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Hematoxylin Staining Reveals a Decrease in Nucleolar Diameter of Pig Oocytes Before Germinal Vesicle Breakdown

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Abstract. During oocyte growth, the morphology of the nucleolus changes into a compact and homogenous structure. The compact nucleoli in full-grown oocytes are not stained by aceto-orcein staining or immunofluorescence staining. In this study, we developed a hematoxylin staining method for pig oocytes in whole-mount preparations to visualize the nucleoli. Nucleoli of growing and full-grown oocytes were stained blue with hematoxylin. Using this staining method, the changes in the oocyte nucleolus during maturation were examined. The nucleolar diameter gradually decreased in maturing oocytes (10.7 ± 0.1 μm to 9.0 ± 0.7 μm, $P<0.05$) before germinal vesicle breakdown (GVBD). The results suggest that the nucleolar volume of oocytes decreases before GVBD.
Key words: Hematoxylin, Maturation, Nucleolus, Oocyte, Pig

During mammalian oogenesis, oocytes are arrested at the diplotene stage of the first meiotic prophase and begin growing. In the pig, oocyte size increases from 30 μm to 120 μm during follicular development from the primordial (40 μm in diameter) to antral stage (8–10 mm) [1]. During the final stage of oocyte growth, the morphology of the oocyte nucleolus changes into a compact and homogenous structure [2]. Electron microscopic observations have revealed that nucleoli of full-grown oocytes have a homogenous structure without well-defined compartments seen in somatic nucleoli [3, 4]. Such a change in nucleolar morphology during oocyte growth has been described in the pig [2], mouse [5], rat [6], cow [7] and human [8]. The relationship between the chromatin and nucleolus also changes during oocyte growth [2]. The growing oocyte nucleolus is invaded by chromatin that moves out of the nucleolus during oocyte growth, and the nucleolus becomes compacted.

Under a light microscope, nucleoli of growing oocytes were stained with aceto-orcein while nucleoli of full-grown oocytes were not stained [9]. Furthermore, nucleoli of full-grown oocytes have a compact structure and are not labeled with any antibodies against nucleolar proteins, such as nucleolin (C23) and nucleoplasmin 2 in whole-mount immunostaining [10, 11]. It has been described that compact nucleoli disappeared at the time of germinal vesicle breakdown (GVBD) during oocyte maturation [12], although there are no reports about the precise changes in the nucleolus before GVBD.

The objective of this study was to develop a staining method for full-grown oocyte nucleoli in whole-mount preparations. We found that hematoxylin positively stains the nucleoli in full-grown oocytes. Using this technique, we further revealed the changes in the nucleolus during the 24 h from the induction of maturation to GVBD.

Growing and full-grown pig oocytes were collected from small antral follicles 0.6–1.0 mm in diameter and large antral follicles 4.0–6.0 mm in diameter, respectively. After cumulus cells were removed by pipetting, oocytes were fixed in 4% paraformaldehyde and immersed in hematoxylin solution for 10 min to stain the nucleolus. In growing oocytes (around 100 μm in diameter), a single large nucleolus was stained blue, and perinucleolar nucleoplasm in the nucleus (germinal vesicle: GV) was slightly stained blue (Fig. 1A). In full-grown oocytes (around 120 μm in diameter), a single nucleolus was stained blue, while nucleoplasm was faintly stained (Fig. 1B). The hematoxylin staining method worked well enough to visualize pig oocyte nucleoli.

Before nucleolus staining of maturing oocytes with hematoxylin, we examined the time course of oocyte maturation under our culture conditions. From antral follicles 4.0–6.0 mm in diameter, three types of full-grown oocytes were prepared: cumulus-oocyte complexes (COCs), denuded oocytes and enucleolated oocytes as the negative control. For enucleolation, nucleoli of denuded oocytes were aspirated out by micromanipulation. Nucleolus removal was checked immunocytologically. The chromatin of intact oocytes showed a ring-like structure around the nucleolus (Fig. 2A) and had a nuclear envelope (Fig. 2E). The nucleoli of intact oocytes were not labeled by anti-C23 antibody (Fig. 2C). The aceto-orcein staining also did not stain the nucleoli (Fig. 2G). In enucleolated oocytes, chromatin formed a clump or clumps (Figs. 2B and 2H), and the nucleolus was not seen (Fig. 2D). However, the oocytes maintained a nuclear envelope (Fig. 2F).

To determine the oocyte maturation stage, conventional aceto-orcein staining was performed. Before culture, full-grown oocytes were at the GV stage (Table 1). In COCs, oocytes remained at the GV stage after 12 h, and GVBD started in some oocytes after 18 h. After 24 h, the oocytes progressed to the late diakinesis stage (LD) or metaphase I (MI). The maturation stage of denuded oocytes progressed a little faster. Half of the oocytes reached MI after 18 h, and 27% of the oocytes matured to metaphase II (MII) after 24 h. The maturation
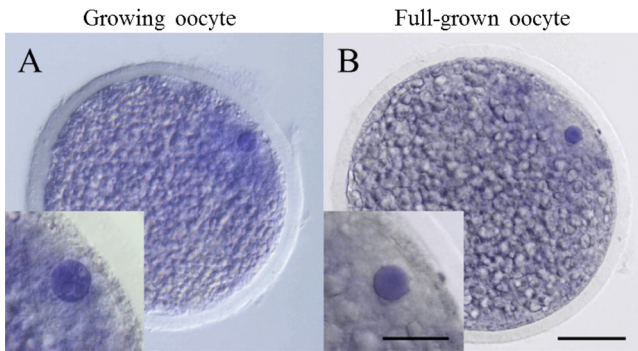
Fig. 1. Differential interference contrast images of growing (A) and full-grown (B) pig oocytes. The oocytes were stained with hematoxylin and whole mounted. A single nucleolus was stained blue and clearly visualized in both oocytes. Insets show the nucleoli of the oocytes at a higher magnification. The scale bars in B represent 40 μm and 20 μm (in the inset).
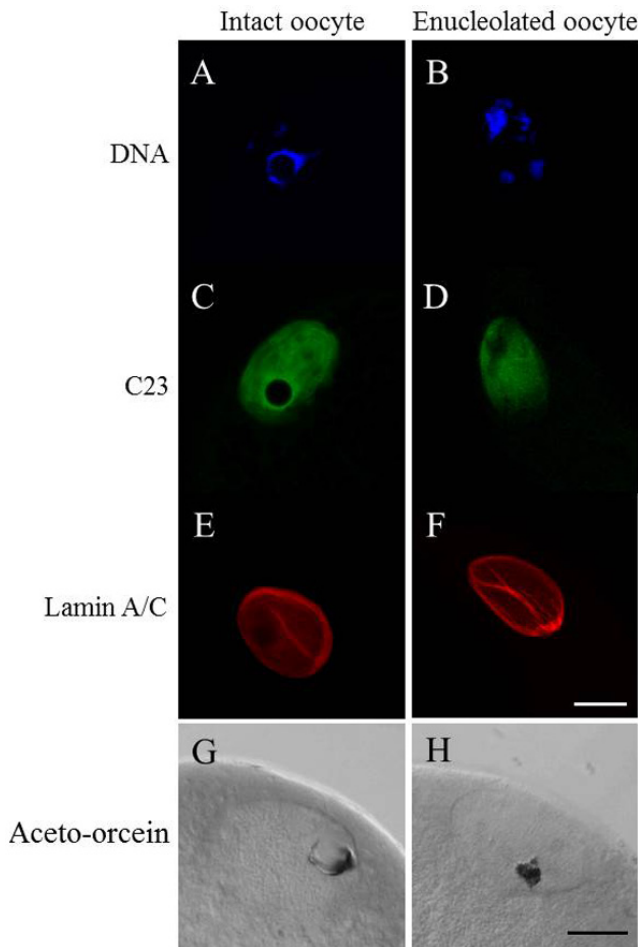

Fig. 2. Immunostaining (A–F) and differential contrast images with aceto-orcein staining (G and H) of pig full-grown oocytes. Immunofluorescence labeling was performed with anti-C23 (in green) and anti-lamin A/C (in red) antibodies on intact (A, C and E) and enucleolated oocytes (B, D and F). Intact oocytes (G) and enucleolated oocytes (H) were also stained with aceto-orcein. Nucleoli of intact oocytes were not labeled by anti-C23 antibody and not stained with aceto-orcein (heterochromatin surrounding the nucleolus was stained). The absence of the nucleolus is evident in enucleolated oocytes. DAPI staining marks chromatin in blue (A and B). Scale bars represent 20 μm.
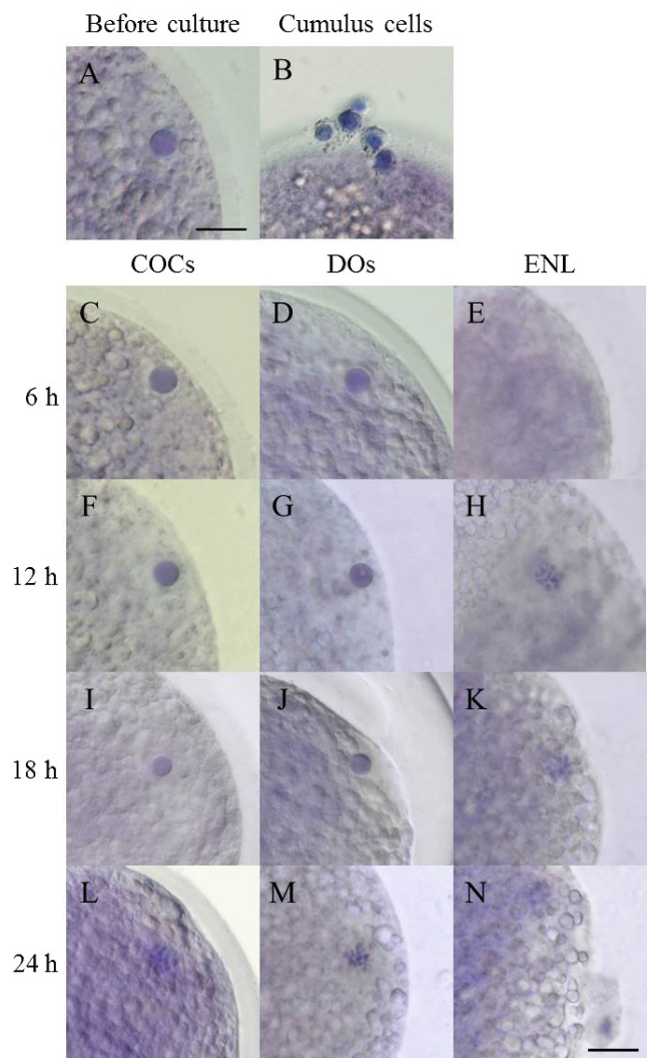

Fig. 3. Differential interference contrast images of maturing pig oocytes before (A) and after culture (C–N) and of cumulus cells (B). Oocytes and cumulus cells were stained with hematoxylin and whole mounted. Cumulus-oocyte complexes (COCs; C, F, I and L), denuded oocytes (DOs; D, G, J and M) and enucleolated oocytes (ENL; E, H, K and N) were cultured for 6 h (C–E), 12 h (F–H), 18 h (I–K) and 24 h (L–N). Nucleoli and chromosomes were stained blue. Nucleolar diameter decreased during maturation in COCs and DOs. Scale bars represent 20 μm.

stage of enucleolated oocytes progressed in a time-course similar to that of denuded oocytes (Table 1). There was no difference in nuclear morphology between denuded oocytes and enucleolated oocytes except in the GV stage. The chromatin in enucleolated oocytes made a clump or clumps in the GV and showed a similar appearance to chromatin at the LD stage. These results correspond to a report that showed maturation of pig and mouse full-grown oocytes progressed normally after enucleolation [10].

Next, we examined staining patterns of maturing oocytes with hematoxylin and measured the nucleolar diameter. Before culture, the mean diameter of oocyte nucleoli was 10.7 ± 0.1 μm (Fig. 3A,

Table 1. Maturation stage of pig full-grown oocytes *in vitro*

Type of oocytes*	Culture time (h)	No. of oocytes examined	No. (%) of oocytes at the stage of **					No. (%) of degenerated oocytes
			GV***	LD	MI	AI-TI	MII	
Before culture	0	30	30 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
COCs	6	31	31 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	12	33	33 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	18	33	27 (82)[a]	5 (15)[a]	0 (0)	0 (0)	0 (0)	1 (3)[a]
	24	34	0 (0)	19 (56)[b]	13 (38)[ab]	0 (0)	0 (0)	2 (6)[ab]
DOs	6	36	34 (94)[a]	0 (0)	0 (0)	0 (0)	0 (0)	2 (6)[ab]
	12	29	22 (76)[a]	5 (17)[a]	0 (0)	0 (0)	0 (0)	2 (7)[ab]
	18	35	6 (17)[bc]	6 (17)[a]	18 (51)[ab]	1 (3)	0 (0)	4 (12)[ab]
	24	30	4 (13)[b]	4 (13)[a]	9 (30)[ab]	3 (10)	8 (27)[ab]	2 (7)[ab]
ENL	6	28	27 (96)[a]	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)[ab]
	12	26	11 (43)[c]	4 (15)[a]	7 (27)[a]	0 (0)	0 (0)	4 (15)[ab]
	18	26	4 (15)[bc]	1 (4)[a]	14 (54)[b]	0 (0)	2 (8)[b]	5 (19)[ab]
	24	25	0 (0)	2 (8)[a]	7 (28)[ab]	2 (8)	8 (32)[a]	6 (24)[b]

* Cumulus-oocyte complexes (COCs) containing full-grown oocytes were collected from antral follicles (4–6 mm in diameter). Denuded oocytes (DOs) were obtained by removing cumulus cells, and enucleolated oocytes (ENL) were prepared by enucleolation of DOs. ** GV, germinal vesicle stage; LD, the late diakinesis stage; MI, metaphase I; AI-TI; anaphase I-telophase I; and MII, metaphase II. *** The enucleolated oocytes had a germinal vesicle that contained a clump or clumps of chromatin. [a–c] Values with different superscripts in the same column differ significantly (P<0.05).

Table 2. Nucleolar diameter of pig oocytes during maturation

Type of oocytes*	Culture time (h)	No. of oocytes examined	Hematoxylin staining (%)**		Diameter of nucleolus (μm)***
			Nucleolus	Chromosome	
Before culture	0	32	32 (100)	0 (0)	10.7 ± 0.1[a]
COCs	6	29	29 (100)	0 (0)	10.8 ± 0.1[a]
	12	33	33 (100)	0 (0)	9.9 ± 0.2[b]
	18	33	28 (85)[a]	5 (15)[a]	9.0 ± 0.7[c]
	24	30	0 (0)	30 (100)	-
DOs	6	32	32 (100)	0 (0)	10.9 ± 0.1[a]
	12	29	26 (89)[a]	3 (11)[a]	9.6 ± 0.2[bc]
	18	32	7 (22)[b]	25 (78)[b]	9.5 ± 0.3[bc]
	24	27	5 (19)[b]	22 (81)[b]	-
ENL	6	25	0 (0)	0 (0)	-
	12	23	0 (0)	11 (48)[c]	-
	18	22	0 (0)	14 (64)[bc]	-
	24	24	0 (0)	17 (71)[bc]	-

* Cumulus-oocyte complexes (COCs), denuded oocytes (DOs) and enucleolated oocytes (ENL) were prepared as described in the Table 1 footnote. ** Oocytes were stained with hematoxylin and observed under a Nomarski interference microscope to measure the nucleolar diameter. *** Values are means ± SEM. [a–c] Values with different superscripts in the same column differ significantly (P<0.05).

Table 2). Nucleoli of cumulus cells were heavily stained blue, while the nuclei were also stained blue (Fig. 3B). In COCs, oocyte nucleoli or chromosomes were stained blue during maturation (Figs. 3C, 3F, 3I and 3L). Nucleolar diameter significantly decreased to 9.9 ± 0.2 μm after 12 h and 9.0 ± 0.7 μm after 18 h (Figs. 3F and 3I, Table 2). These values show that the nucleolar diameter and volume decreased to 85 and 60%, respectively, during maturation. Nucleoli disappeared in some oocytes after 18 h and in all oocytes after 24 h. After nucleoli disappeared, chromosomes at the LD and MI stages were stained with hematoxylin (Fig. 3L). In denuded oocytes, the nucleolar diameter significantly decreased to 9.5 ± 0.3 μm after 18 h (Fig. 3J, Table 2). Nucleoli disappeared in most of the oocytes, and chromosomes at MI were observed after 18 h and 24 h (Fig. 3M). Some oocytes progressed to MII after 24 h, and the chromosomes and condensed chromatin in the polar body were stained blue (data not shown). In enucleolated oocytes, chromosomes in the oocytes and chromatin in the first polar body were observed similarly to denuded oocytes after culture (Figs. 3H, 3K and 3N). However, no nucleoli

were observed in any oocytes throughout the maturation stage.

In the present study, we used hematoxylin staining to visualize oocyte nucleoli. Hematoxylin is a dye often used with eosin for hematoxylin and eosin staining (HE staining). Hematoxylin is oxidized to hematein, which forms a positively-charged complex with metal (aluminium in Mayer's hematoxylin solution in this study). The hematein-metal complexes bind to negatively charged parts in histological sections, such as nuclei and ribosomes [13]. The hematein-metal complexes bind to DNA in nuclei and RNA in cytoplasm and nucleoli. The nucleolus is the site of rRNA synthesis and ribosome production, and its staining with hematoxylin has been well-known in somatic cells [14]. Since oocytes actively synthesize RNA during the growth phase, it is expected that oocyte nucleoli contain synthesized rRNA and small nucleolar RNA (snoRNA) as somatic nucleoli [3]. Therefore, hematein-metal complexes probably bind to RNAs in nucleoli of growing oocytes. Since chromatin spreads in the GV of growing oocytes [9], hematoxylin stained DNA in the spreading chromatin in the GV. However, as the oocytes approach their full-grown size, RNA synthetic activity decreases, and coincidently the nucleolar structure becomes compact [2]. Although the compact nucleoli of full-grown oocytes are not engaged in RNA synthesis, they were stained with hematoxylin in the present study. The composition of the nucleoli has not been determined yet. However, it is thought that RNA accumulated in the nucleoli during oocyte growth, and nucleoli of full-grown oocytes were stained with hematoxylin. In a previous study, we reported that oocyte nucleoli can be visualized by centrifuging the oocytes to locate lipid droplets to on side within the oocytes [15]. The method of centrifugation is simple, although nucleoli cannot be observed when lipid droplets hide the nucleoli in whole-mount preparations. Moreover, centrifuging oocytes or embryos causes the fusion of nucleoli [16]. Oocyte nucleoli were stained with Gimsa stain, although they were not observed clearly since ooplasm was also stained (data not shown). We think that hematoxylin staining is a simple method to visualize oocyte nucleoli clearly.

Recent research has shown that the oocyte nucleolus is required for early embryonic development, while it is not necessary for oocyte maturation [10]. In the present study, enucleolated oocytes maintained maturation competence similar to intact oocytes. However, throughout the course of maturation, no nucleoli were observed in enucleolated oocytes. These results indicate that enucleolated oocytes are capable of carrying the maturation stage forward but incapable of forming nucleoli in the GV again during maturation culture.

The present study shows a decrease in nucleolar diameter of pig oocytes before GVBD in both COCs and denuded oocytes. Maturation of DOs progressed faster than that of COCs, and the decrease in nucleolar diameter seemed to be faster in DOs at 12 h after maturation culture, although the mean diameter was not significantly different from that of COCs. The nucleolar diameter of COCs decreased more after 18 h, while the nucleolar diameter of DOs did not. This result is thought to reflect that some DOs did not undergo GVBD, although 100% of COCs underwent GVBD under our culture conditions. The nucleolus is a non-membrane bound organelle, and its assembly and disassembly in somatic cells is thought to be controlled by CDK1 [17]. CDK1 activity increases at MI in pig oocytes [18]. However, it seems that CDK1 is not involved in the decrease in nucleolar volume before GVBD during maturation, since nucleoli injected into cytoplasm disappear in both GV and MII oocytes soon after injection [19]. Some factors other than cell cycle regulators might be engaged in nucleolar disassembly and the decrease in volume. Compact nucleoli of full-grown oocytes contain proteins [20], and nucleoplasmin 2 has been described as the major component of nucleoli in mouse oocytes [11, 21]. In addition, proteasome inhibitors increase the nucleolar size in full-grown oocytes of pigs [15]. The reason for the decrease in nucleolar diameter is unclear; however, the decrease is possibly associated with a change in localization of nucleolar proteins. Alternatively, the ubiquitin-proteasome system may degrade nucleolar proteins during oocyte maturation.

In summary, the present study demonstrates that compact nucleoli of pig full-grown oocytes were stained with hematoxylin and clearly visualized in whole-mount preparations. This hematoxylin staining method revealed that the volume of the compact nucleolus of the oocyte decreases before GVBD.

Methods

Oocyte collection and culture

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. The ovaries were washed once in 0.2% (w/v) cetyltrimethylammonium bromide and three times in Dulbecco's phosphate-buffered saline (PBS) containing 0.1% (w/v) polyvinyl alcohol (PBS-PVA; Sigma-Aldrich, St. Louis, MO, USA). Growing oocytes and full-grown oocytes were collected from small antral follicles (0.6–1.0 mm in diameter) and large antral follicles (4.0–6.0 mm in diameter), respectively. To collect small follicles, ovarian cortical slices (1–1.5 mm in thickness) were cut from the ovarian surface by using a surgical blade (No.21; Keisei Medical Industrial, Tokyo, Japan) and a pair of forceps. Under a dissecting microscope, small antral follicles were dissected from the cortices, and the tissues surrounding the follicles were torn off. Large antral follicles were dissected from ovaries using two blades (No.11; Keisei Medical Industrial), and the follicles that had a spherical oocyte that was surrounded by cumulus granulosa cells and adhered to the follicle were selected [22]. After the follicles were opened in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate and 0.08 mg/ml kanamycin sulfate (Sigma-Aldrich), cumulus-oocyte complexes (COCs) were isolated from the follicles. Then the cumulus cells were completely removed from some growing and full-grown oocytes by pipetting to obtain denuded oocytes (DOs).

Following two washes with HEPES-199, COCs from large antral follicles were cultured with theca of the follicles in 2.0 ml of the basal medium supplemented with 0.1 IU/ml human menopausal gonadotropin (hMG Pergonal; Aska Pharmaceutical, Tokyo, Japan) in a plastic dish (#1008, Falcon Labware, BD, Franklin Lakes, NJ, USA) on a rocking system at 38.5 C under an atmosphere of 5% CO_2 in humidified air for 24 h. The basal medium was bicarbonate-buffered medium 199 (TCM-199) supplemented with 10% (v/v) fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate and 2.2 mg sodium bicarbonate. Full-grown DOs were enucleolated as described below. DOs and enucleolated oocytes were cultured in 0.5 ml of the basal medium in a four-well dish (4-well Multidish,

Nunclon, Denmark) at 38.5 C under an atmosphere of 5% CO_2 in humidified air for 24 h.

Enucleolation of full-grown oocytes

Enucleolation of oocytes followed the method described by Fulka *et al.* [23]. After 4 h of recovery culture following denudation, full-grown DOs were transferred into HEPES-199 containing 12.5 μg/ml cytochalasin B (Sigma-Aldrich) for 10 min and then centrifuged at 4,000 *g* for 10 min to move the lipid droplets to one side of the oocytes. A plastic dish (#1007, 60×15 mm, Falcon Labware, BD) was used as a micromanipulation chamber. The dish was put on a stage warmed to 38.5 C in an inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a Hoffman modulation system (Modulation Optics, Greenvale, NY, USA).

Each oocyte was held by a holding pipette (inner diameter 10 μm, angle 30°) at the 9 o'clock position, and then the oocyte was rotated until the GV was at the 3 o'clock position. The zona pellucida was punctured with a square-ended injection pipette (inner diameter 8–10 μm) and piezo pulses using a micromanipulator (Narishige Group, Tokyo, Japan) equipped with a Piezo Micro Manipulator Controller (PMAS-CT150; Prime Tech, Ibaragi, Japan). After penetration through the zona pellucida, the injection pipette was positioned against the GV membrane. Thereafter, gentle suction was applied, which resulted in suction of the nucleolus into the mouth of the pipette. The injection pipette was then slowly withdrawn from the oocyte cytoplasm. When the pipette opening with the captured nucleolus was just outside the vitelline membrane, additional suction was applied. The nucleolus thereafter penetrated the GV membrane, leaving the entire GV content within the nuclear envelope.

Fixation and staining of oocytes

After culture of COCs, cumulus cells were removed from oocytes by gentle pipetting with a small-bore pipette in HEPES-199. Before and after culture, the oocytes of all groups were fixed and stained with 1% (w/v) aceto-orcein to examine the maturation stage under a Nomarski interference microscope (BX51; Olympus Optical). The oocytes were classified by the morphology of the chromatin and nuclear envelope (GV, full-grown oocytes at the GVI-IV stage; LD, the late diakinesis stage; MI, metaphase I; AI, anaphase I; TI, telophase I; and MII, metaphase II) according to the classification of Motlik and Fulka [12].

For hematoxylin staining, full-grown oocytes before and after culture and growing oocytes were washed twice in PBS-PVA and fixed in 4% (w/v) paraformaldehyde in PBS-PVA for 40 min. The oocytes were washed twice in PBS-PVA for 15 min each and were then immersed in Mayer's hematoxylin solution (Wako Pure Chemical Industries, Osaka, Japan) for 10 min. After being washed in PBS-PVA for 20 min, they were mounted on glass slides with PBS-PVA and observed under a Nomarski interference microscope. Photos of oocytes and an objective micrometer were taken at the same magnification using Viewfinder Life (Pixela, Osaka, Japan), and the diameters of nucleoli were measured.

For immunofluorescence staining, oocytes were washed twice in PBS-PVA and stained as follows. The oocytes were fixed and permeabilized in 4% (w/v) paraformaldehyde in PBS-PVA containing 0.2% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 40 min.

They were washed twice in PBS-PVA for 15 min each and were then blocked in PBS-PVA containing 1 mg/ml bovine serum albumin (BSA, Wako Pure Chemical Industries) (PBS-PVA-BSA) and 0.2% (v/v) Triton X-100 at 4 C overnight. The oocytes were then incubated with the primary antibody diluted with PBS-PVA-BSA at 4 C overnight. The primary antibodies were mouse monoclonal anti-nucleolin antibody (1:200, C23, sc-8031; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-Lamin A/C antibody (1:200, sc-7292; Santa Cruz Biotechnology). After being washed three times in PBS-PVA-BSA for 15 min each, the oocytes were reacted with Alexa Fluor 488-labeled donkey anti-mouse IgG (1:200, A21202, Molecular Probes, Eugene, OR, USA) or Alexa Fluor 568-labeled goat anti-mouse IgG (1:200, A11004, Molecular Probes) for 40 min at room temperature. After the oocytes were washed three times in PBS-PVA-BSA for 15 min each, they were mounted on glass slides with ProLong Gold Antifade Reagent with DAPI (P36931, Molecular Probes) and observed under a confocal laser scanning microscope (FV1000-KDM, Olympus Optical).

Statistical analysis

The frequencies of oocytes at each stage of maturation or showing different staining patterns with hematoxylin were analyzed using the Chi-square test. Statistical differences in the mean (± SEM) diameters of nucleoli were analyzed by the Student's *t*-test. Values of $P<0.05$ were considered to indicate statistical significance.

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