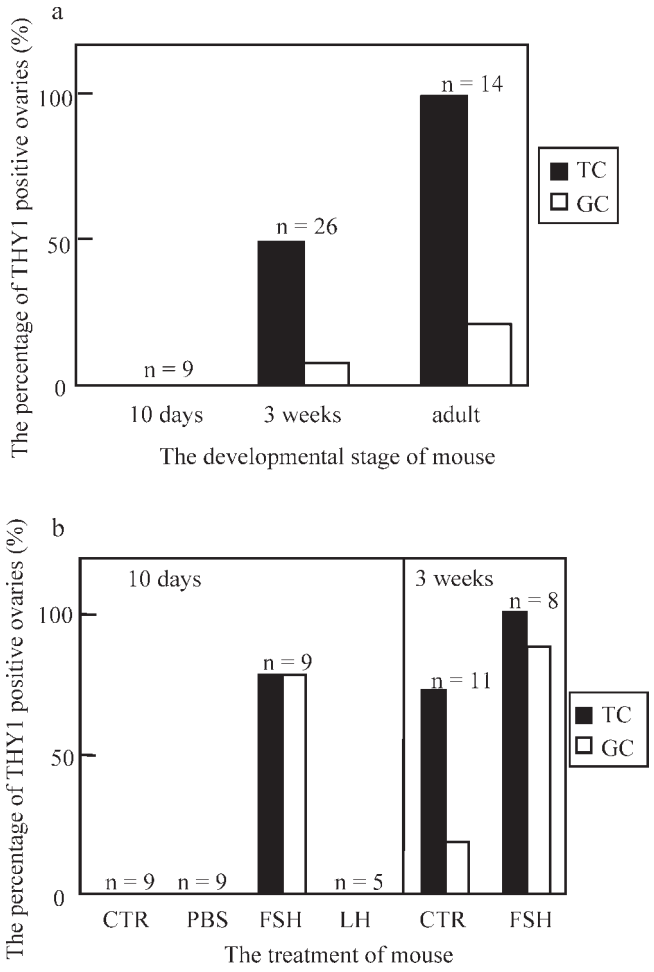

FIG. 4. THY1 localization in the ovary during mouse development and the effect of hormones on THY1 localization. THY1 localization in mouse ovaries during development (**a**) or following treatment with FSH, LH, and E_2 (**b**) was analyzed by immunostaining of ovarian sections. The number of THY1-positive ovaries at the indicated times was then manually counted, and the specific localization of THY1 within the ovary was determined. Numbers above the bars indicate the number of mice examined. Solid bars indicate theca cells (TC); open bars indicate granulosa cells (GC). Changes in the percentage of THY1-positive ovaries during mouse development are shown (**a**), as are hormonal effects on the percentage of THY1-positive ovaries (**b**). The left side indicates 7-day-old mice treated with FSH at 0.8 μg/animal or LH at 0.25 μg/animal and for which ovaries were fixed 3 days later. The right side indicates 3-wk-old mice for which ovaries were fixed 2 days after injection with FSH at 2 μg/animal.

The answers to these questions might be key for elucidating the function of THY1 during folliculogenesis in mouse ovary.

One candidate counterpart for THY1 during theca cell adhesion is integrin beta3 [12, 27, 28]. It has been reported that THY1 and integrin beta3, which are expressed in neurons and astrocytes, respectively, interact to form focal adhesion structures and, thereby, mediate neuronal extension [27]. In addition, THY1 and integrin alphaV beta3, expressed in endothelial cells and melanoma cells, respectively, interact to facilitate cell adhesion and to promote extravasation [28]. Our immunohistochemical analysis indicated a strong integrin beta3 signal in theca cells from preantral to antral follicles in adult ovaries (Fig. 1, e, g, and i). When double staining of THY1 and integrin beta3 was carried out, both signals were observed at the same localization (Fig. 1, j–m). These data suggested that

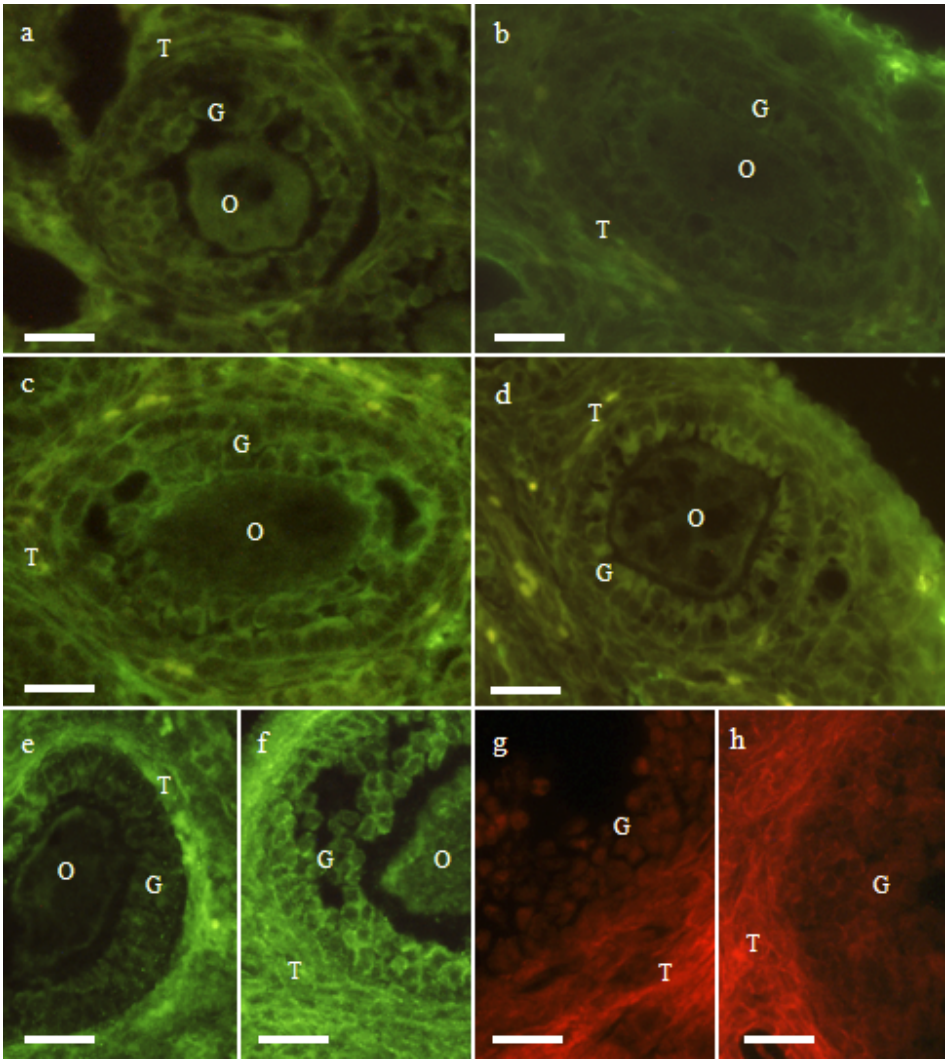

FIG. 5. Hormonal effects on THY1 and integrin beta3 localization in ovaries. Hormonal treatments were performed on 7-day-old mice, from which ovaries were fixed 3 days later. Ovaries from untreated 10-day-old mice (**a**), mice treated with PBS (**b**), mice treated with FSH at 0.8 μg/animal (**c**), or mice treated with LG at 0.25 μg/animal (**d**) are shown. In 3-wk-old mice, ovaries were fixed 2 days after hormonal treatment. Ovaries from untreated 3-wk-old mice (**e** and **g**) or from mice treated with FSH at 2 μg/animal (**f** and **h**) are shown. Ovaries were immunostained for THY1 (**a–f**) or integrin beta3 (**g** and **h**). The immunostaining procedure was identical to that described for Figure 1. The same ovary is shown in **f** and **h**. G, granulosa cell; O, oocyte; T, theca cell. Bar = 25 μm.

THY1 may influence cell adhesion in mouse ovaries through an interaction with integrin beta3.

Although an intense THY1 signal was observed in other follicular stages, it was less intense in antral follicles (Fig. 1, f and h). In contrast, a continuous, intense integrin beta3 signal was observed in secondary through antral follicles. It has been suggested that cell adhesion can be divided into two steps. The first step is the initial binding step, and the second step is a subsequent strengthening response, which is accompanied by actin polymerization [34]. During adhesion of thymocytes and epithelial cells, THY1 is involved in the initial binding step, and other mechanisms act during the strengthening response [16]. This situation might be applicable to theca cell adhesion. THY1 may act as a trigger of theca cell adhesion in secondary follicles, and THY1 function might be replaced by other adhesion mechanisms as folliculogenesis advances. The extracellular matrix is a likely source of candidate molecules, such as fibronectin, that might mediate these other adhesion mechanisms. It has been shown that fibronectin modulates cell adhesion in cooperation with other cell adherent molecules [5] and also localizes at theca cells [3–5]. Furthermore, it has been reported that the expression of THY1 facilitates the production of fibronectin from human ovarian cancer [35]. In addition, an interaction between integrin beta3 and fibronectin in mouse peri-implantation blastocysts that influences cell adhesion has also been reported [36]. We observed that strong integrin beta3 signals remained until the antral follicle stage (Fig. 1i). Thus, integrin beta3 may trigger cell adhesion through interaction with THY1 in early stage follicles and through interaction with extracellular matrix molecules in antral follicles. Based on these observations, we suspect that THY1 expression in theca cells might facilitate the production of fibronectin and trigger cell adhesion with integrin beta3, and its function of reinforcing follicle structure might then be replaced by fibronectin. Interactions between these cell adhesion molecules and extracellular matrices require further study.

On the other hand, the possibility that another receptor might be required for THY1 and integrin beta3 interaction has been raised. It has been reported that neuron-to-astrocyte adhesion was the result of THY1 in neuron and integrin beta3 in astrocyte interaction [27]. However, this adhesion was only partially inhibited by antibodies to integrin alpha5 beta3 or RLD peptide and RGD-like peptide found in THY1 structure [27, 37]. Avalos et al. [38] showed that neuronal THY1 stimulated astrocyte adhesion via engagement of integrin alpha5 beta3 and the proteoglycan syndecan-4. In addition, syndecan-4 was demonstrated to be expressed in growing mouse follicles at the stages (type 4–5b) [39] in which intense THY1 signal was observed during the present experiment. Whether syndecan-4 might be involved in the adhesion of theca cells via THY1-integrin beta3 binding remains to be determined.

Integrins are a family of heterodimeric cell adhesion molecules, and they not only mediate cell adhesion to their counterpart but also facilitate cell survival, proliferation, and motility [40]. Although the presence of integrins in mammalian ovaries has been reported, it is not adequately understood whether the integrins have important roles in ovarian function—that is, folliculogenesis, ovulation, and atresia. With regard to integrin beta3 in mammalian ovary, few reports have appeared. Burns et al. [30] reported that integrin beta3 mRNA was expressed in theca and interstitial cells in mouse ovary, in agreement with our data in the present study. Coincubation with blocking antibody against integrin alpha5 beta3 increased apoptosis and prevented migration in human luteinized granulosa cells grown on fibronectin, suggesting that integrin beta3 might promote survival and migration of granulosa cells [41]. The integrin beta3-deficient mouse shows defects in platelet aggregation and clot retraction, prolonged bleeding times, and cutaneous and gastrointestinal bleeding [42]. These mice are viable and fertile, but they have reduced survival because of abnormal placental development and hemorrhage during prenatal and postnatal stages. Although the ovulation seems to occur in their ovary, the characterization of their folliculogenesis has not been reported. The role of integrin beta3 on folliculogenesis remains to be elucidated.

In the present study, it is suggested that THY1 might be a candidate counterpart of integrin beta3 in mouse ovaries. In addition, the adhesion of human luteinized granulosa cells to fibronectin is mediated by integrin beta3 [41]. In addition, in other cells and tissues, other counterparts of integrin beta3 have been revealed as fibrinogen, fibronectin, von Willebrand factor, collagen, and vitronectin [40], and among these, fibronectin and collagen are reported to exist in the mammalian ovary [3, 5–7]. Individual integrins can often bind to more than one counterpart, and individual counterparts of integrins are often recognized by more than one integrin. In ovaries, therefore, the network of integrin-counterpart adhesion might regulate cell proliferation, survival, apoptosis, and differentiation, resulting in appropriate folliculogenesis. The relationship between integrins and their counterparts in ovaries is a subject for future study.

THY1 is sometimes localized not only in theca cell layers but also in granulosa cell layers of mouse ovaries (Fig. 1, j and k). When such THY1 localization was detected in the present study, integrin beta3 was also localized in both of these cell types. Following THY1 antibody treatment of the follicle cultures, we observed that granulosa cells became rounded in shape and that individual cells were separated from each other. In addition, the oocytes showed irregular shapes, with an obscure outline (Fig. 2, e and f). The combined data suggest that THY1 and integrin beta3 also affect cell adhesion between granulosa cells, between granulosa cells and oocytes, and between theca cells.

Factors that Control THY1 Expression in Mouse Ovaries

The present results suggest that the localization of THY1 in ovaries might be regulated by factors that affect folliculogenesis throughout ovaries. Thus, THY1 signals were not observed in the ovaries of 10-day-old mice (Fig. 5a). However, the percentage of THY1-positive ovaries increased during development, being 50% in 3-wk-old mice and reaching 100% in adults (Fig. 4a). Furthermore, THY1 signals were simultaneously detected throughout a single ovary. Moreover, in immature mice, THY1 localization in ovaries seemed to be affected more by the maturity of the individual mouse than by the stage of the respective follicles. In addition, the percentage of THY1-expressing ovaries increased as ovulation approached (Fig. 4a). Therefore, the combined data suggested hormones that induce sexual maturity in the mouse can induce THY1 expression.

This hypothesis was further supported by experiments in which we analyzed the effects of hormonal treatment of 7-day-old and 3-wk-old mice on THY1 localization in ovaries. FSH administration to 7-day-old mice induced THY1 localization in the ovaries (Figs. 4b and 5c). Ovaries of control mice at the same stage did not show any THY1 signal (Figs. 4b and 5a). Furthermore, administration of LH or E_2 did not induce THY1 localization (Figs. 4b and 5d). These results suggested that FSH might specifically induce THY1 expression in ovaries. Unfortunately, changes in the serum FSH concentration during mouse development were not examined in the present study. However, it has been reported that FSH concentration in untreated mouse serum peaks at 10 days of age and gradually falls thereafter [43]. This high concentration of serum FSH in 10-day-old mice might affect THY1 expression in the follicles. The serum FSH concentration is believed to start to change in a cyclical manner in mice at 3 wk of age. Following this change to cyclic FSH expression, an LH surge begins to trigger ovulation. THY1 signal gradually appeared in all mice during development and was observed in all mice by adulthood. To date, many reports have concerned the localization and function of THY1. However, to our knowledge, the present study is the first concerning factors that might regulate THY1 expression.

In 3-wk-old mice treated with FSH, integrin beta3 signals, as well as THY1 signals, were detected in granulosa cells (Fig. 5, f and h). It has been reported that integrin beta3 mRNA is not expressed in the ovaries of FSH-beta-knockout mice [30]. These data suggest the possibility that expression of integrin beta3 in ovaries might also be controlled by FSH.

The pattern of THY1 localization differed between theca cells and granulosa cells. THY1 signal was always observed in the theca cell layer in all adult mice (Fig. 4a). In contrast, a THY1 signal was rarely observed in granulosa cells (Fig. 1, j and k). This result suggested that THY1 localization in granulosa cells is more variable than its localization in theca cells. In addition, the regulation of THY1 expression may differ between theca cells and granulosa cells. Thus, because granulosa cells express the FSH receptor [44, 45], changes in FSH concentration during the estrous cycle might directly affect THY1 expression in granulosa cells. If so, then theca cells might be indirectly affected by FSH through granulosa cells, although this mechanism remains unclear. THY1 localization throughout the ovary of normal mice (Fig. 1, j and k) was very similar to its localization in FSH-treated ovaries (Fig. 5, c and f). Therefore, FSH treatment of the mice may mimic a specific stage of the estrous cycle in normal mice. Because the FSH concentration fluctuates during the estrous cycle, THY1 expression may also vary in individual mice, depending on the timing of their estrous cycle.

In THY1-deficient mouse, the active transforming growth factor (TGF)-beta protein was increased, and the increased TGF-beta inhibited epithelial and endothelial cell proliferation and led to impaired alveolar development [33]. In the rat kidneys, repeated injection of anti-THY1 antiserum induced chronic glomerulonephritis, in which the elevated TGF-beta was detected [46]. These results therefore suggested that a decrease in THY1 level might increase the active TGF-beta level. In the ovaries, TGF-beta protein is expressed in granulosa cells [47–50] and theca cells [51, 52]. As for the function of TGF-beta in ovaries, it inhibited FSH-stimulated progesterone and E_2 production in bovine granulosa cells [53].

In addition, combined treatment with FSH and TGF-beta increased the number of apoptotic follicles [54]. FSH is known to act as a survival factor to induce granulosa cell proliferation and prevent apoptosis [55–58]. These results indicate that TGF-beta might attenuate FSH-stimulated follicle development and play a role in determining the fate of a developing follicle to atresia. In the present study, THY1 expression was stimulated by FSH in mouse ovaries. Although the relationship among FSH, THY1, and TGF-beta remains unclarified, the hypothesis that periodical expression of THY1 induced by FSH might reduce active TGF-beta and, in turn, that increased THY1 might recruit follicles into growth is fascinating. Supposing that THY1 regulates active TGF-beta level in mouse ovaries, THY1 might be an important factor for determining the fate of follicles in the ovary.

The present data suggest that THY1 might play a role in cell adhesion via binding to integrin beta3 in mouse ovaries. In particular, THY1 may be involved in cell-cell adhesion during formation of the theca cell layer. However, the mechanism of theca cell layer formation is not fully understood. Therefore, to understand the role of THY1 in cell adhesion, the factors that regulate cell adhesion in the theca cell layer and the interactions between theca cells must be further studied. Moreover, elucidating the combined interaction of THY1 with other factors through cell adhesion should result in a fuller understanding about folliculogenesis, especially the determination of follicular fate.

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