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Original Article

Histological Observation of the Development of Follicles and Follicular Atresia in Immature Rat Ovaries

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To clarify the development of follicular growth and atresia in the immature ovary, rats' ovaries and blood were removed at fixed points during the period from 0 to 35 days after birth (Day 0 to Day 35). The ovaries were immunohistochemically examined, and blood concentrations of serum follicle-stimulating hormone (FSH) and estrogen (E) were measured. We investigated how time-course changes in follicular cell proliferation, estrogen receptor β (ERβ), apoptosis, and FSH and E concentrations are connected with follicular growth and atresia. Apoptosis was found in the ova from Day 0 to Day 3. On Day 15, apoptosis occurred in some granulosa cell nuclei in some follicles, but BrdU uptake and the presence of cyclin D2 and ERβ could be observed in other granulosa cells. From Day 17, apoptosis increased in the follicular granulosa cells, and BrdU uptake and the presence of cyclin D2 and ERβ were decreased. Follicular atresia continued, reaching a peak on Day 30. Serum FSH and E concentrations increased until Day 15, then markedly decreased after Day 17. The mechanism of apoptosis in the ova from Day 0 to 3 has not been clarified. However, the onset of follicular atresia was caused by apoptotic degeneration from Days 15 to 17. These results showed that the oocytes were selected by apoptosis at 2 points in the time-course of the maturation of the ovary.

Key words: histology, apoptosis, proliferation, estrogen, follicle-stimulating hormone

The number of germ cells in a 5-month-old human fetus reaches about 7 million, the highest number at any time in the ovary, but decreases to around 2 million at birth, and to some 400,000 in juveniles. Still, the total number of germ cells that an average woman ovulates during her life is only 400 [1]. Specifically speaking, during female reproductive life, most ovarian follicles (more than 99.9%) undergo a degenerative process called atresia at some stage of their development, and only a few follicles reach the ovulatory stage [2].

The development of ovarian follicles is considered to be regulated by various factors such as gonadotropins [3–6], steroid hormones [7, 8], cytokines [9], growth factors *etc.* [10, 11]. The mechanism of growth from primordial into primary follicles has not been clarified, but it is clear that follicle-stimulating hormone (FSH) [6, 12], estrogen, and androgen receptors [13–15] emerge once granulosa cells have become lamellar. The theca cells developing at this time have luteinizing hormone (LH) receptors and produce androgen [6, 12]. The hormone FSH

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changes androgen into estrogen in the granulosa cells. Estrogen increases the number of FSH receptors by working on granulosa cells and enhances the sensitivity of the granulosa cells to FSH [7, 8, 16]. In granulosa cells that cannot quickly change androgen into estrogen, the androgen accumulates, leading to apoptosis in the ova, which in turn causes atresia [17, 18].

As follicles grow, local factors in the ova such as cytokines (IL-1, *etc.*) [9] and various growth factors (EGF, FGF, IGF, *etc.*) *etc.* [10, 11] are produced from granulosa cells and theca cells. These local factors promote or inhibit follicular growth via autocrine and/or paracrine mechanisms related to steroid hormone production.

The expression of Fas mRNA in granulosa cells was first observed by Quirk *et al.* [19]. Numerous oocytes (in the immature period) and granulosa cells are eliminated by apoptosis accompanied by expression of the Fas-Fas ligand system [20, 21]. Atresia occurs in the majority of ovarian follicles from the preantral to early antral stages. The morphological changes that occur in granulosa cells of follicles undergoing atresia match all apoptosis-related morphological characteristics [22]. In addition, internucleosomal DNA fragmentation, which is a hallmark of cells undergoing apoptosis, occurs in atretic follicles [22, 23].

Despite numerous studies on atresia [22, 24], the exact mechanisms that determine whether a follicle will continue to grow or to undergo atresia are still unknown. Moreover, there have been no histochemical studies on how the maturation of immature ovarian follicles is connected with the steroid hormone (estrogen) and follicle stimulating hormone (FSH) concentrations in the sera during in the immature period.

In this study, the time-course changes in follicular development and atretic formation in the ovaries were immunohistochemically observed (expression of BrdU uptake, cyclin D2, Fas, TUNEL, estrogen receptor α and β (ERα and β)) using the respective antibodies, and those of FSH and estrogen concentrations in the sera were measured by the ELIAS method in immature female rats (0 to 35 days after birth (Days 0 to Day 35)). These experiments were done to examine how FSH and estrogen concentrations in the sera affected the follicular development and atretic formation.

Materials and Methods

All experiments with animals were performed according to the guidelines for Animal Experiments of Okayama University.

Animals and materials. Pregnant Wistar rats purchased from Charles River Japan, Inc. (Yokohama, Japan) or born in our laboratory were used. The animals were housed under controlled lighting (14L: 10D) and temperatures and had free access to rat chow and tap water.

The reagents used in this study were Mayer's hemalum solution (Merk, Darmstadt, Germany); a 4% paraformaldehyde and 1% eosin Y solution (Muto Pure Chemicals, Tokyo, Japan); the Apoptosis *in situ* Detection Kit "Wako" (Wako Pure Chemical Industries, Ltd., Osaka, Japan), anti-mouse or anti-rabbit immunoglobulins conjugated to a peroxidase-labeled dextran polymer (EnVision+), anti-5-bromo-2'-deoxyuridine (BrdU), a 3,3'-diaminobenzidine (DAB) substrate kit, and pentobarbital sodium and Block Ace™ (skim milk) (Dainippon Pharmaceutical, Osaka, Japan); anti-cyclin D2 and estrogen receptor α (ERα) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); estrogen receptor β (ERβ) antibody (Affinity BioReagents, Golden, CO, USA); Fas antibody (BD Pharmingen, San Diego, CA, USA); Histofine Simple Stain Rat MAX-PO (M) (NICHIREI CORPORATION, Tokyo, Japan); Tween® 20 (Sigma-Aldorich, St. Louis, MO, USA); a Rat FSH ELISA kit (Biocode-Hycel, Liege, Belgium); and an estrogen (E1, E2, E3) ELISA kit (Japan EnviroChemicals, Tokyo, Japan).

Reagents of the best quality were purchased in addition to those mentioned above.

Tissue preparation. Rats were divided into 11 groups according to days after birth. The day of birth was considered Day 0. Rats were killed on Days 0, 3, 5, 7, 10, 15, 17, 20, 25, 30 and 35 (5 rats each; 55 in total). On each of these days, the animals were anesthetized with 0.5% pentobarbital, and the ovaries were quickly removed. The left ovary was immersed and fixed in 4% paraformaldehyde at 4 °C for 24 h, dehydrated according to standard procedures, and subsequently embedded in paraffin. To study the cellular proliferation of follicles, BrdU (50 mg/kg) dissolved in 1 ml of 10 mM phosphate-buffered saline (pH 7.0) (PBS) was intraperitoneally

administered 2 h before the follicles were removed. The removed ovaries were embedded in paraffin, and each paraffin block was cut into 5 μm-thick sections which were mounted on silane-treated glass slides. Tissue sections were prepared from 5 rats of each group.

Morphological and immunohistochemical study. Arbitrarily selected sections were dewaxed and subjected to hematoxylin and eosin (HE) staining. For detecting cyclin D2, ERα, ERβ and BrdU uptake, arbitrarily selected sections were dewaxed and immersed in 3% H_2O_2/methanol at room temperature (RT) for 10 min to dispose of the intrinsic peroxidase, and autoclaved for 15 min at 121 °C in a 10 mM citrated buffered solution (pH 6.0). After sections had cooled at RT, nonspecific binding of antibodies was blocked by applying PBS containing 5% skim milk to the sections for 10 min at RT. Two of the 4 sections were treated with anti-ERα (1:500) and β (1:200) antibodies for 1 h at RT. The other 2 sections were treated with anti-cyclin D2 (1:200) or with anti-BrdU (1:10) antibodies for 30 min at RT. These sections were washed 3 times with 0.1% Tween® 20/PBS for 5 min. EnVision + (rabbit) for detecting ERα and β, and cyclin D2, or EnVision + (mouse) for detecting BrdU uptake was applied to each section for 20 min at RT and used as a secondary antibody. Adult rat ovaries were used to test for a positive or negative reaction to cyclin D2 or ERα and β antibodies, and adult rat small intestines were used to test for a positive or negative reaction to BrdU uptake.

Arbitrarily selected sections were utilized to examine apoptotic cells (DNA fragmentation) in the ovary using terminal deoxyribonucleotidyl transferases (TdT) and deoxyuridine triphosphate-biotin nick end labeling (TUNEL) (Apoptosis *in situ* Detection Kit *wako*). The detection was carried out according to the instructions provided with the kit. The tissue sections were dewaxed in xylene, dehydrated in a graded series of ethanol solutions, and immersed in TdT for 15 min at 37 °C after treatment with a protein digestion enzyme (proteinase K 20 mg/l) at 37 °C. After 3 washes with PBS and inhibition of the endogenous peroxidase activity with 3% H_2O_2 for 5 min at RT, tissue sections were incubated with a peroxidase-conjugated antibody for 10 min at 37 °C. Peroxidase activity was visualized by incubating the sections with DAB/DAB enhancer solution for 5 min at RT. As a control, some of the sections were reacted without biotinylated 16-dUTP. Counterstaining was performed with kernechtrot. Adult rat small intestines were used to test for a positive or negative reaction to TUNEL.

For the detection of Fas, remaining sections were dewaxed and immersed in 3% H_2O_2/methanol for 10 min at RT, and nonspecific binding of antibodies was blocked with PBS containing 5% skim milk for 10 min at RT. Anti-Fas/CD95/APO-1 mAb antibody (1:200) was applied to the sections for 2 h at RT and used as the primary antibody. Then Histofine Simple Stain Rat MAX-PO(M) was applied as the secondary antibody. The spleen was used to test for a positive or negative control reaction to Fas. The sections were treated with the secondary antibody using a DAB substrate kit for 5 min at RT to cause the target cells to emit colors.

Classification of follicles and atretic follicles in immature rats. Variously stained sections were observed under an Axioplan Zeiss microscope with an ×10, ×20 or ×40 Plan Neofluar Lens. Photos taken with an AxioCam CCD camera were saved in the tagged-image files format (TIFF) at a size of 1300 × 1030 pixels (~3.8MB). All follicular sizes (diameter) (in the cross-section containing the oocyte nucleolus) were determined by measuring 2 perpendicular axes (diameters at right angles) using an analysis (scaling tools) system linked to the AxioCam CCD camera.

The follicular sizes were classified into 8 classes by a partially modified classification of Gaytán *et al.* [25] as follows: Primordial follicles, showing one layer of flattened pregranulosa cells surrounding the oocyte; Unilaminar primary follicles, showing at least one single enlarged pregranulosa cell; Multilaminar class a (Ma) follicles, showing 1–3 layers of granulosa cells and having a diameter of up to 75 μm; Multilaminar class b (Mb) follicles, measuring from 76 to 150 μm in diameter; Multilaminar class c (Mc) follicles, 151 to 200 μm in diameter; Multilaminar class d (Md) follicles, 201 to 274 μm in diameter; and Multilaminar class e (Me) follicles, having a diameter larger than 275 μm. In this study, follicular changes were observed in Ma to Me follicles.

The class Ma~Me follicular numbers were calculated with HE-stained ovarian sections prepared from

the removed ovaries of rats. The number of each class follicle (Ma～Me) was expressed as incidence (%) obtained by calculating the ratio of each class Ma～Me follicular number to that of the total follicles in each ovarian section. Five ovarian pieces were taken from 5 different parts of the rat ovary at each sampling time. As 5 rats were used at each time point and the sampling was performed 11 times, 275 ovarian sections were prepared ($5 \times 5 \times 11$). The numbers of follicles counted were: Day 5 (5d), 1127; 7d, 962; 10d, 1016; 15d, 1091; 17d, 1079; 20d, 1149; 25d, 953; 30d, 1261; and 35d, 1016. The data are expressed as means ± SD values of the follicular numbers at each measuring time. The mean values of the results obtained by ANOVA and Tukey's tests are expressed as percentages.

Atretic follicles were classified according to Osman [26] as follows: Stage I, degenerative changes are present only in the granulosa wall which shows cell shrinkage, pyknosis, and karyorrhexis. At this stage the follicles are still spherical or ovoid. Stage II, changes are also present in the oocyte which shows signs of the resumption of meiosis, such as the breakdown of the nuclear membrane with or without the formation of a pseudomaturation spindle, and oocyte fragmentation. In antral follicles, the oocytes are able to resume meiosis spontaneously after the granulosa cells degenerate. At this stage, most follicles show deformities.

To detect atretic follicles, HE-stained ovarian sections prepared for calculating the incidence of class Ma～Me follicles were used. Incidence of atretic follicles was obtained by calculating the ratio of the number of stage I and stage II atretic follicles classified according to Osman's atretic classification to that of ovarian follicles in 275 ovarian sections each.

Localization of BrdU uptake. To find the BrdU uptake (labeling) index as a percentage of labeled cells among granulosa cells or theca layer cells, the numbers of labeled immunostained cells and unlabeled cells stained with hematoxylin were counted. The numbers of labeled and unlabeled cells were estimated by counting small areas and then multiplying by the total area of the section according to Gaytán *et al.* [25]. The total number of cells was calculated by adding the number of labeled cells to the number of unlabeled cells, and then the percentage of labeled cells among the granulosa cells or theca layer cells was estimated by dividing the number of labeled cells by the total number of cells. The labeling index was used to determine the numbers of class Ma to Me follicles at each of the time points that ovaries were removed. At each sampling time, 2 follicles taken from each of 2 sections prepared from each of 5 rats each (20 follicles in total) were used. The data are expressed as means ± SD.

Analysis in serum FSH and estrogen values. With the removal of ovaries, blood was collected under pentobarbital anesthesia from the left ventricle. Sera were obtained after centrifuging (× 3,000 rpm, 4 °C, 10 min) the blood, and was stored at −20 °C until FSH or estrogen values were measured. FSH and estrogen values in rats from Day 5 to Day 35 were measured in the sera collected from 5 rats using FSH, and Estrogen (E1, E2, E3) ELISA kits.

The measuring limits for the FSH and estrogen ELISA kits are 50 ng/serum ml and 3 ng/ml serum, respectively, so actual measurements were done by diluting the serum 50 times for FSH and 10 times for estrogen ELISA. FSH and estrogen concentrations in the blood measured with a measuring kit had less than a 10% coefficient of variation.

Statistical analysis. Results are expressed as the means ± SD. Statistical analyses were performed to obtain the mean values of the results calculated by ANOVA and Tukey's tests. The values were considered significant when the p value was less than 0.05.

Results

Development of follicles. The development of follicles in the ovary was examined using immature rats (Days 0 to 35) and HE staining (data not shown), and the follicles were classified into 5 size classes by a partially modified version of the system of classification of Gaytán *et al.* [25] (Fig. 1).

On Day 0, groups of several oocytes were enveloped in stromal cells forming cylindrical pocket-like structures [27]. On Day 3, pregranulosa cells began to envelop each oocyte to make follicles ranging from primordial to primary. Then, in the central area of the ovary, several pregranulosa cells grew into granulosa cells. On Day 5, in the central area of the ovary, the granulosa cells of larger follicles increased to form 2–3 layers, and theca cells began to surround the growing follicles. Until Day 5, most follicles were

of the Ma class, and on Day 5, class Mb follicles developed in the central area of the ovary. On Day 7, the number of class Ma follicles significantly decreased and those of class Mb follicles significantly increased. On Day 10, the number of class Ma follicles significantly decreased while that of class Mb follicles was significantly greater than on Day 7 and in fact peaked; meanwhile, in the central area of the ovary, class Mc follicles developed and began to show formation of follicular antra. On Day 15, most follicles in the ovary were of the Mb class and in the central area of the ovary, class Mc and Md follicles with antra had developed. No pyknosis was observed in granulosa cells during the period between Day 5 and Day 15 with HE staining.

On Day 17, the numbers of class Ma and Mb follicles were significantly decreased, and class Mc and Md follicles were more numerous than on Day 15. The number of class Mc follicles peaked at Day 17 and then decreased. Class Me follicles developed, and some of the granulosa cells in several follicles began to be eliminated due to pyknosis. On Day 20, in some follicles, follicular granulosa cells began to be eliminated, and corona radiate cells were disarranged around the oocyte. On Day 25, pyknosis of granulosa cells was seen in various follicles, and follicles were seen to be undergoing atresia. In some follicles, the granulosa cell layers became thinner. Very few class Ma follicles were observed, and the numbers of class Md and Me follicles were greatest (approximately 25% and 35% of all follicles, respectively) in the immature period. After Day 30, numerous primary, class Ma~Me follicles and atretic follicles were observed in the ovary. In the atretic follicles, granulosa cell layers became thinner, and the antra grew larger in those follicles from which the oocyte had completely disappeared.

Detection of apoptosis, and development of atretic follicles. Detection of apoptosis was immunohistochemically observed using the anti-TUNEL antibody and the anti-Fas antibody from Day 0 to Day 35 in the ovary of rats (Fig. 2: TUNEL

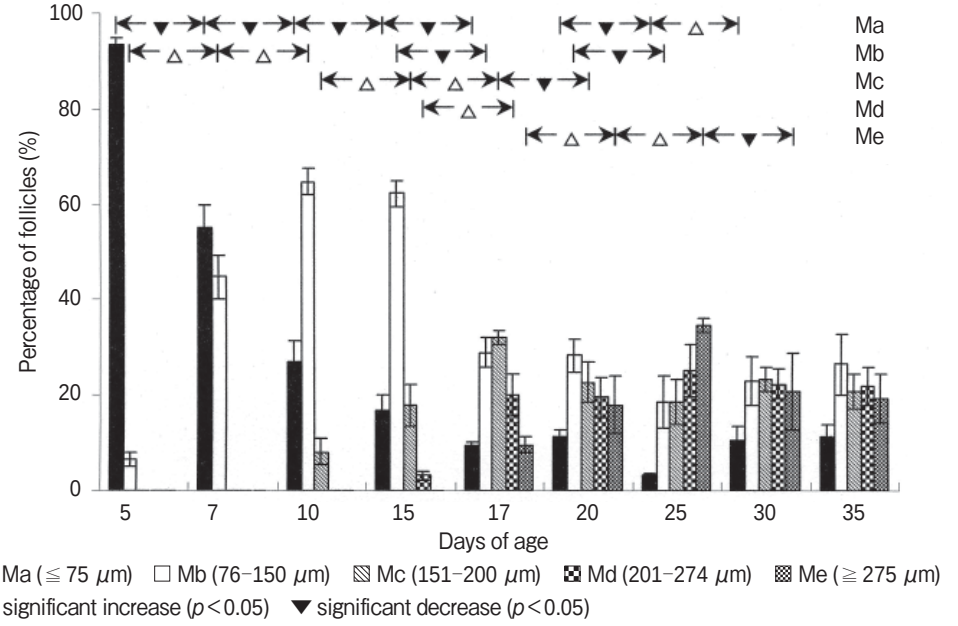

Fig. 1 Follicular maturity grades from Day 5 to Day 35 in rats.
Details of the Ma~Me follicular classification are given in the text. On Day 5, most follicles were of the Ma class. On Day 10, the number of class Ma follicles significantly decreased in number while the number of class Mb follicles peaked and class Mc follicles also developed. On Day 15, class Ma follicles significantly decreased in number, and class Mb follicles slightly decreased compared to Day 10; meanwhile, class Md follicles developed. On Day 17, the numbers of class Ma and Mb follicles significantly decreased while those of class Mc and Md follicles increased from their Day 15 levels; meanwhile, class Me follicles developed. On Day 25, the number of class Ma follicles observed was very few, but the number of class Md and Me follicles reached a peak.

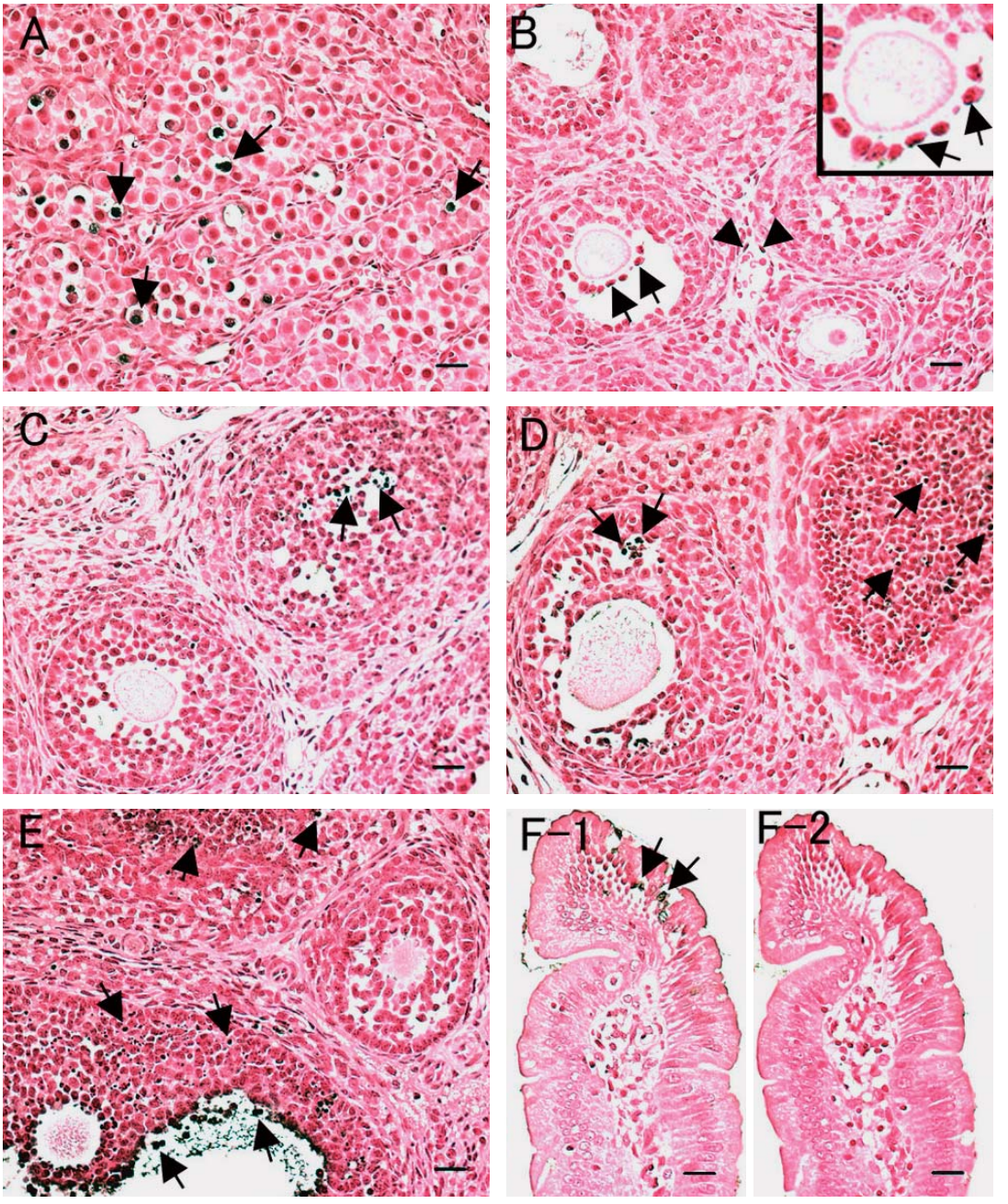

Fig. 2 Detection of cells reacting positively to anti-TUNEL antibody in various developmental stages from Day 0 to Day 35 in rats. Photos **A**, **B**, **C**, **D** and **E** show images of follicles on Day 0, 15, 17, 20 and 25, respectively. On Day 0, numerous oocytes were positively stained with the anti-TUNEL antibody (TUNEL) (arrows). On Day 15, some granulosa cell nuclei in some follicles (arrows) and interstitial cells (arrow heads) reacting positively to TUNEL began to be formed. The photo in the right upper portion shows the results of some granulosa cell nuclei reacting positively to TUNEL. From Day 17, the early stage (Stage I) of atresia [26] began to be found. Numerous granulosa cells showed a positive reaction to TUNEL (arrows). After Day 20, Stage II atresia [26] began to be found. Numerous granulosa cells in the larger atretic follicles showing a positive reaction to TUNEL were observed (arrows). An image of the positive control of proliferating cells in control rats reacting to TUNEL in the adult rat's small intestine (**F-1**) (arrows) and an image of the negative control of the photo (**F-2**) are shown. (Magnification: **A**, **B**, **C**, **D**, **E** and **F**, bar = 20 μm)

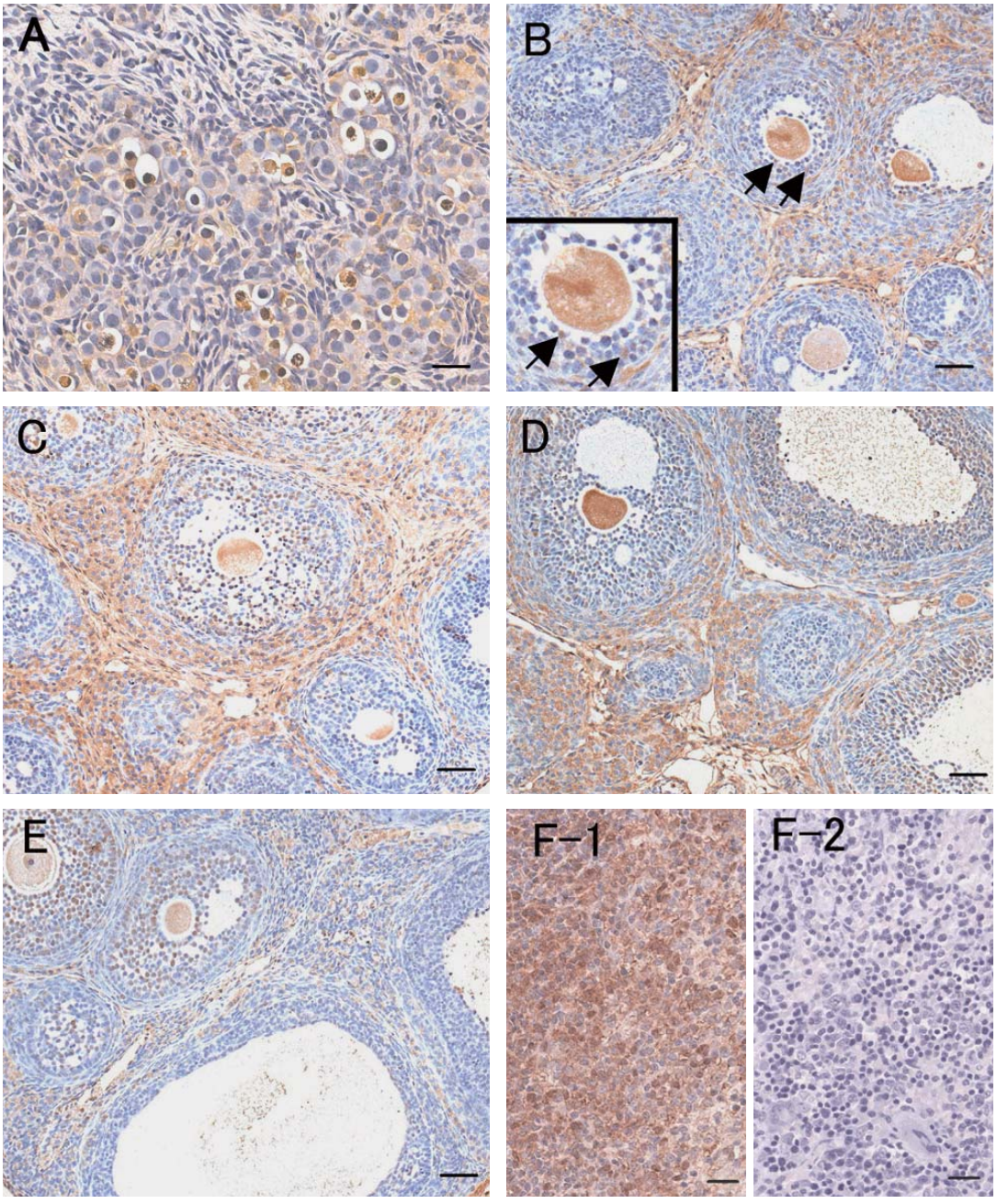

Fig. 3 Detection of cells reacting positively to anti-Fas antibody in various developmental stages from Day 0 to Day 35 in rats. Photos **A**, **B**, **C**, **D** and **E** show the images of follicles on Day 0, 15, 17, 20 and 25, respectively. On Day 0, numerous oocytes showed a positive reaction to the anti-Fas antibody (Fas) (dark brown). From Day 5 to Day 35, numerous oocytes and theca cells in each follicle showed a strong positive reaction to Fas (dark brown). On Day 15, some positive reactions to Fas in granulosa cells began to be found in some follicles (arrows). The photo in the left under portion shows the results of some granulosa cells reacting positively to Fas. From Day 17 to Day 35, granulosa and theca cells of some larger follicles (Mc～Me) in Stage I atresia showed a positive reaction to Fas. In stage II atretic follicles which had thinner granulosa cell layers, the granulosa cells showed a negative reaction to Fas. The adult rat's spleen was used to test for positive (**F-1**) and negative (**F-2**) control reactions to Fas. (Magnification: **A** and **F**, bar = 20 μm; **B**, **C**, **D** and **E**, bar = 50 μm)

reaction, and Fig. 3: Fas reaction).

On Day 0, numerous oocytes forming groups of cylindrical pocket-like structures enveloped with stromal cells showed a positive reaction to TUNEL, and these cells were restricted to the medullary region. However, from Day 5 to Day 10, neither oocytes nor granulosa cells in the ovary showed any labeling. On Day 15, some granulosa cell nuclei in some follicles and interstitial cells reacting positively to TUNEL began to be formed. On Day 17, evidence of pyknosis began to be clearly found in granulosa cells and interstitial cells in some ovarian follicles. These granulosa cells showed a positive reaction to TUNEL. These findings are consistent with the early stage of (Stage I) atresia according to Osman [26]. After Day 20, pyknosis occurred in numerous granulosa cells in many larger atretic follicles, and these cells were observed to show a positive reaction to TUNEL (Stage II atresia [26]).

On Days 0 to 3, several oocytes enclosed with stromal cells in cylindrical pocket-like structures showed a positive reaction to anti-Fas antibody (Fas). From Day 5 to Day 35, primordial follicles began to grow from primary to Me class follicles, and numerous oocytes and theca cells in each follicle showed a strong positive reaction to Fas. From Day 5 to 15, the granulosa cells in follicles showed no positive reaction to Fas. On Day 15, granulosa cells reacting positively to Fas began to be formed in some follicles. From Day 20 (Fig. 4D) to Day 35, some larger follicles showed Stage I atresia. In stage II atretic follicles which have thinner granulosa cell layers, the granulosa cells showed no reaction to Fas.

Fig. 4 shows the incidence of atretic development obtained by calculating the ratio of follicles in atresia to all the follicles classified according to Osman [26] using the data from 275 HE-stained sections. At this time TUNEL and Fas-stained sections were also observed as a reference to atretic development. As shown in Fig. 4, development of stages I and II follicular atresia began to be found from Day 17 and Day 20, respectively. Stage I follicular atresia was found significantly more than stage II atresia until Day 25. At that point, stage I atresia began to decline whereas stage II follicular atresia kept increasing until Day 35.

Detection of BrdU uptake and cyclin D2. The immature ovary was immunohistochemically stained with anti-BrdU antibody (Fig. 5). On Days 0 to 3, BrdU uptake was observed in the epithelial cells enveloping several oocytes. On Day 5 to Day 35, BrdU uptake was observed in granulosa cells in the primary and class Ma～Me follicles and in the theca cells surrounding each healthy follicle. On Day 17, BrdU uptake was observed in granulosa and theca cells in healthy follicles, but in several follicles, BrdU uptake was not observed, nor was it observed in some of the granulosa cells in which pyknosis had begun to occur, nor in some of the theca cells. On Day 20, BrdU uptake was observed in granulosa and theca cells in healthy follicles; however, in follicles in which numerous granulosa cells had been undergoing pyknosis (causing atretic follicles in the ovary), BrdU uptake was not found either in the granulosa cells or in the theca cells around the follicles. Moreover, on Days 25 to 30, in the atretic follicles, no BrdU uptake was found either in the granulosa cells or in the theca cells.

As follicles grew from Day 10 to Day 25, the dynamics of BrdU uptake in follicular cells was quantitatively measured according to Gaytán *et al.* [25]. As a result (Table 1), from Day 10 to Day 25, the indices (%) of BrdU uptake of granulosa cells in fol-

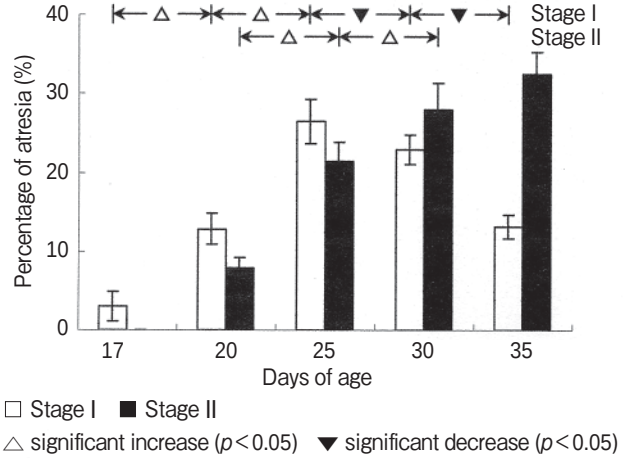

Fig. 4 Incidence of atretic follicles from Day 17 to Day 35 in rats. Details of the classification of stage I and stage II atresia [26] and of the calculation methods are given in the text. From Day 17, stage I atretic follicles began to appear, reaching a peak on Day 25, but decreasing after that. Stage II atretic follicles began to appear from Day 20 and kept growing until Day 35.

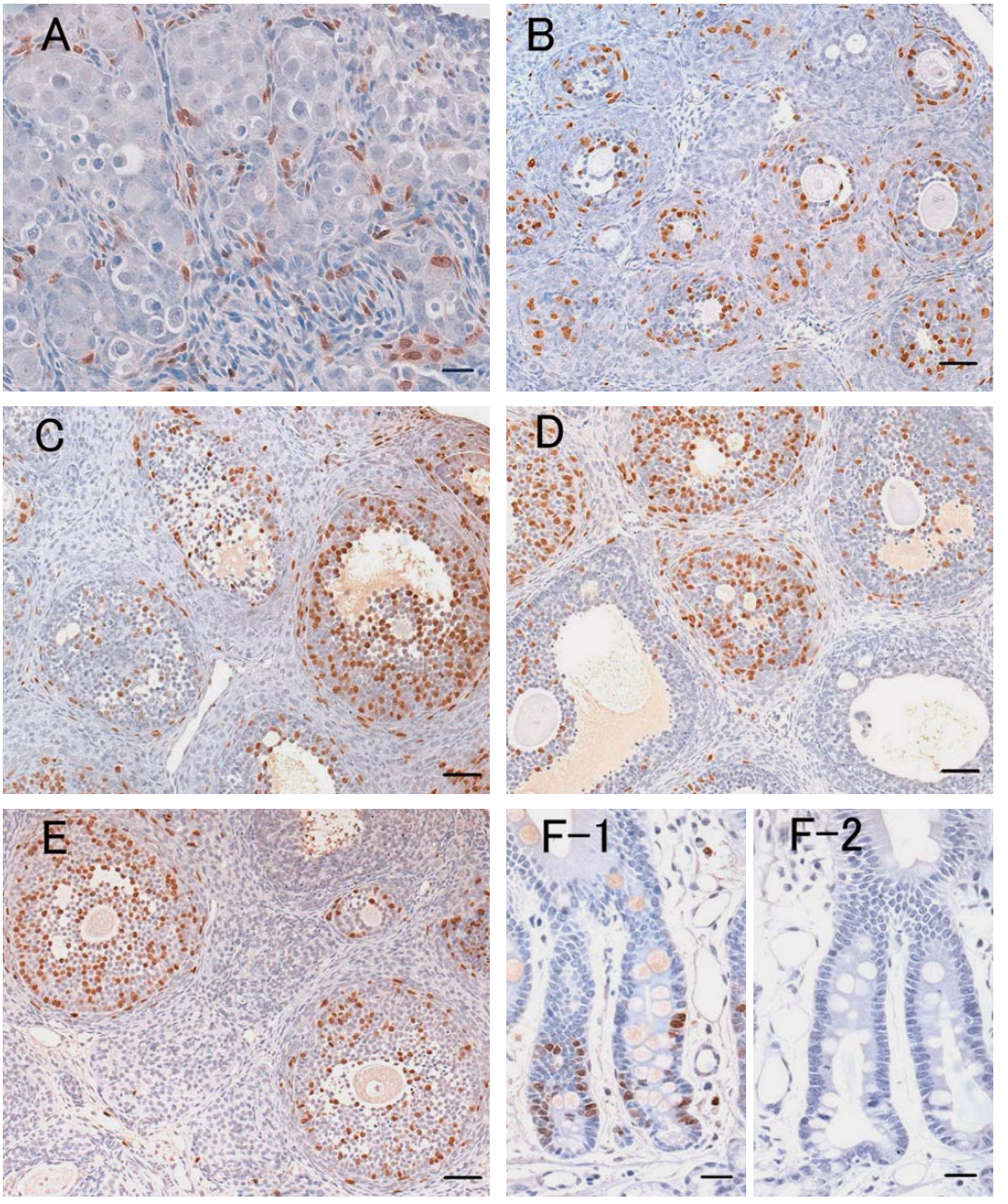

Fig. 5 Images of BrdU uptake from Day 0 to Day 35 in rats.
Photos **A**, **B**, **C**, **D** and **E** show images of follicles on Day 0, 15, 17, 20 and 25, respectively. On Days 0 to 3, BrdU uptake was observed in the epithelial cells enveloping several oocytes. From Days 5 to 30, primary to class Me follicles developed, and BrdU uptake was observed in the granulosa and theca cells in healthy follicles. However, from Day 17 to 30, BrdU uptake was not observed either in some granulosa cells in which pyknosis had begun to occur or in theca cells in several follicles. The adult rat's intestine was used to examine positive (**F-1**) and negative (**F-2**) reactions to anti-BrdU antibodies. (Magnification: **A** and **F**, bar = 20 μm; **B**, **C**, **D** and **E**, bar = 50 μm)

Table 1 Labeling index (%) of BrdU uptake

Granulosa cells		10 days	15 days	17 days	20 days	25 days
Ma (≦ 75 μm)	(n = 20)	11.05 ± 1.50	11.14 ± 1.58	11.27 ± 1.28	11.51 ± 1.21	11.15 ± 1.65
Mb (76–150 μm)	(n = 20)	16.32 ± 1.65a	16.01 ± 1.98a	16.21 ± 2.03a	16.66 ± 2.27a	16.48 ± 1.93a
Mc (151–200 μm)	(n = 20)	19.64 ± 1.61a	19.56 ± 1.53a	20.16 ± 1.80a	20.51 ± 1.44a	20.41 ± 1.66a
Md (201–274 μm)	(n = 20)	–	23.38 ± 1.43a	23.31 ± 1.33a	23.59 ± 1.19a	23.39 ± 1.23a
Me (≧ 275 μm)	(n = 10)	–	–	33.34 ± 1.90a	33.74 ± 1.96a	33.79 ± 1.46a
Atretic follicles						
Stage I （≧ 275 μm)	(n=10)	–	–	17.61 ± 1.94b	17.98 ± 1.58b	17.99 ± 1.16b

Theca cells		10 days	15 days	17 days	20 days	25 days
Ma (≦ 75 μm)	(n = 20)	5.51 ± 4.18	5.26 ± 4.45	6.08 ± 3.97	6.20 ± 4.11	6.19 ± 4.10
Mb (76–150 μm)	(n = 20)	10.40 ± 2.30	10.46 ± 2.67	10.44 ± 1.54	10.49 ± 1.46	10.65 ± 1.81
Mc (151–200 μm)	(n = 20)	12.37 ± 1.43	12.44 ± 1.48	11.43 ± 1.56	11.58 ± 1.35	11.58 ± 1.36
Md (201–274 μm)	(n = 20)	–	13.34 ± 1.59	13.39 ± 1.74	13.47 ± 1.10	13.51 ± 1.19
Me (≧ 275 μm)	(n = 10)	–	–	15.89 ± 1.38	15.79 ± 1.89	15.97 ± 1.11
Atretic follicles						
Stage I （≧ 275 μm)	(n = 10)	–	–	8.82 ± 1.46b	9.02 ± 1.30b	9.04 ± 1.13b

a: $p < 0.05$ compared among different follicular classes, b: $p < 0.05$ compared to the healthy Me class follicles.

licles were found to have significantly increased in number as compared between follicles of different sizes of each class (Ma to Me) on each day, but theca cells did not significantly increase. However, from Day 10 to Day 25, the index (%) of BrdU uptake was not significantly different between follicles of the same sizes of each class (Ma to Me), either in granulosa or theca cells. However, after Day 17, in class Me follicles undergoing atresia, both granulosa and theca cells showed less BrdU uptake than did those of healthy follicles.

The immature ovary was immunohistochemically stained with anti-cyclin D2 antibody (cyclin D2) (Fig. 6). On Days 0 to 3, oocytes and stromal cells showed a very weak positive reaction and epithelial cells enveloping oocytes showed a positive reaction to cyclin D2. On Days 5 to 15, the granulosa cells in the primordial, primary, and class Ma～Md follicles strongly reacted to cyclin D2. Theca cells showed a very weak reaction to cyclin D2. On Day 17, the granulosa cells in healthy larger follicles showed a strong positive reaction to cyclin D2, and oocytes and theca cells showed a weak positive reaction. However, some pyknosis began to occur in granulosa cells in several class Mc～Me follicles (atretic follicles) showing a weak reaction. On Day 20, in some class Mc～Me follicles, pyknosis began to occur more extensively in granulosa cells, and a proportion of these cells showed no reaction to cyclin D2. In large follicles, negatively reacted granulosa cells began to be observed opposite the cumulus in the follicles. From Days 25 to 30, the granulosa cells in the healthy follicles showed a positive reaction to cyclin D2, but oocytes and granulosa cells of the atretic follicles showed no staining with cyclin D2.

Detection of ERβ and ERα. The immature ovary was immunohistochemically stained with anti-ERβ antibody (ERβ) (Fig. 7). On Days 0 to 3, numerous oocytes showed a very weak positive reaction while epithelial cells enveloping oocytes showed a positive reaction to ERβ. On Days 5 to 15, the granulosa cells of the primordial, primary, and class Ma～Md follicles showed a strong positive reaction to ERβ (Fig. 7B, arrows), while oocytes and theca cells showed a weak reaction. On Day 17, some of the granulosa cells in some primordial, primary and class Ma～Me follicles showed a negative reaction to ERβ (Fig. 7C, arrowheads). Oocytes and theca cells showed a weak positive reaction. On Day 20, a number of granulosa cells in some class Mc～Me follicles

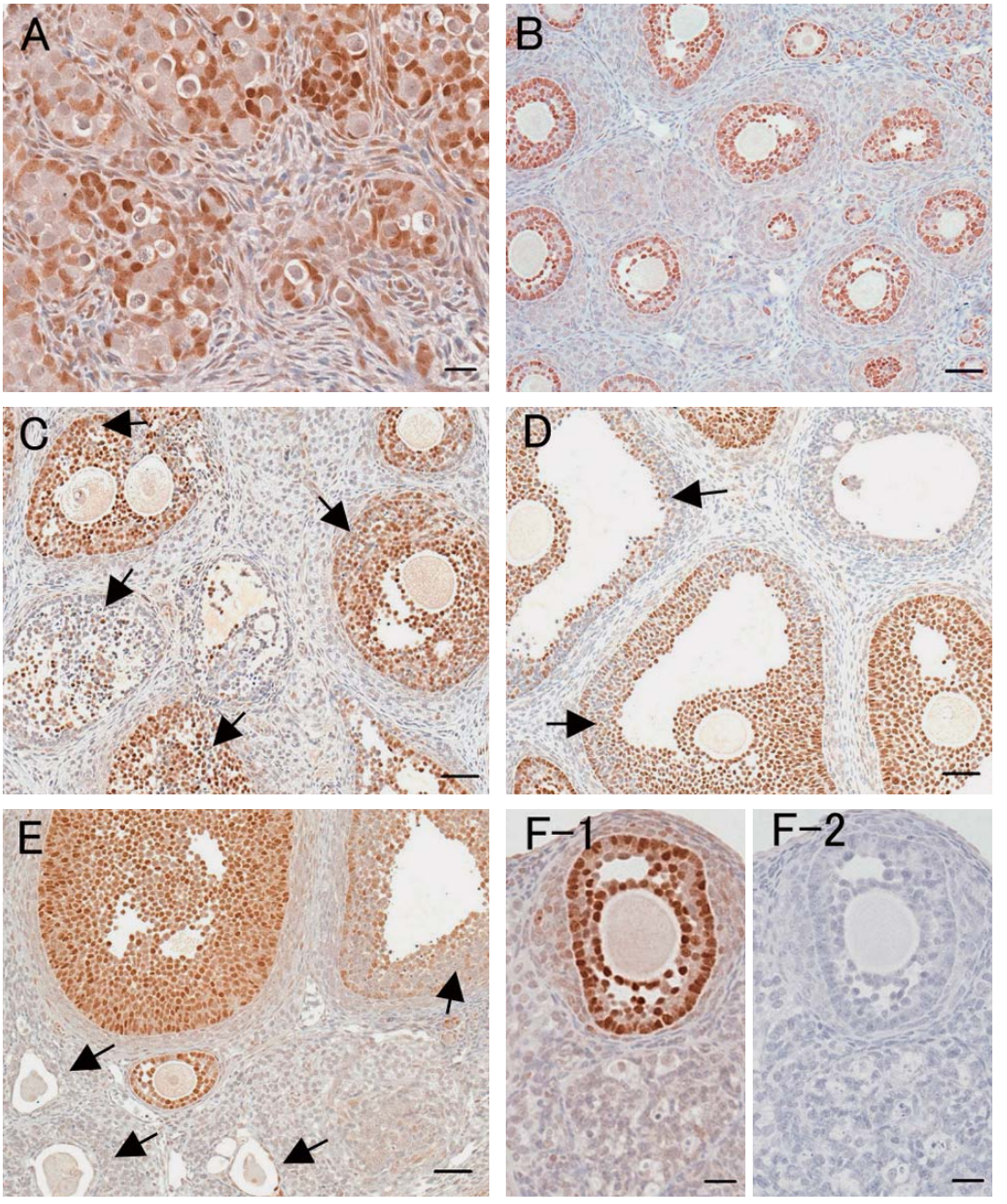

Fig. 6 Detection of the anti-cyclin D2 antibody from Day 0 to Day 35 in rats.
Photos **A**, **B**, **C**, **D** and **E** show images of follicles on Day 0, 15, 17, 20 and 25, respectively. On Days 0 to 3, oocytes and stromal cells showed a very weak positive reaction while epithelial cells enveloping oocytes showed a positive reaction to the anti-cyclin D2 antibody (cyclin D2). From Days 5 to 35, the granulosa cells of the primordial, and primary to class Me follicles showed a strong positive reaction to cyclin D2, and theca cells showed a very weak positive reaction. However, on Day 17, some granulosa cells in the several class Mc～Me follicles showed a weak positive reaction to cyclin D2 (arrows). On Day 20, in some class Mc～Me follicles, a number of granulosa cells, oocytes and theca cells were observed to show negative reactions to cyclin D2. Some negatively reacted granulosa cells began to be observed opposite the cumulus in the follicles (arrows). Oocytes and theca cells showed a weak positive reaction. From Day 25, oocytes and granulosa cells of the atretic follicles showed negative staining with cyclin D2 (arrows). Adult rat ovaries were used for testing positive (**F-1**) and negative (**F-2**) control reactions to cyclin D2. (Magnification: **A** and **F**, bar = 20 μm; **B**, **C**, **D** and **E**, bar = 50 μm)

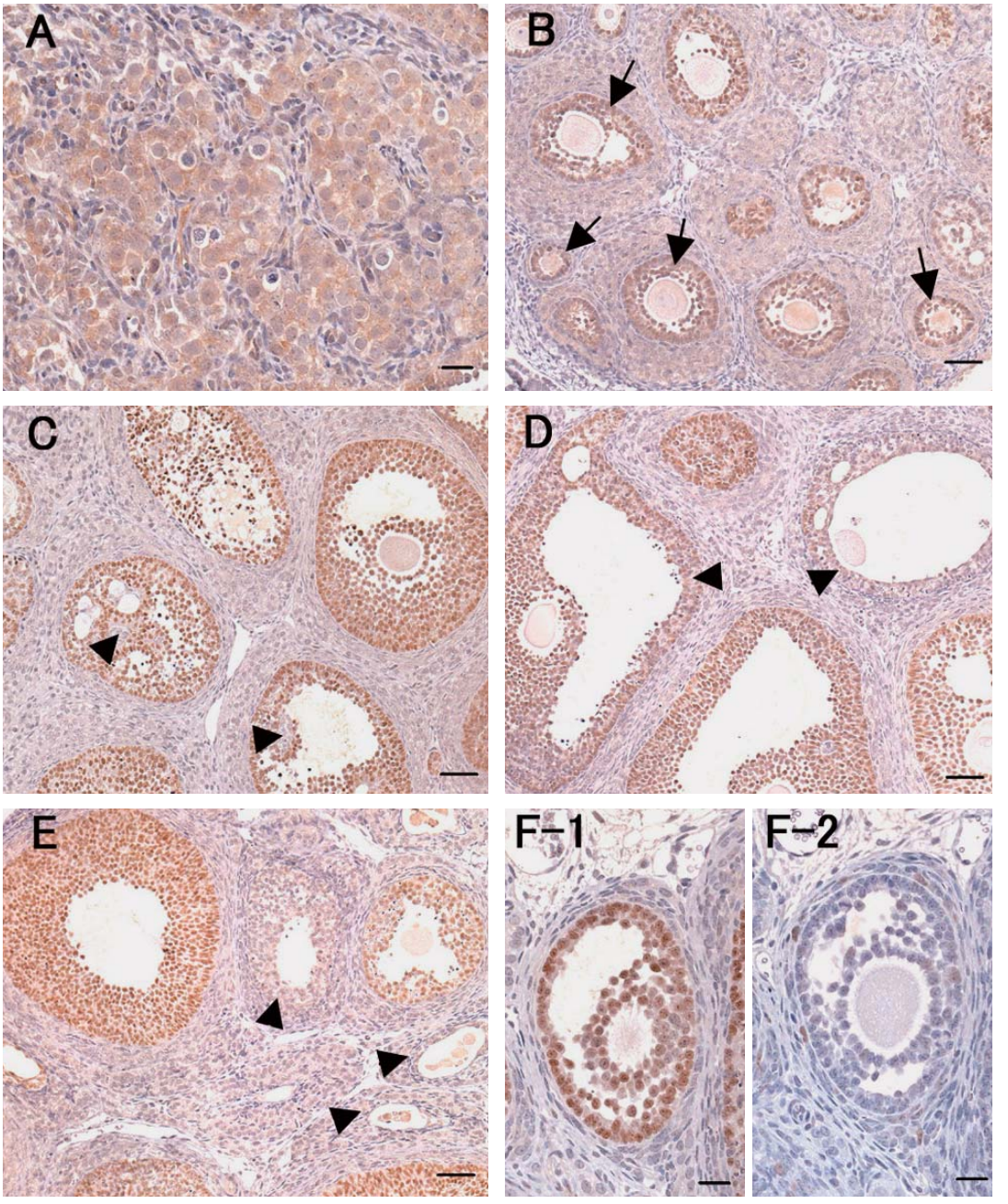

Fig. 7 Detection of the anti-estrogen receptor β antibody (ERβ) from Day 0 to Day 35 in rats.
Photos **A**, **B**, **C**, **D** and **E** show images of follicles on Day 0, 15, 17, 20 and 25, respectively. The time-course changes of the reaction to ERβ in granulosa cells were similar those of cyclin D2. Details of the time-course changes in reaction to ERβ are given in the text. Arrows and arrowheads in photos indicate positive and negative reactions to ERβ, respectively. Adult rat ovaries were used for testing positive (**F-1**) and negative (**F-2**) control reactions to ERβ. (Magnification: **A** and **F**, bar = 20 μm; **B**, **C**, **D** and **E**, bar = 50 μm)

and in atretic follicles showed a negative reaction to ERβ (Fig. 7D, arrowheads). Oocytes and theca cells showed a weak positive reaction. On Days 25 to 30, in the healthy follicles, granulosa cells showed a positive reaction to ERβ, but granulosa cells of the atretic follicles showed a negative reaction. Oocytes and theca cells showed a weak positive reaction to ERβ (Fig. 7E, arrowheads).

The staining dynamics of ERβ were very similar to those of cyclin D2 for granulosa cells, and theca cells were stained with ERβ but not stained with cyclin D2. ERα was observed only in theca cells and not in granulosa cells in the follicles from Day 0 to Day 35 (Data not shown).

Serum FSH and estrogen concentrations. As shown in Fig. 8 (left scale), the serum FSH concentration significantly increased from 738 to 1,050 ng/ml from Day 5 to Day 10 and reached a peak on Day 15 (1,061 ng/ml); however, on Day 17 it decreased significantly to 695 ng/ml compared with those of Day 15. After Day 20, the serum FSH concentration significantly and markedly decreased to under 256 ng/ml.

As shown in Fig. 8 (right scale), the serum estrogen concentration significantly increased from 542 to 854 pg/ml between Day 5 and Day 10 and reached a peak (1,262 pg/ml) on Day 15. On Day 17, the serum estrogen concentration significantly decreased (816 pg/ml) compared with that on Day 15; after Day 20, it decreased to under 560 pg/ml.

Discussion

There have been no attempts to clarify the regulatory mechanism for oocyte and follicular maturation in the immature ovary. In this study, the authors immunohistochemically examined the time-course of dynamic changes in follicular maturation and atresia of immature female rats from Day 0 to Day 35, and clarified that oocytes in the ovary were selected by apoptotic degeneration twice during the immature period (Days 0 to 3 and Days 15 to 17).

In the immature ovary (Fig. 1), changes in follicular size were found on Days 0 to 3, when most follicles were smaller than class Ma follicles (under 75 μm in diameter) and from Day 5 to Day 15, when the

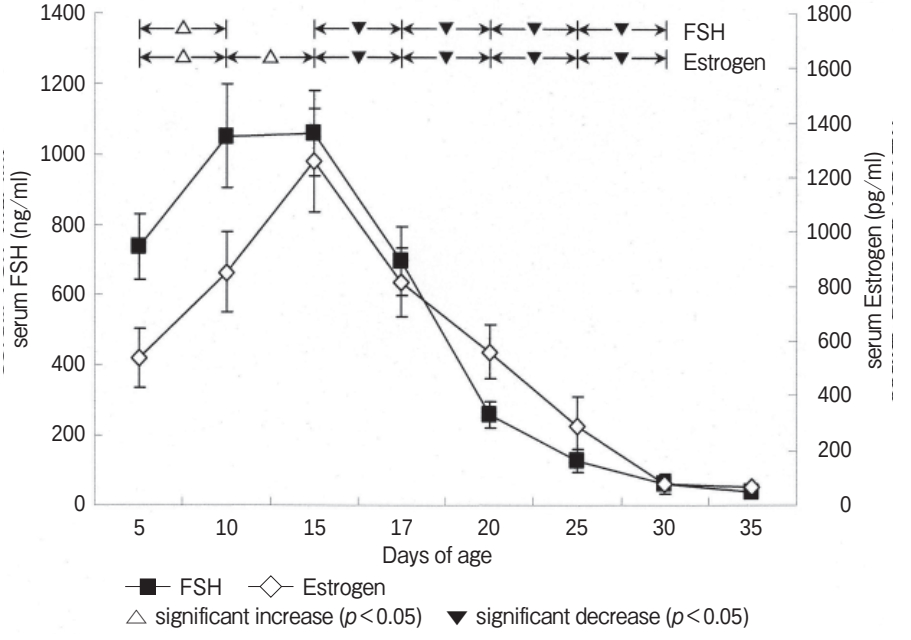

Fig. 8 Changes of serum FSH and estrogen concentrations from Day 5 to Day 35 in rats.
The serum FSH concentrations significantly increased from Day 5 to Day 15; after that, serum FSH concentrations markedly decreased. Serum estrogen concentrations significantly increased from Day 5 to Day 10 and reached a peak on Day 15. After Day 17, serum estrogen concentrations markedly decreased.

number of class Mb follicles (from 76 to 150 μm in diameter) expanded, reaching a peak on Day 10. However, on Day 17, class Mb follicles were significantly less numerous than on Day 15. From Day 15, class Md (from 201 to 274 μm in diameter) and Me (larger than 274 μm in diameter) follicles were expressed. On Day 25, the number of class Ma follicles was very small, but the number of class Md and Me follicles reached a peak, and after that decreased.

As seen in Figs. 2 and 3, from Day 0 to Day 3 in numerous oocytes in the ovary, apoptosis developed showing a positive reaction to anti-TUNEL and Fas antibodies (TUNEL and Fas) and was restricted to the medullary region. The apoptosis with a positive reaction to Fas seen from Day 0 to Day 3 in this study was consistent with the results of Kondo *et al.* [20] and Hakuno *et al.* [21]. It is unclear whether or not TUNEL-positive cells are the same as Fas-positive cells, and the mechanism of apoptosis in ova has not been clarified.

In this study, we have estimated the proliferative activity of different ovarian tissue compartments using BrdU and cyclin D2, and the follicle-growing activity by ERβ. BrdU was used as a cell-cycle activated marker during S and G1. From Day 10 to Day 25, the dynamics of BrdU uptake in follicular cells was the same that found by Gaytán *et al.* [25]. It was reported by Robker and Richards [28] that cyclin D2 was specifically localized to granulosa cells of growing follicles, while cyclin D1 and D3 were restricted to theca cells. Furthermore, ERβ was expressed predominantly in the granulosa cells of the primordial, primary and secondary follicles growing into preovulatory follicles [29].

From Day 5 to Day 15, a strong positive reaction to the antibodies for BrdU, cyclin D2 and ERβ was observed in the remaining follicular cells of the central area of the ovary (Figs. 5, 6 and 7). From Day 5 to Day 10, no apoptosis was found in any follicular cells (Figs. 2 and 3). On Day 15 (Fig. 2B), some granulosa cell nuclei in some follicles and interstitial cells reacting positively to TUNEL began to be formed. These results are consistent with the results of Gaytán *et al.* [30] and Guigon *et al.* [31]. It was considered that from Day 5 to Day 10, follicles were in a state of cellular proliferation and Ma and Mb class follicles began to develop. That granulosa cells reacted positively to ERβ from Day 5 was consistent with the results of Drummond *et al.* [32].

From Day 17, class Me follicles were found. In some of these follicles, granulosa cells began to test negative for BrdU uptake, cyclin D2, and ERβ. In some of these cells, pyknosis began to develop and pyknotic cells reacted positively to the TUNEL antibody (Figs. 2, 6 and 7). These results indicated the beginning of follicular atresia as reported by Osman [26]. Our results that follicular granulosa cells reacted negatively to cyclin D2 and ERβ were consistent with the results of Cheng *et al.* [33]. In our results from Day 0 to Day 35, the number of atretic follicles (stage I + II) in the immature ovary reached a peak from Day 25 to Day 30, but after that it was significantly less than on Day 30 (Fig. 4). The staining dynamics of cyclin D2 and ERβ was very similar in granulosa cells, but theca cells were only stained with ERβ.

As seen in Fig. 8, serum FSH and estrogen concentrations increased from Day 5 to Day 15, reaching a peak, but after that, markedly decreased. These results (changes in FSH and estrogen concentrations from Day 5 to Day 35) are consistent with the results of Ojeda and Ramirez [3], Döhler and Wuttke [4], and Uilenbroek *et al.* [5].

As estrogens are said to promote the cell cycle by inducing stage G1 [34] and forming gap junctions in granulosa cells [35], progression to stage G1 may not be promoted if FSH or estrogen is not sufficiently supplied to the follicles for their maturation. That estrogen increased to high levels in the immature ovary (from Day 5 to Day15) with a high serum FSH concentration suggests that estrogen has a positive feedback effect on the hypothalamo-pituitary-axis and that increased serum FSH levels are necessary for vascular formation and normal follicular development as reported by Döhler and Wuttke [4], Uilenbroek *et al.* [5], and Jiang *et al.* [36]. As the serum FSH concentration decreased from Day 17, it was considered that some follicles did not receive sufficient FSH.

As for the reason why pyknosis of granulosa cells caused follicular atresia: According to the two-cell, two-gonadotropin theory [37, 38], a decrease in the supply of FSH to the follicles causes a decrease in the production of estrogen in the granulosa cells in the follicles. Then, insufficient supply of estrogen to the follicles triggers the arrest of follicular maturation. Therefore, a decrease of gonadotropins in the sera

affects the release of steroid hormones and cell cycling factors, *etc.*, and causes cell disarrangement, inducing apoptosis in the follicular granulosa cells and development of follicular atresia in the ovary.

In this study, on Days 5 to 15, the ovary was in the stage of cellular proliferation as indicated by marked by increases in BrdU uptake, cyclin D2 activity, ERβ activity and FSH and estrogen concentrations in the sera, and no apoptosis was observed in any follicular cells in the ovary. On Day 15, the concentrations of FSH and estrogen showed the highest levels in the blood (Fig. 8) (consistent with Döhler and Wuttke [4]), and pyknosis of the granulosa cells could not be clearly observed in HE-stained sections. However, very few granulosa cells began to react positively to the both TUNEL and anti-Fas antibody (consistent with Gaytán *et al.* [30], and Guigon *et al.* [31]). On Day 17, FSH and estrogen concentrations decreased.

From these results, before the FSH and estrogen concentrations in the sera decreased, part of granulosa cell nuclei reacting positively to TUNEL began to be formed. We hypothesized that the follicular control mechanism or onset mechanism of apoptosis cannot be explained with hormones (the two-cell, two-gonadotropin theory) and is influenced by many other factors (gonadotropins, EGF, IGF, HGF, *etc.*). The reason why the time lag occurred between the beginning of positive reaction of granulosa cells to TUNEL and that of the decrease in FSH concentration in the blood remains a question for the future.

On Day 25, the number of large (Md and Me) class follicles reached a peak and then on Days 25 to 30, the number of atretic follicles reached a peak. It was also observed that follicular granulosa cells reacted positively to cell cycle markers and that many of these follicles remained in the ovary. In adult rats, the balance between follicular growth and atresia was considered to be maintained by cyclic variation in the concentrations of FSH and estrogen in blood.

There have been reports that a DNA ladder (apoptosis formation) was detected in cultured follicular cells by Hughes and Gorospe [39], McGee *et al.* [40], Kim *et al.* [41]. In this study, we tried to biochemically examine the DNA ladder and caspase activity of apoptosis in granulosa cells of some larger follicles using the entire ovary. However, no satisfactory results were obtained, leaving us questions for further study in the future.

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