



Nucleolus Inter-replacement between Oocytes and Embryos in the Pig

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D o c t o r a l D i s s e r t a t i o n

Nucleolus Inter-replacement between Oocytes and Embryos in the Pig

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博士論文

Nucleolus Inter-replacement between Oocytes and Embryos in the Pig

ブタ卵母細胞および胚の核小体置換

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Index

Chapter I	
General Introduction	1
Chapter II	
Nucleoli from Growing Oocytes Support the Development of Enucleolated Full-grown Oocytes	
Introduction	7
Materials and methods	9
Results	19
Discussion	32
Chapter III	
Nucleoli from Growing Oocytes Inhibit the Maturation of Enucleolated Full-grown Oocytes	
Introduction	37
Materials and methods	39
Results	47
Discussion	57
Chapter IV	
Nucleoli from 2-cell Embryos Support the Development of Enucleolated Full-grown Oocytes	
Introduction	62
Materials and methods	64
Results	73
Discussion	81
Chapter V	
General Summary	86

Acknowledgements	97
References	98

Abbreviations

AD: actinomycin D
BSA: bovine serum albumin
COCs: cumulus-oocyte complexes
DAPI: 4',6-diamidino-2-phenylindole
DIC: differential interference contrast
dbcAMP: dibutyryl cyclic AMP
DFCs: dense fibrillar components
ENL: enucleolation
FBS: fetal bovine serum
FC: filamentous chromatin stage
FCs: fibrillar centers
fNL: full-grown oocyte nucleolus
GCs: granular components
gNL: growing oocyte nucleolus
GV: germinal vesicle
GVBD: germinal vesicle breakdown
HEPES-199: HEPES-buffered medium 199
LD: late diakinesis
MI: metaphase I
MII: metaphase II
NL: nucleolus
NPM2: nucleoplasmin 2
PBS: phosphate-buffered saline
PVA: polyvinyl alcohol
rDNA: ribosomal DNA
rRNA: ribosomal RNA
SC: stringy chromatin stage
UBF: upstream binding factor

Chapter I

General Introduction

The somatic cell nucleolus was discovered by Fontana in 1781 (Fontana 1781). The oocyte nucleolus was first documented in 1835 in the jerryfish (Wanger 1835), and the mammalian oocyte nucleolus was first reported in the rabbit (Bischoff 1842). In 1952, nucleoli were first isolated from starfish oocytes (Vincent 1952). In 1964, one report showed that the development of anucleolate (no nucleoli) mutant *Xenopus* embryos was arrested due to their inability to synthesize new ribosomes, suggesting that the nucleolus is the site of ribosomal RNA (rRNA) synthesis and nascent ribosome assembly (Brown and Gurdon 1964). Electron microscopic study on the mammalian oocyte nucleolus was also reported in 1960s (Parsons 1962).

Oocytes have the specific nucleoli which are different from somatic cells. During oogenesis in mammals, oocytes become arrested at the diplotene stage of the first meiotic prophase and start growing at this stage. During the oocyte growth in the pig, primordial follicles (40 μm in diameter) develop to antral follicles (8–10 mm), and

the size of oocytes increases from 30 μm to 120 μm (Miyano and Manabe 2007). The nucleoli of non-growing and growing oocytes are invaded by chromatin, although the chromatin is moved out of the nucleoli during oocyte growth, and the nucleoli become compacted in the full-grown oocytes.

At the ultrastructural level (Fig. 1), nucleoli of somatic cells and growing oocytes in mammals are composed of fibrillar centers (FCs), dense fibrillar components (DFCs), and granular components (GCs) (Crozet 1989; Olson *et al.* 2000). Pig oocyte nucleoli in early antral follicles are composed of GCs, vacuoles, a few FCs, and DFCs. The nucleoli in growing oocytes are the site of active rRNA synthesis and ribosome production (Hyttel 2011) similar to nucleoli in somatic cells (Olson *et al.* 2000). The rRNA synthesis, however, decreases as oocytes approaching the final size; for example rRNA synthesis of pig oocytes starts to decrease in the early antral follicle stage, when the oocyte size is around 95 μm (Crozet 1989). The decrease coincides with inactivation of the nucleolus, as visualized by the transformation of the fibrillogranular nucleolus to an electron-dense fibrillar sphere with remnants of the FCs at the surface (Bjerregaard *et al.* 2004). Consequently, the nucleoli of full-grown oocytes are compacted and separated from chromatin, and have no rRNA synthetic activity. However, functions of the nucleoli in full-grown oocytes have not been understood.

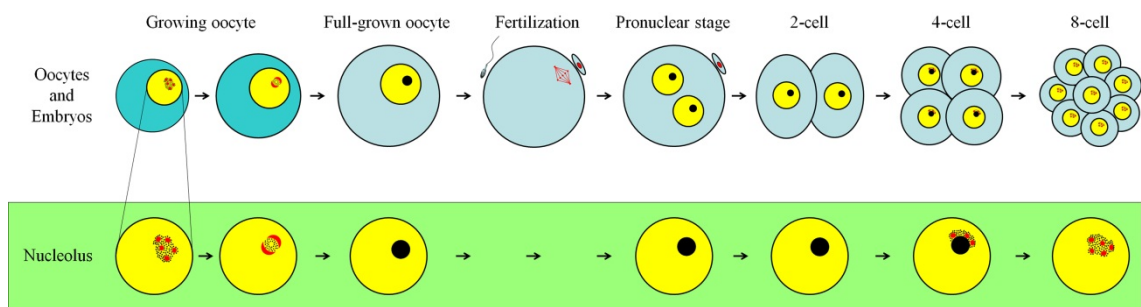


Fig. 1 The nucleoli of growing oocytes in mammals are composed of fibrillar centers (red), dense fibrillar components, and granular components (black dots). During oocyte growth in the pig, nucleoli actively synthesize rRNA. At the end of the growth phase, nucleoli are inactivated to a dense nucleolar remnant (black) by marginalization of the fibrillar centers. During oocyte maturation, nucleoli disappear, and upon fertilization, nucleoli (black) are formed in pronuclei. At the 4- and 8-cell stage, fibrillar centers and fibrillar components (red), and granular components (dots) develop on the surface of nucleoli.

Full-grown oocytes resume meiosis after gonadotropic stimulation, and mature to metaphase II (MII). During oocyte maturation, the nucleoli disappear in the cytoplasm of oocytes, and upon fertilization, nucleoli are formed in male and female pronuclei (Fig. 1). During the pronuclear and 2-cell stages, condensed chromatin forms a ring-like structure around the nucleoli, and in pig embryos, the chromatin separates from the nucleoli composed of electron-dense fibrillar sphere by marginalization of the FCs (Tomanek *et al.* 1989). The morphology and some nucleolar components of pronuclear and 2-cell pig embryos are similar to those of oocytes at the germinal vesicle (GV) stage (Hyttel 2011). Based on their morphological and functional differences compared to nucleoli of somatic cells, the nucleoli in GV oocytes and zygotes have been often referred to as the “nucleolus-like body” (Szollosi *et al.* 1991) and “nucleolus precursor body” (Tomanek *et al.* 1989). After 2-cell stage, FCs, DFCs and GCs develop on the surface of nucleoli and the nucleoli are transformed to somatic cell-type nucleoli before the morula stage (Tomanek *et al.* 1989).

Recent two reports have suggested new roles of the oocyte nucleolus other than rRNA synthesis. The first one was reported by Fulka and his colleagues (Fulka *et al.* 2003). When the nucleolus was removed from pig growing oocytes which were incompetent to resume meiosis, some of them underwent germinal vesicle breakdown

(GVBD). This result suggests that nucleoli in growing oocytes have an inhibitory role in meiotic resumption of the oocytes. The role of nucleoli in cell cycle regulation has been documented in yeasts (Cockell and Gasser 1999; Visintin and Amon 2000; Visintin *et al.* 1999) and somatic cells (Hudson *et al.* 2001). However, further evidence has not been provided on the role of the nucleolus of growing oocytes.

The second one has shown that the oocyte nucleolus is required for early embryonic development (Ogushi *et al.* 2008). They showed that the nucleoli isolated from full-grown oocytes did not contain visible amount of chromatin and that enucleolated oocytes injected with nucleoli from full-grown oocytes developed to live mice. Thus, enucleolation was done without irreparable damage to the chromosome. Enucleolated pig and mouse oocytes were able to mature to MII. However, enucleolated oocytes neither formed nucleoli in the pronucleus nor developed to blastocysts after fertilization or parthenogenetic activation. On the other hand, when nucleoli were injected into enucleolated oocytes at MII and the oocytes were then activated, they formed nucleoli in the pronuclei and developed to blastocysts. Thus, the oocyte nucleolus is not necessary for oocyte maturation, but it is necessary for the formation of pronuclear nucleoli after fertilization or parthenogenetic activation and is essential for further embryonic development.

In full-grown oocytes and embryos before 2-cell stage, nuclei include compacted nucleoli which do not contain chromatin and have no RNA synthetic activity. In this study, nucleolus removal and injection have been carried out to pig growing and full-grown oocytes and 2-cell embryos. However, as nucleoli of growing oocytes are not compacted, it is difficult to enucleolate. Several reports have shown that actinomycin D which is an inhibitor of RNA polymerase I and II causes the chromatin out of the nucleoli, and makes nucleoli compact in growing oocytes (Crozet 1983; Schultz *et al.* 1981). After actinomycin D treatment, nucleoli of growing oocytes were micromanipulated in this study. The nucleoli of growing and full-grown oocytes and 2-cell embryos were removed and inter-replaced among oocytes/embryos using micromanipulation to determine the ability of each stage nucleoli in terms of supporting embryonic development. In Chapter II, I examined whether growing oocyte nucleoli support early embryonic development as full-grown oocyte nucleoli. In Chapter III, effect of growing oocyte nucleoli on maturation of enucleolated full-grown oocytes was examined. In Chapter IV, I conducted nucleolus replacement experiments to determine the ability of nucleoli from 2-cell embryos to substitute for nucleoli of GV oocytes.

Chapter II

Nucleoli from Growing Oocytes Support the Development of Enucleolated Full-grown Oocytes

Introduction

Change in the nucleolus morphology during oocyte growth has been reported in the mouse (Chouinard 1971), rat (Takeuchi 1984), pig (Crozet 1989), cow (Crozet *et al.* 1986), and human (Tesarik *et al.* 1983). The association between chromatin and the nucleolus changes as the oocytes grow progressively and acquire the competence to mature (Crozet 1989). Several reports have shown that actinomycin D which is an inhibitor of RNA polymerases I and II causes the chromatin out of nucleoli, and makes them compact in growing oocytes (Crozet 1983; Schultz *et al.* 1981). Beside the difference in chromatin morphology, the nucleolus in growing oocytes synthesizes RNAs, while the nucleolus in full-grown oocytes shows decreased RNA synthesis (Crozet 1989; Motlik *et al.* 1984b). However, the other functional differences between the nucleoli of growing and full-grown oocytes are not well understood.

Recent research has shown that the full-grown oocyte nucleoli are required for early embryonic development, but are not necessary for oocyte maturation (Ogushi *et al.* 2008). However, it is not known that the growing oocyte nucleoli support the early embryonic development as the full-grown oocyte nucleoli. The objective of this chapter is to determine the ability of nucleoli from growing pig oocytes to substitute for nucleoli of full-grown oocytes in terms of supporting embryonic development. In the preliminary experiment, the morphology of growing oocyte nucleoli was different from that of full-grown oocytes. Since growing oocyte nucleoli were invaded by the chromatin and not compacted, the chromatin of growing oocytes was aspirated with the nucleoli during the enucleolated process. Growing oocytes were thus treated with actinomycin D to release the chromatin from their nucleoli, and nucleoli from growing oocytes were collected and injected into enucleolated and matured full-grown MII oocytes. The formation of pronuclear nucleoli in the nucleolus-transferred enucleolated oocytes and their development to blastocysts were examined after electro-stimulation.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Collection and Culture of Oocytes

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. The ovaries were washed once in 0.2% (w/v) cetyltrimethylammonium bromide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and three times in Dulbecco's phosphate-buffered saline (PBS; Wako Pure Chemical Industries) containing 0.1% (w/v) polyvinyl alcohol (PBS-PVA). Growing oocytes and full-grown oocytes were collected from small and large antral follicles, respectively. To collect small follicles of 0.6–1.0 mm in diameter, cortical slices (1–1.5 mm in thickness) were cut from the ovarian surface using a surgical blade (No. 21) 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 of 4–5 mm in diameter were dissected using two surgical blades (No. 11), and the follicles containing a

spherical oocyte that was surrounded by cumulus granulosa cells and adhered to the follicle wall were selected (Moor and Trounson 1977). After the follicles were opened in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate (Wako Pure Chemical Industries), and 0.08 mg/ml kanamycin sulphate, cumulus-oocyte complexes were isolated from the follicles. Then the cumulus cells were completely removed from growing and full-grown oocytes by pipetting to obtain denuded oocytes.

Full-grown denuded oocytes were enucleolated as described below and cultured in 0.5 ml of culture medium in a 4-well dish (4 Well Multidish; Thermo Fisher Scientific K.K., Yokohama, Japan) at 38.5°C under an atmosphere of 5% CO₂ in air for 30 hr. The culture medium was bicarbonate-buffered medium 199 supplemented with 10% (v/v) fetal bovine serum (FBS; ICN Biomedicals, Inc., Aurora, OH, USA), 0.1 mg/ml sodium pyruvate (Nacalai Tesque, Inc., Kyoto, Japan), 0.08 mg/ml kanamycin sulphate, and 2.2 mg/ml sodium bicarbonate (Wako Pure Chemical Industries).

Before and after culture, the oocytes were fixed and stained with 1% (w/v) aceto-orcein (Wako Pure Chemical Industries), and examined the maturation stage using Nomarski or differential interference microscopy. The oocytes were classified by the morphology of the chromatin and nuclear envelope (FC: growing oocytes at the

filamentous chromatin stage, SC: growing oocytes at the stringy chromatin stage, GV: full-grown oocytes at the GVI–IV stage, MI: metaphase I, and MII: metaphase II) according to the classification of Motlik and Fulka (1976) and Hirao *et al.* (1995).

Enucleation and Nucleolus Injection

Enucleation of oocytes followed the method described by Fulka *et al.* (2003) (Fig. 2-a–d). After 4 hr of recovering culture following denudation, full-grown oocytes were transferred into HEPES-199 containing 12.5 µg/ml cytochalasin B 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 Becton Dickinson Labware, Franklin Lakes, NJ, USA) was used as a micromanipulation chamber. The dish was put on a warmed stage (38.5°C) in an inverted microscope (Olympus Optical Co., Tokyo, Japan) equipped with a Hoffman modulated 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,

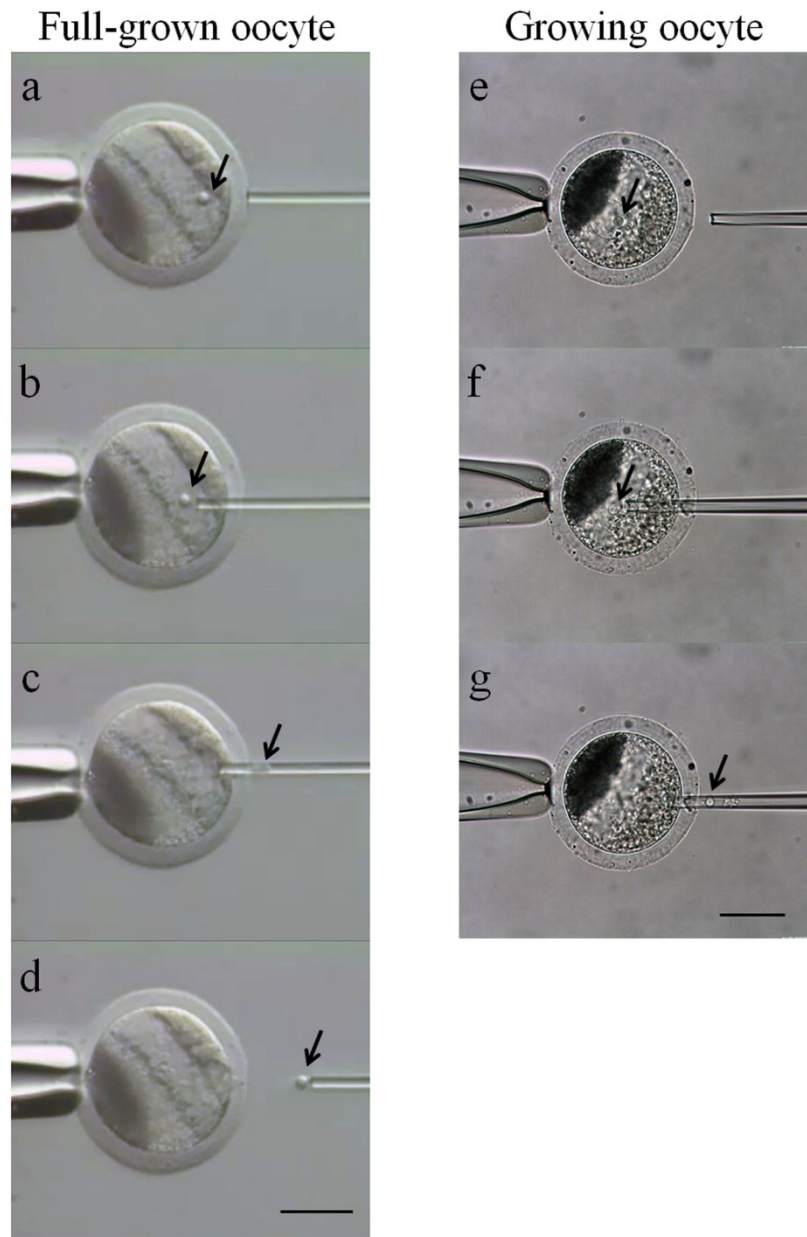


Fig. 2 Enucleation of pig full-grown (a–d) and growing oocytes (e–g) at the germinal vesicle stage. Arrows indicate the nucleoli. An injection pipette penetrates through the zona pellucida and its tip is then pushed against the germinal vesicle membrane. With mild suction, the nucleolus is preferentially aspirated into the injection pipette. The nucleolus can be then collected from the oocytes. Scale bars show 50 μm .

Tokyo, Japan) equipped with a PIEZO drive (PNAS-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 oocyte plasma membrane, additional suction was applied. The nucleolus thereafter penetrated the GV membrane, leaving the entire GV content within the nuclear envelope. After enucleation, the oocytes were washed 9 times in HEPES-199. They were then cultured for 25–26 hr (total 30 hr) as described above. Some denuded oocytes were centrifuged, manipulated in the same manner involving the suction of a small volume of nucleoplasm but not the nucleolus, and cultured for 30 hr as sham-operated oocytes (Fig. 3).

Growing and full-grown oocytes, as the nucleolus donors, were cultured in the medium supplemented with 5 μ g/ml actinomycin D and 2 mM dibutyryl cyclic AMP (dbcAMP) for 24 hr before enucleation. After being washed several times, they were transferred into HEPES-199 containing 12.5 μ g/ml cytochalasin B for 10 min, and then centrifuged at 3,500 g for 10 min to move the lipid droplets to one side of the oocytes. The nucleoli of the oocytes were aspirated using the same method used in full-grown

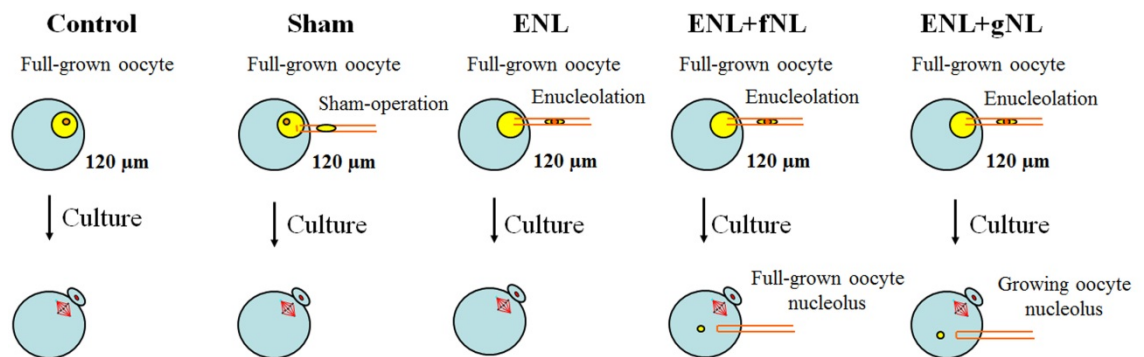


Fig. 3 Experimental scheme of oocyte enucleation and nucleolus injection. Nucleoli were microsurgically aspirated from germinal vesicles to produce enucleolated oocytes (ENL). Oocytes aspirated small amount of nucleoplasm served as control (Sham). The isolated nucleoli from full-grown and growing oocytes were injected into enucleolated-matured oocytes at MII (ENL+fNL and ENL+gNL, respectively). Five hour after nucleolus injection, oocytes were activated by electro-stimulation. Nucleolus donor oocytes were treated with actinomycin D for 24 hr before enucleation.

oocytes (Fig. 2-e–g).

Into some of the enucleolated and matured full-grown oocytes, collected nucleoli were injected. Enucleolated full-grown oocytes that showed normal morphology and had the first polar body (MII oocytes) were selected after 30 hr of maturation culture and were used as recipient oocytes. Nucleoli collected from full-grown oocytes (fNL) or growing oocytes (gNL) were injected into the MII oocytes (Fig. 3). The injection of nucleoli was performed in a similar way to intracytoplasmic sperm injection (Katayama *et al.* 2005). 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 first polar body was at the 6 o'clock position. The zona pellucida was punctured by several applications of piezo-pulse. Then the nucleolus, which had been aspirated into a glass micropipette (inner diameter 8–10 μm), was ejected into the deep cytoplasm of the oocyte. The injection pipette was then slowly withdrawn. The oocytes were cultured for 5 hr before electro-stimulation.

Electro-stimulation and Development Culture

The enucleolated and sham-operated oocytes with the first polar body after maturation culture, and the enucleolated, matured and nucleolus-injected oocytes

described above were washed three times with 0.3 M mannitol (Nacalai Tesque, Inc.) solution containing 0.1 mM MgSO₄ (Wako Pure Chemical Industries), 0.05 mM CaCl₂ (Wako Pure Chemical Industries) and 0.01% PVA. They were transferred to the same solution between parallel electrodes, separated by 2 mm in a chamber (FTC-03; Shimadzu Co. Ltd., Kyoto, Japan), and subjected to a single square pulse of direct current for 100 µsec at 1,500 V/cm from an electric cell fuser (LF101; BEX Co., Tokyo, Japan). After electro-stimulation, the oocytes were washed and cultured for 4 hr in PZM3 (Yoshioka *et al.* 2002) containing 0.5 mg/ml cytochalasin B to prevent the emission of the second polar body. After being washed in PZM3, the oocytes were further cultured for 7 days in PZM3 at 38.5°C under an atmosphere of 5% CO₂ in air.

Fluorescence Microscopy

Enucleolated oocytes were collected after enucleolation and the subsequent 1 hr culture so that the absence of the nucleolus was determined by staining with 12 µg/ml Hoechst 33342 or immunocytoologically. The formation of the nucleolus in pronuclei was examined immunocytoologically for the oocytes 10 hr after electro-stimulation.

For immunofluorescence staining, these 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, Inc.) for 40 min. The oocytes 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 % Triton X-100 at 4°C overnight. The oocytes were 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, Inc., Santa Cruz, CA, USA) and mouse monoclonal anti-lamin A/C antibody (1:200, sc-7292; Santa Cruz Biotechnology, Inc.). 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 (1:400, A21202; Molecular Probes, Eugene, OR, USA) or Alexa Fluor 568-labeled goat anti-mouse IgG (1:400, A11004; Molecular Probes) for 40 min at room temperature. After the oocytes were washed three times in PBS-PVA-BSA for 15 min each, DNA was counterstained with 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 20 min at room temperature. The oocytes were washed three times in PBS-PVA-BSA before being mounted on glass slides with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), and observed under a fluorescence microscope (IX70-S1F2; Olympus Optical Co.) with an emission filter of

330–385 nm (Hoechst 33342) or a confocal laser scanning microscope (FV1000-KDM; Olympus Optical Co.) for Alexa Fluor 488, Alexa Fluor 568 and DAPI.

Statistical Analysis

The frequencies of oocytes and embryos at each stage of maturation or development were analyzed using the chi-square test. P values less than 0.05 were considered to indicate statistical significance.

Results

Confirmation of Enucleolation

Full-grown oocytes were collected from large antral follicles that were 4–5 mm in diameter. Their mean diameter excluding the zona pellucida was $118.6 \pm 5.3 \mu\text{m}$ (n=21). After 4 hr of recovering culture following denudation, oocytes were treated with cytochalasin B, centrifuged, and enucleolated by micromanipulation. Nucleolus removal was checked immunocytoologically. The chromatin of sham-operated oocytes from which a small volume of nucleoplasm was aspirated, showed a ring-like structure around the nucleolus (Fig. 4-a) and had a nuclear envelope (27/27; Fig. 4-b). In enucleolated oocytes, chromatin formed a clump, and the nucleolus was absent from the oocytes (25/25; Fig. 4-d). However, the oocytes contained a nuclear envelope (Fig. 4-e).

By Hoechst staining, enucleolated oocytes were examined to observe their remaining chromatin. Sham-operated oocytes had a nucleolus in the GV (26/26; Fig. 5-a). The nucleolus that had been aspirated from the oocyte was placed under the zona pellucida (Fig. 5-d). The nucleolus did not show any Hoechst fluorescence, while the enucleolated oocytes retained the chromatin in the GV (24/24; Fig. 5-e and f). This suggests that the isolated nucleoli did not contain visible amounts of DNA.

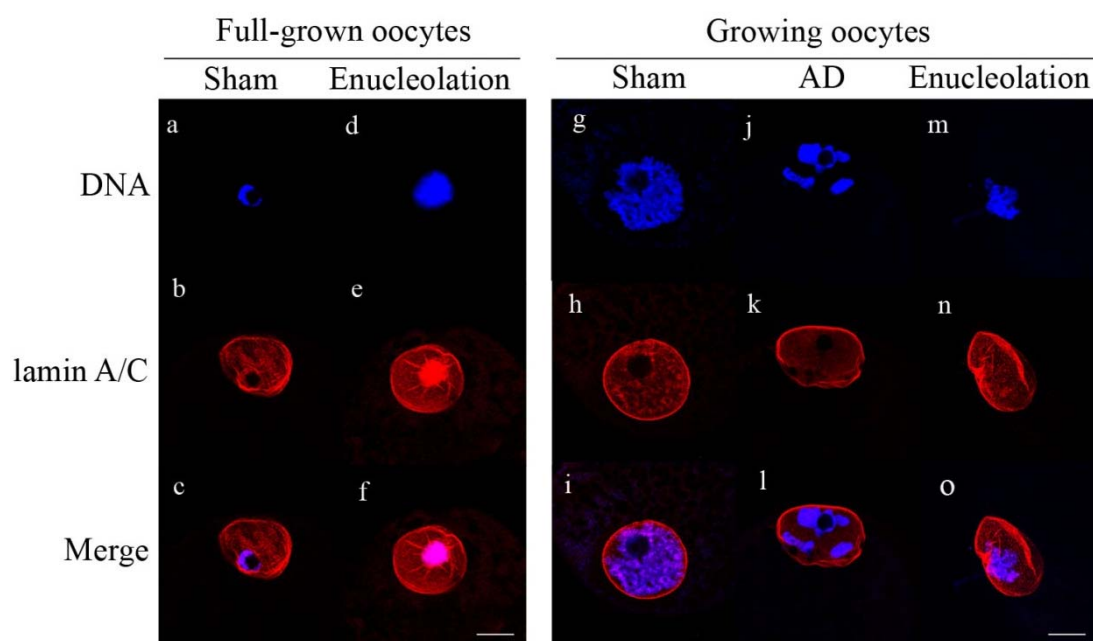


Fig. 4 After enucleolation, immunofluorescence labeling was performed with anti-lamin A/C antibody (in red) on sham-operated (Sham, a–c) and enucleolated full-grown pig oocytes (Enucleolation, d–f). Sham-operated growing oocytes (Sham, g–i), growing oocytes treated with 5 $\mu\text{g/ml}$ actinomycin D for 24 hr (AD, j–l), and enucleolated growing oocytes after treatment with actinomycin D (Enucleolation, m–o) were also stained immunocytoologically. The absence of the nucleolus is evident in enucleolated oocytes. DAPI staining marks chromatin in blue. Scale bars show 20 μm .

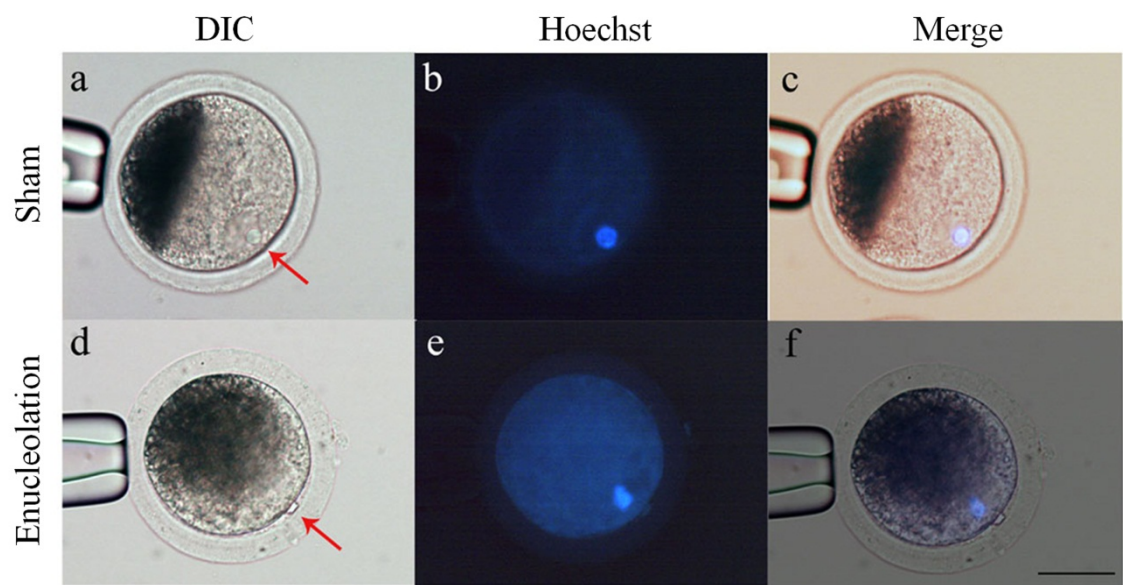


Fig. 5 Enucleolated full-grown pig oocytes maintained the chromatin. The nucleolus was placed between the oocyte plasma membrane and the zona pellucida. Arrows indicate the nucleoli. Hoechst staining marks chromatin in blue. Scale bar shows 50 μ m.

Maturation of Enucleolated Oocytes

Enucleolated oocytes were cultured for maturation. They progressed to metaphase I (MI) after 18 hr and metaphase II (MII) after 30 hr, in a time-course similar to that of sham-operated oocytes (Table 1). There was no difference in nuclear morphology between the enucleolated and sham-operated oocytes except in the GV stage. The chromatin in enucleolated oocytes made a clump in the GV as described above, and was similar in appearance to chromatin at the late diakinesis stage (Motlik and Fulka 1976).

Enucleolation of Growing Oocytes

Growing oocytes were collected from small antral follicles 0.6–1.0 mm in diameter. Their mean diameter was $100.1 \pm 3.8 \mu\text{m}$ (n=16). When the growing oocytes were enucleolated in the same enucleolation procedure to full-grown oocytes, the chromatin was aspirated together with the nucleolus from the oocytes. It was thought that the chromatin of growing oocytes was localized in the nucleolus as well as the nucleoplasm (Crozet *et al.* 1981). Several reports have shown that the chromatin is moved out of the nucleoli of growing oocytes by transcription inhibitors such as actinomycin D, which is an inhibitor of RNA polymerases I and II (Crozet 1983;

Table 1. Maturation stage of enucleolated pig oocytes

Type of oocytes	Culture time (hr)	Total No. of oocytes examined*	No. (%) of oocytes at the stage** of			No. (%) of degenerated oocytes
			GV***	MI	MII	
Sham	4	30	30 (100)	0 (0)	0 (0)	0 (0)
	18	32	7 (22) ^a	19 (59) ^a	0 (0)	6 (19)
	30	35	1 (3) ^b	9 (26) ^b	22 (62)	3 (9)
ENL	4	30	30 (100)	0 (0)	0 (0)	0 (0)
	18	30	6 (20) ^a	18 (60) ^a	0 (0)	6 (20)
	30	30	7 (23) ^a	0 (0)	16 (54)	7 (23)

*Sham-operated oocytes (Sham) and enucleolated oocytes (ENL) were cultured for 30 hr and examined by aceto-orcein staining.

**GV: germinal vesicle stage, MI: metaphase I, and MII: metaphase II.

***All of the enucleolated oocytes had a germinal vesicle which contained a clump of chromatin.

^{a,b} Values with different superscripts in the same column differ significantly ($P<0.05$).

Schultz *et al.* 1981). Thus, the growing oocytes were treated with actinomycin D (0.1, 5 and 10 $\mu\text{g/ml}$) for different culture time (6, 18 and 24 hr), to examine the chromatin that was expelled from the nucleolus (Table 2). During the culture, control oocytes (0 $\mu\text{g/ml}$ actinomycin D) were at the filamentous chromatin (FC) or stringy chromatin (SC) stage (Fig. 6-a, and b), and the nucleolus was stained by aceto-orcein as the oocytes before culture (Fig. 7-c). When oocytes were treated with any concentrations of actinomycin D, the nucleolus began to be compacted in the GV. After 18 or 24 hr with 5 or 10 $\mu\text{g/ml}$ actinomycin D, oocyte nucleoli were compacted and became aceto-orcein negative, and their chromatin became completely condensed (Fig. 6-d). However, at the concentration of 0.1 $\mu\text{g/ml}$, oocyte chromatin was still dispersed in the GV (Fig. 6-c), and at 10 $\mu\text{g/ml}$ actinomycin D, some of the oocytes degenerated. Based on this result, growing oocytes were cultured with 5 $\mu\text{g/ml}$ actinomycin D for 24 hr (Fig. 7-b and d) and their nucleoli were aspirated and used in the following experiments. Full-grown oocytes were also treated with actinomycin D in a similar manner, although the nucleolus morphology was not changed (Fig. 6-e).

Nucleolus aspiration from growing oocytes by manipulation was checked immunocytoologically. The chromatin of growing oocytes was dispersed in the GV (Fig. 4-g), and the oocytes had an intact nuclear envelope before actinomycin D treatment

Table 2. Effect of actinomycin D on nucleolus and chromatin morphology of growing pig oocytes

Inhibitor	(μg/ml)	Culture time (hr)	Total No. of oocytes examined*	No. (%) of oocytes						deg (%)
				orcin staining		type of chromatin**				
				+	-	FC	SC	specific 1	specific 2	
Control	-	0	10	10 (100)	0 (0)	10 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	-	6	17	17 (100)	0 (0)	13 (76)	4 (24)	0 (0)	0 (0)	0 (0)
	-	18	18	18 (100)	0 (0)	13 (72)	4 (28)	0 (0)	0 (0)	0 (0)
	-	24	16	11 (69)	0 (0)	7 (44)	4 (25)	0 (0)	0 (0)	5 (31)
AD	0.1	0	10	10 (100)	0 (0)	10 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	5		10	10 (100)	0 (0)	10 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	10		10	10 (100)	0 (0)	10 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	0.1	6	20	0 (0)	20 (100)	0 (0)	0 (0)	20 (100)	0 (0)	0 (0)
	5		19	0 (0)	17 (89)	0 (0)	0 (0)	16 (84)	1 (5)	2 (11)
	10		20	0 (0)	20 (100)	0 (0)	0 (0)	20 (100)	0 (0)	0 (0)
	0.1	18	19	0 (0)	15 (79)	0 (0)	0 (0)	15 (79)	0 (0)	4 (21)
	5		20	0 (0)	18 (90)	0 (0)	0 (0)	0 (0)	18 (90)	2 (10)
	10		20	0 (0)	19 (95)	0 (0)	0 (0)	0 (0)	19 (95)	1 (5)
	0.1	24	17	0 (0)	16 (94)	0 (0)	0 (0)	16 (94)	0 (0)	1 (6)
	5		19	0 (0)	17 (89)	0 (0)	0 (0)	0 (0)	17 (89)	2 (11)
	10		19	0 (0)	15 (79)	0 (0)	0 (0)	0 (0)	15 (79)	4 (21)

*Growing pig oocytes were cultured with actinomycin D (AD) for 6–24 hr. Control oocytes were treated with 0.5% (v/v) dimethyl sulfoxide. The oocytes were stained with aceto-orcin, and nucleolus status was classified into orcin (+) and orcin (-).

**FC: oocyte at the filamentous chromatin stage, SC: oocytes at the stringy chromatin stage, Specific 1: inhibitor-specific morphology of chromatin: nucleolus was compacted and the chromatin dispersed in GV, and Specific 2: the chromatin became more condensed than Specific 1 oocytes. See Fig. 6. deg: degeneration.

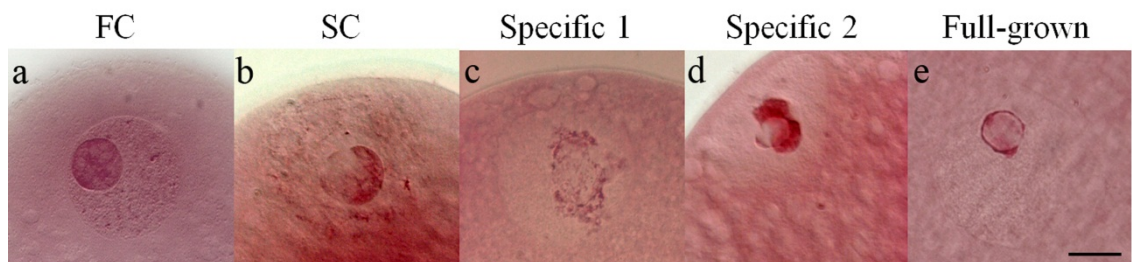


Fig. 6 Effect of actinomycin D on nucleolus and chromatin morphology of growing pig oocytes. Growing oocytes from small antral follicles were cultured in the medium supplemented with AD up to 24 hr. Control oocytes were cultured in the medium supplement with only solvent. After culture, oocytes were fixed with acetic acid and ethanol (1:3, v/v), stained with 1% aceto-orcein, and examined by the Nomarski interference microscopy. Control oocytes were at the filamentous chromatin (FC, a) or stringy chromatin (SC, b) stage, and the nucleolus was stained by aceto-orcein. After treatment with 0.1 µg/ml actinomycin D, nucleolus was not stained by aceto-orcein, however, the chromatin was still dispersed in the GV (Specific 1, c). When growing oocytes were cultured with 5 µg/ml actinomycin D for 24 hr, nucleoli were compacted and aceto-orcein negative, and their chromatin was completely condensed (Specific 2, d). Full-grown oocytes were also treated with actinomycin D in a similar manner, although the nucleolus morphology was not changed (e). Scale bar shows 10 µm .

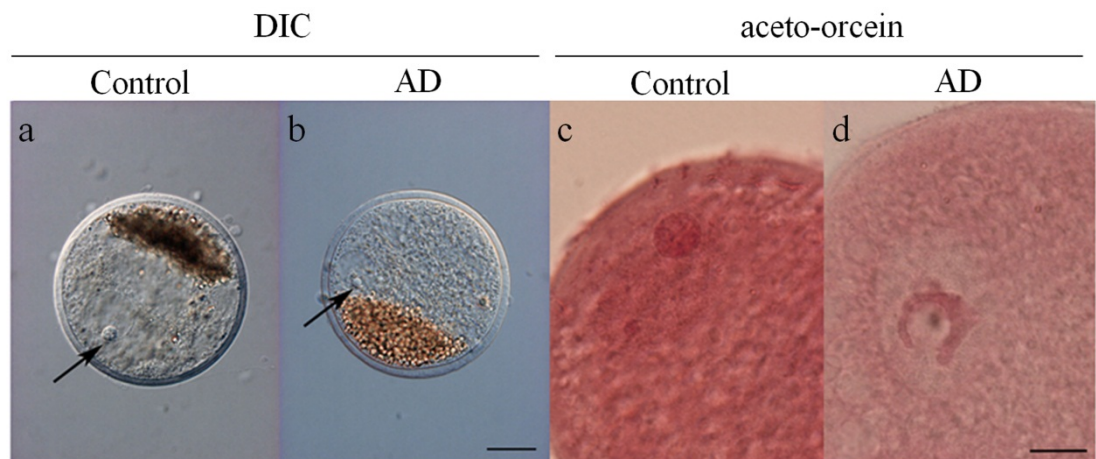


Fig. 7 Differential interference images of growing pig oocytes in whole-mount preparation after centrifugation (a and b), and staining with aceto-orcein (c and d). Growing oocytes (Control, a and c) were treated with 5 $\mu\text{g/ml}$ actinomycin D for 24 hr (AD, b and d). Arrows indicate the nucleoli. Scale bars in a,b and c,d show 40 and 10 μm , respectively.

(15/15, Fig. 4-h). Growing oocytes treated with actinomycin D formed clumps of chromatin in the GV, and their nucleoli were compacted (13/13, Fig. 4-l). After enucleolation, oocyte chromatin formed a clump, and the nucleolus was absent from the GV (16/16, Fig. 4-m). The oocyte nuclear envelope remained (Fig. 4-n).

Nucleolus Formation in Pronuclei of Enucleolated Oocytes

The nucleoli were collected from growing or full-grown oocytes, both of which had been treated with actinomycin D, were transferred to enucleolated and matured full-grown oocytes. The oocytes were then activated by electro-stimulation, and collected at 10 hr post-electro-stimulation to examine the nucleolus formation in the pronuclei using the anti-C23 (nucleolin) antibody (Ogushi *et al.* 2008) immunocytochemically. Sham-operated oocytes formed prominent nucleoli in pronuclei (Sham, 10/10; Fig. 8-a–c). No nucleolus was formed in enucleolated oocytes (ENL, 18/18; Fig. 8-d–f). On the other hand, enucleolated oocytes injected with nucleoli from full-grown oocytes formed nucleoli in pronuclei (ENL+fNL, 9/11; Fig. 8-g–i). Furthermore, nucleoli were formed in enucleolated oocytes injected with nucleoli from growing oocytes (ENL+gNL, 9/11; Fig. 8-j–l). Some oocytes injected with nucleoli formed pronuclei without a nucleolus. In these oocytes, the injected nucleoli remained

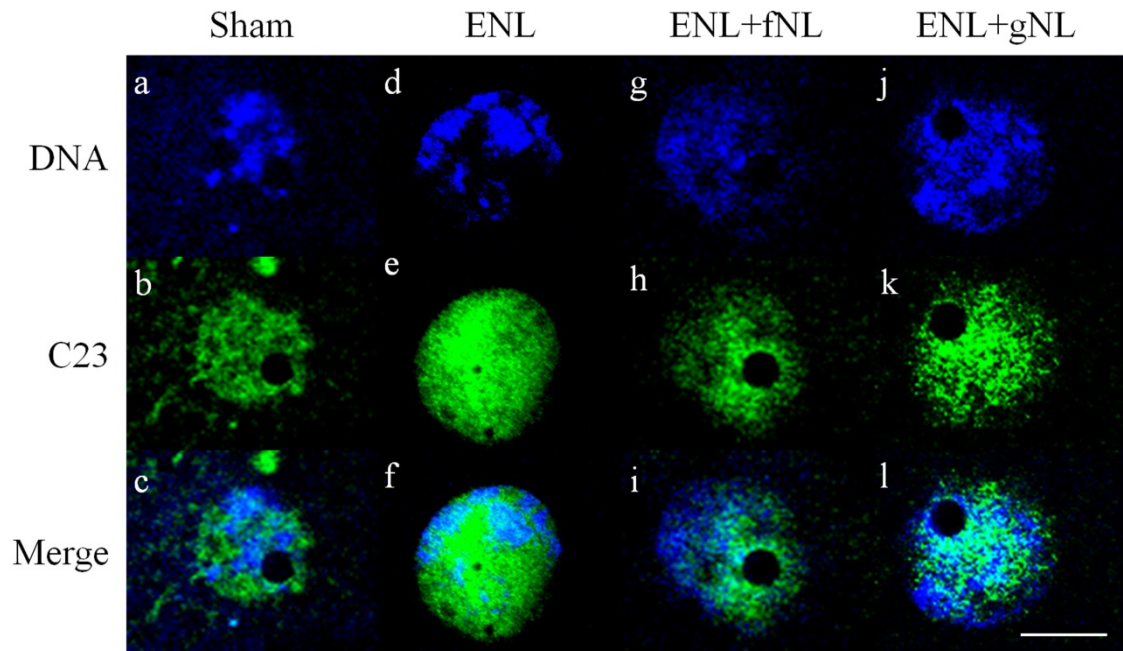


Fig. 8 Nucleolus formation in pronuclei of electro-activated sham-operated oocytes (Sham, a–c) and enucleolated oocytes after injection of the nucleolus from full-grown (ENL+fNL, g–i) or growing oocytes (ENL+gNL, j–l). No nucleolus was formed in enucleolated oocytes (ENL, d–f). Nucleoli in pronuclei were detected by immunofluorescence labeling with the antibody against C23 (in green). DAPI staining marks chromatin in blue. Scale bar shows 10 μ m.

in the cytoplasm, and C23 was localized around them as well as in the pronuclei.

Development of Enucleolated Oocytes

Table 3 shows the development of enucleolated full-grown oocytes that were matured, injected with nucleoli from full-grown oocytes (ENL+fNL) or growing oocytes (ENL+gNL), and thereafter electro-stimulated. Enucleolated full-grown oocytes (ENL) as well as sham-operated oocytes (Sham) and untreated oocytes (Control) were matured and electro-stimulated as controls. In all types of oocytes, the total percentages of oocytes cleaved 2 days after electro-stimulation (Day 2) were similar (Control: 90%, Sham: 84%, ENL: 91%, ENL+fNL: 80%, and ENL+gNL: 76%). The percentages of control and sham-operated oocytes that had developed to blastocysts at Day 7 were 53% and 43%, respectively. In contrast, no enucleolated oocytes developed to blastocysts. Some enucleolated oocytes were degenerated (12/31; 39%), while other oocytes remained at the 4-cell stage (15/31; 48%) at Day 7. The percentages of enucleolated oocytes injected with nucleoli from full-grown oocytes (ENL+fNL) or growing oocytes (ENL+gNL) that developed to blastocysts were 15% and 18%, respectively.

Table 3. In vitro development of enucleolated pig oocytes after reinjection of the nucleolus from growing oocytes

Type of oocytes	Total No. of oocytes examined*	No. (%) of oocytes at the stage of						
		Day 2				Day 7		
		1 cell	2-3 cell	4 cell	5-8 cell	deg	morula	blastocyst deg
Control	30	2 (7)	2 (7)	23 (76) ^{ab}	2 (7)	1 (3)	6 (20)	16 (53) ^a 0 (0)
Sham	30	4 (13)	0 (0)	24 (81) ^a	1 (3)	1 (3)	11 (37)	13 (43) ^a 2 (7)
ENL	31	1 (3)	3 (10)	25 (81) ^a	0 (0)	2 (6)	0 (0)	0 (0) 12 (39)
ENL+fNL	34	7 (21)	6 (18)	21 (62) ^{ab}	0 (0)	0 (0)	2 (6)	5 (15) ^b 10 (29)
ENL+gNL	34	3 (9)	7 (21)	18 (52) ^b	1 (3)	5 (15)	4 (12)	6 (18) ^b 6 (18)

* Enucleolated (ENL), sham-operated (Sham) and intact full-grown pig oocytes (Control) at the GV-stage were cultured for 30 hr in the maturation medium. The oocytes which had a first polar body were selected, and some of the ENL oocytes were injected with the nucleolus from full-grown oocytes (ENL+fNL) or growing oocytes (ENL+gNL) after treatment of actinomycin D. Then the oocytes were subjected to a single electric pulse (100 μ sec, 1,500 V/cm) and further cultured for 7 days in PZM3 (Yoshioka *et al.* 2002). Developmental stages were examined 2 and 7 days after electro-stimulation. deg: degeneration.

^{a,b} Values with different superscripts in the same column differ significantly (P<0.05).

Discussion

Transcription of the rRNA genes and the subsequent processing of rRNA to the formation of preribosomal particles are spatially organized within the nucleolus (Leary and Huang 2001; Maddox-Hyttel *et al.* 2007). At the ultrastructural level, the nucleoli of somatic cells and growing oocytes in mammals are composed of 3 components (FCs, DFCs, and GCs) (Crozet 1989; Olson *et al.* 2000). Pig oocyte nucleoli in early antral follicles are composed of GCs, vacuoles, a few FCs, and DFCs. In those oocytes, upstream binding factor (UBF) and the RNA polymerase I-associated factor PAF53 are localized to numerous FCs in the nucleolus, where rRNA synthesis occurs (Bjerregaard *et al.* 2004). The rRNA synthesis of pig oocytes starts to decrease in the early antral follicle stage, when the oocyte size is around 95 μm (Crozet 1989). The decrease in rRNA synthesis coincides with inactivation of the nucleolus, as visualized by the transformation of the fibrillogranular nucleolus to an electron-dense fibrillar sphere with remnants of the FCs at the surface (Bjerregaard *et al.* 2004). Finally, the nucleolus becomes completely compacted, and it stops the rRNA synthesis. Thus, the nucleoli of full-grown oocytes have no function of rRNA synthesis; however, a recent report has shown that the nucleoli of full-grown oocytes are essential for early embryonic

development (Ogushi *et al.* 2008).

In the present study, enucleolated full-grown pig oocytes matured similarly to sham-operated oocytes, a finding which confirmed that the maternal nucleolus was not necessary for oocyte maturation in the pig. Furthermore, enucleolated oocytes cleaved to the 4-cell stage similarly to sham-operated oocytes, whereas no enucleolated oocytes developed to blastocysts but remained at the 4-cell stage. It has been reported that the pig embryos without nucleoli at 4-cell stage were viable, having the ability to synthesize proteins at similar levels when compared to control embryos originating from sham-operated oocytes (Ogushi *et al.* 2008). After fertilization, embryos drive their development for one or several cell cycles using the stockpile of molecules originating from the oocytes. Therefore, it is speculated that early protein synthesis uses maternally stored ribosomes and RNAs. Functional nucleoli are assembled later on, when the embryonic genome becomes activated in the pig (Viuff *et al.* 2002). The nucleolar materials originating from oocytes may represent the precursor molecules required for the assembly of fully functional nucleoli at the later stage (Tesarik *et al.* 1987). The results in the present study indicate that the nucleolus is not essential for the first several cleavages after activation, although it is essential for the subsequent embryonic development as shown in the previous study (Ogushi *et al.* 2008). When the

enucleolated pig oocytes were injected with the nucleolus from full-grown oocytes, they recovered their ability to develop to blastocysts. Using this nucleolus injection system, ability of nucleoli from growing oocytes to support the development of enucleolated oocytes was examined.

When pig growing oocytes (100 μm in diameter) from small antral follicles were enucleolated, the chromatin was aspirated together with the nucleolus. Their nucleoli were larger than those in full-grown oocytes (120 μm in diameter) and the nucleolus is thought to contain ribosomal DNAs (rDNAs) which are localized on chromosomes with nucleolar organizing regions (Maddox-Hyttel *et al.* 2007). Several reports have shown that nucleoli in growing oocytes become compacted after being treated with actinomycin D (Crozet 1983; Schultz *et al.* 1981). After treatment of pig growing oocytes with actinomycin D in the present study, the nucleoli became compacted enough to be aspirated from the oocytes and the oocytes kept the chromatin as it was. Therefore, growing oocytes were enucleolated following actinomycin D treatment. The actinomycin D treatment also improved survival rate of enucleolated growing oocytes. Compacted nucleoli from actinomycin D-treated growing oocytes were used as in a comparison of their function to that of nucleoli in full-grown oocytes. Such compacted nucleoli from growing oocytes were able to substitute for the nucleoli

of full-grown oocytes. In the enucleolated oocytes that were injected with the nucleolus from growing oocytes, nucleoli were formed in pronuclei after electro-stimulation, although enucleolated oocytes did not form any nucleoli in the pronuclei. Moreover, the oocytes recovered their ability to develop to the blastocyst stage. These results suggest that the compacted nucleolus, from either full-grown oocytes or actinomycin-D-treated growing oocytes, is necessary for nucleolus formation in the pronucleus and for the development of embryos beyond 4-cell stage. The enucleolated oocytes that were injected with the nucleolus from full-grown oocytes treated with actinomycin D developed to blastocysts at a rate of 15%. On the other hand, the enucleolated oocytes that were injected with the nucleoli from non-treated full-grown oocytes developed to blastocysts at a rate of 25% (9/36 data not shown in Table 3). This result indicates that the developmental rate is decreased by actinomycin D treatment. I assume that the nucleolus treated with actinomycin D was more compacted than the nucleolus in non-treated oocytes, and that some of the injected oocytes were not able to reassemble the nucleolus properly.

Oocyte genomes also acquire the ability to support embryonic development during the oocyte growth. Bovine and mouse full-grown oocytes reconstructed with chromosomes from growing oocytes (bovine: 90–99 μm , mice: 50–60 μm in diameter)

developed to full term (Bao *et al.* 2000; Bao *et al.* 2003). In their experiment, the nuclei from growing oocytes were transferred to enucleated full-grown GV-oocytes, the reconstructed oocytes were matured, and the resulting MII chromosomes were re-transferred to MII cytoplasm. The reconstructed MII oocytes are thought to contain the nucleolus materials that originated from full-grown oocytes in the cytoplasm. Since the chromosomes from much smaller growing oocytes did not have the ability to support the embryonic development because of incomplete genome imprinting (Obata and Kono 2002), oocytes establish the genomic ability to support embryonic development during the growth phase. In the present experiment, the nucleoli of pig growing oocytes, which were treated with actinomycin D, supported embryonic development to a similar extent as the nucleoli in full-grown oocytes. Similar to the oocyte genomes, the oocyte nucleoli may prepare sufficient materials for early embryonic development before the completion of oocyte growth. However, growing oocytes still have to synthesize the proteins and RNAs that support their maturation and fertilization, and their nucleoli maintain the morphology that characterizes RNA and ribosome production in the ovary.

Chapter III

Nucleoli from Growing Oocytes Inhibit the Maturation of Enucleolated Full-grown Oocytes

Introduction

The growth of oocytes and their acquisition of meiotic competence are accompanied by a series of ultrastructural and biochemical changes (Eppig *et al.* 1996; Gosden *et al.* 1997; Picton *et al.* 1998). Profound change is also detected in the nucleolus (Zatsepina *et al.* 2000). Growing oocytes have nucleoli resembling to those in somatic cells, *i.e.* a typical fibrillogranular structure with intensive RNA synthesis, while nucleoli in full-grown oocytes differ completely. During the final stage of oocyte growth, the nucleoli have monotonous fibrillar morphology and become compact with decreased RNA synthesis (Crozet 1989; Motlik *et al.* 1984b).

The results of Chapter II showed that growing oocyte nucleoli recovered the embryonic developmental ability of enucleolated full-grown oocytes, indicating that the nucleoli of growing oocytes have accumulated the materials required for early

embryonic development similar to full-grown oocyte nucleoli. However, the role of the growing oocyte nucleoli in oocyte maturation remains to be revealed.

Growing pig oocytes from small antral follicles of 0.6–1.0 mm in diameter have limited meiotic competence under the culture condition where full-grown oocytes from large antral follicles of 4–5 mm in diameter show full meiotic competence to mature to MII. After removal of the nucleolus from growing oocytes, some of them underwent germinal vesicle breakdown (GVBD) (Fulka *et al.* 2003), suggesting the possible inhibitory role of the growing oocyte nucleoli in meiotic resumption.

The objective of the present chapter is to show the role of growing oocyte nucleoli in oocyte maturation in the pig. Nucleoli from growing oocytes were injected into enucleolated growing and full-grown oocytes at the GV stage, and maturation of the oocytes was examined after injection. Nucleolus reassembly in GV after injection of the nucleolus into oocyte cytoplasm was also examined in enucleolated oocytes.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Collection of Cumulus-oocyte Complexes

The collection methods of small (0.6–1.0 mm in diameter) and large (4.0–5.0 mm in diameter) antral follicles from pig ovaries were described in Chapter II. After the follicles were opened in 25 mM HEPES-buffered medium 199 (HEPES-199) containing 0.1% PVA and 50 µg/ml gentamicin (GIBCO Invitrogen, Prague, Czech Republic), cumulus-oocyte complexes (COCs) were isolated from the follicles.

Preparation of Nucleolus Donor Oocytes

COCs containing growing and full-grown oocytes, as the nucleolus donors, were cultured in the medium supplemented with 5 µg/ml actinomycin D and 2 mM dbcAMP for 24 hr before enucleation. The culture medium was bicarbonate-buffered medium 199 supplemented with 4 mg/ml growth protein of bovine serum (GPBoS; Sevapharma, Prague, Czech Republic), 0.5 µg/ml FSH (from pig pituitary), 0.5 µg/ml

LH (from bovine pituitary), 40 µg/ml sodium pyruvate, 50 µg/ml gentamicin, and 70 µg/ml L-cysteine.

Experimental Design

The following three experiments were conducted to elucidate the role of growing oocyte nucleoli in oocyte maturation.

i) Maturation of Enucleolated Growing Oocytes

To compact oocyte nucleoli, COCs containing growing oocytes were treated with actinomycin D for 24 hr except the control group and then cumulus cells were removed completely before micromanipulation (Fig. 9). After enucleolation, each enucleolated oocyte was injected with a nucleolus from growing or full-grown donor oocytes. After micromanipulation, all types of oocytes were cultured for maturation for 30 hr. Enucleolation and nucleolus injection methods are described below.

ii) Maturation of Full-grown Oocytes Injected with Nucleoli from Growing Oocytes

After cumulus cells were removed from COCs containing full-grown oocytes, the oocytes were pre-cultured for 4 hr (Fig. 10-A). Then each oocyte was injected with a nucleolus isolated from full-grown or growing donor oocytes. After micromanipulation, oocytes were cultured for maturation for 30 hr.

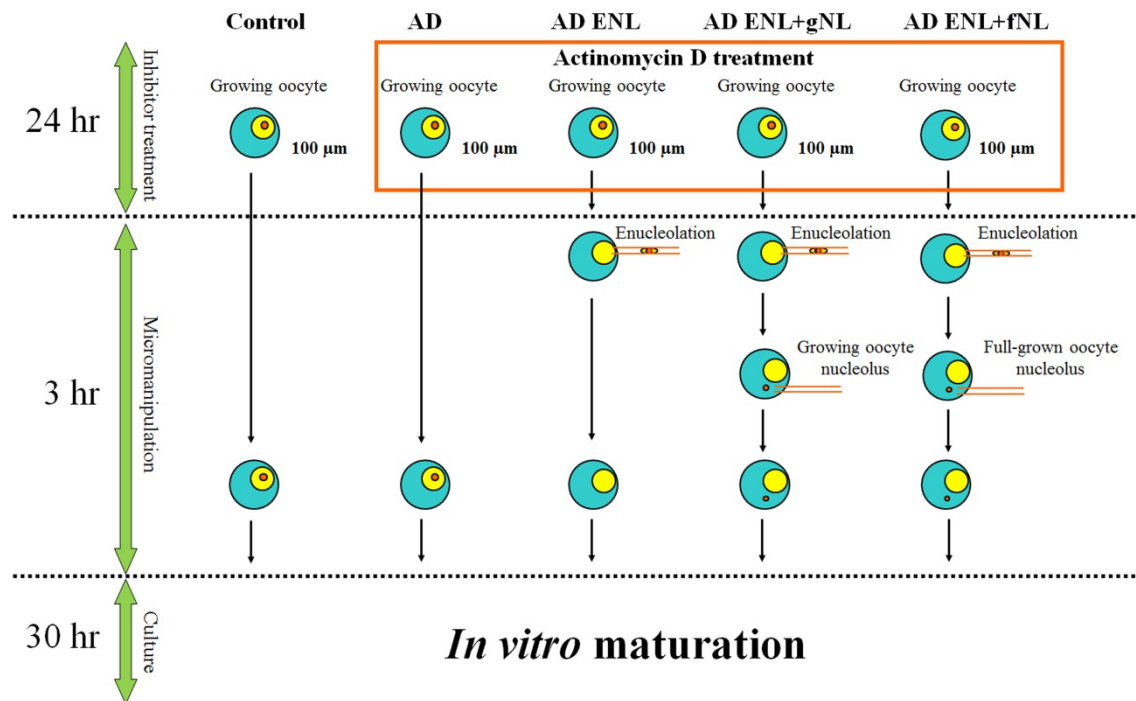


Fig. 9 Experimental scheme of growing oocyte enucleation and nucleolus injection. Growing oocytes were treated with actinomycin D (AD) for 24 hr before enucleation. Nucleoli were microsurgically aspirated from growing oocytes to produce enucleolated oocytes (AD ENL). Isolated nucleoli from growing or full-grown oocytes were injected into cytoplasm of enucleolated growing oocytes (AD ENL+gNL and AD ENL+fNL, respectively). Nucleolus donor oocytes were treated with actinomycin D for 24 hr before collection of the nucleoli.

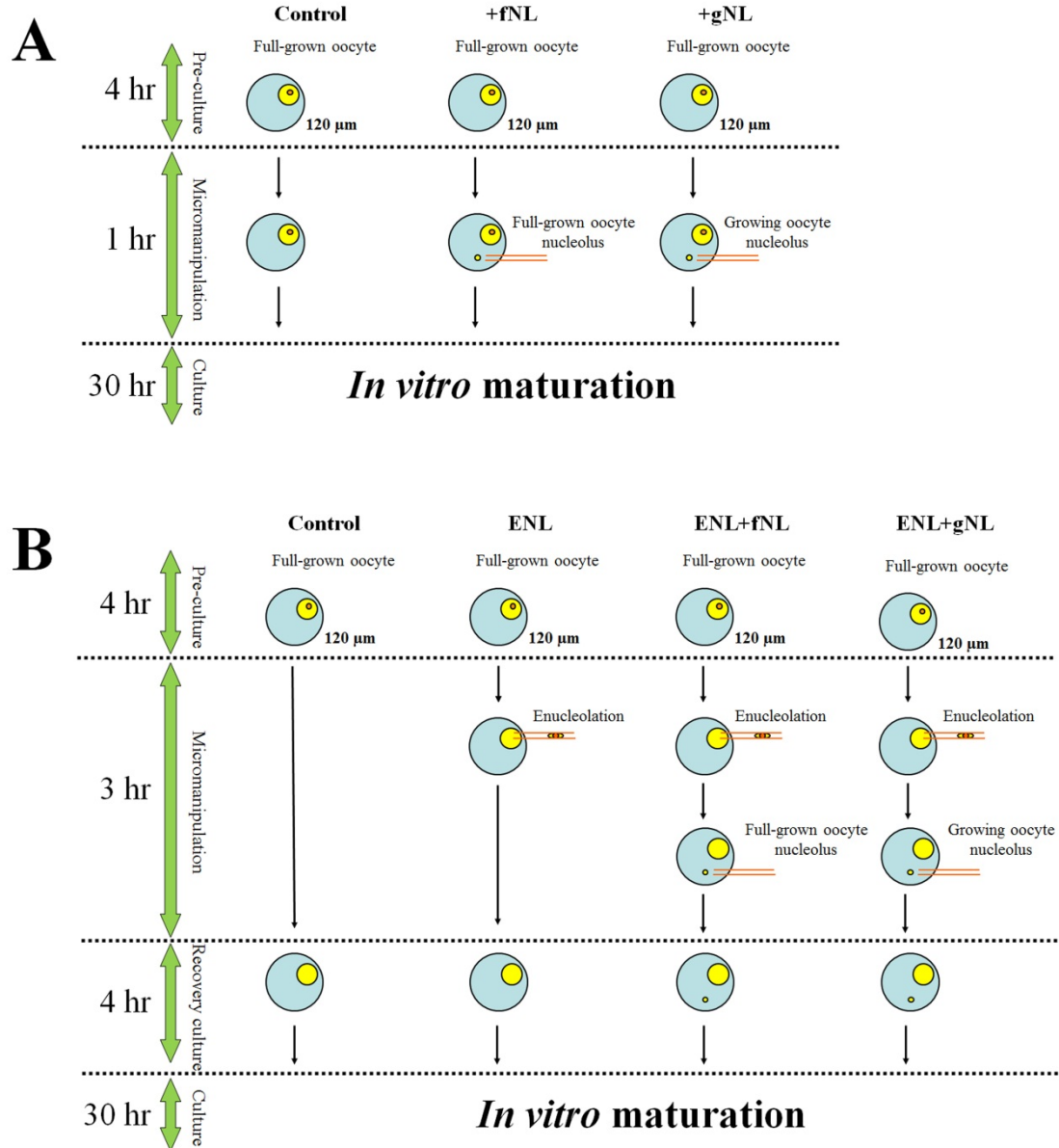


Fig. 10 Experimental schemes of nucleolus injection using intact (A) and enucleolated (B) full-grown oocytes. A: Isolated nucleoli from full-grown or growing oocytes were injected into cytoplasm of full-grown GV oocyte (+fNL and +gNL, respectively). B: Nucleoli were microsurgically aspirated from full-grown oocytes to produce enucleolated oocytes (ENL). Isolated nucleoli from full-grown or growing oocytes were injected into cytoplasm of enucleolated full-grown oocytes (ENL+fNL and ENL+gNL, respectively). Nucleolus donor oocytes were treated with actinomycin D for 24 hr before collection of the nucleoli.

iii) Maturation of Enucleolated Full-grown Oocytes Injected with Nucleoli from Growing Oocytes

After cumulus cells were removed from COCs containing full-grown oocytes, the oocytes were pre-cultured for 4 hr before enucleolation (Fig. 10-B). Then each full-grown oocyte was enucleolated and injected with a nucleolus isolated from full-grown or growing donor oocytes. After micromanipulation, all types of oocytes were recovery-cultured for 4 hr in culture medium supplement with 2 mM dbcAMP, followed by maturation culture for 30 hr.

Enucleolation and Nucleolus Injection

Oocytes were enucleolated as described in Chapter II with small modification. Briefly, full-grown and growing oocytes were transferred into HEPES-199 containing 12.5 µg/ml cytochalasin B and 0.01 µg/ml demecolcine for 10 min. Then the full-grown and growing oocytes were centrifuged at 4,000 g and 3,500 g, respectively, for 10 min to move the lipid droplets to one side of the oocytes. Oocytes were enucleolated in a plastic dish (60×15 mm, Nunclon) used as a micromanipulation chamber which was put on a warmed stage (38.5°C) in an inverted microscope (Olympus Optical, Co.).

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) equipped with a PIEZO drive. After penetration through the zona pellucida, the injection pipette was positioned against the plasma and GV membranes. Thereafter, gentle suction was applied, which resulted in suctioning 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 plasma membrane, additional suction was applied. The nucleolus thereafter penetrated the GV membrane, leaving the entire GV content within the nuclear envelope. After enucleation, the oocytes were washed 9 times in HEPES-199.

After enucleation, the oocytes were recovery-cultured for at least 1 hr. The nucleoli collected from nucleolus donor oocytes were injected into the cytoplasm of enucleolated growing oocytes and intact or enucleolated full-grown oocytes at the GV stage as described above. Injection of the nucleoli was performed in a similar way to intracytoplasmic sperm injection (Katayama *et al.* 2005). Each oocyte was held by a holding pipette (inner diameter 10 μm , angle 30°) at the 9 o'clock position. The zona pellucida was punctured by several applications of piezo-pulse. Then the nucleolus,

which had been aspirated into an injection pipette (inner diameter 8–10 μm), was ejected into the deep cytoplasm of the oocyte. After injection, the oocytes were washed 9 times in HEPES-199.

Maturation Culture of Oocytes

After micromanipulation, growing and full-grown oocytes were cultured for maturation in 0.5 ml of culture medium in a 4-well dish (4 Well Multidish; Nunclon) at 38.5°C under an atmosphere of 5% CO_2 in air for 30 hr. Before and after culture, the oocytes were fixed and stained with 1% aceto-orcein, and examined the maturation stage using Nomarski interference microscopy. The oocytes were classified by the morphology of the chromatin and nuclear envelope as described in Chapter II.

Fluorescence Microscopy

Some of the enucleolated and nucleolus-injected full-grown oocytes were collected after 4 hr of recovery culture (Fig. 10-B) to chase the injected nucleoli. The oocytes were fixed in 3.7% paraformaldehyde in PHEM buffer (60 mM PIPES, 20 mM HEPES, 10 mM EGTA, and 1 mM MgCl_2) for 40 min. After the oocytes were washed twice in PHEM-buffer for 15 min each, they were mounted on glass slides with

ProLong Gold Antifade Reagent with DAPI (P36931, Molecular Probes) and observed under a fluorescence microscope (IX70-S1F2; Olympus Optical Co.).

Statistical Analysis

The frequencies of oocytes at each stage of maturation were analyzed using the chi-square test. P values less than 0.05 were considered to indicate statistical significance.

Results

Maturation of Enucleolated Growing Oocytes

Growing oocytes were treated with actinomycin D (AD) for compacting their nucleoli for 24 hr, enucleolated and cultured for maturation for 30 hr. Table 4 shows the maturation stage of enucleolated growing oocytes (AD ENL), and enucleolated growing oocytes that were injected with nucleoli from full-grown oocytes (AD ENL+fNL) or growing oocytes (AD ENL+gNL). Before culture, all oocytes were at the GV stage (32/32, Fig. 11-a). Most of control oocytes (Control) arrested at the GV stage after maturation culture (41/47, 87%, Fig. 11-b). Actinomycin D-treated oocytes (AD) were also arrested at the GV stage (60/68, 88%) whose nucleolus was compacted (Fig. 11-c). However, 46% (21/46) of enucleolated growing oocytes underwent GVBD and progressed to the late diakinesis (LD) stage or beyond (Fig. 11-d). These results agree with the previous results that showed enucleolated growing oocytes underwent GVBD (Fulka *et al.* 2003). Enucleolated growing oocytes injected with nucleoli from growing oocytes arrested at the GV stage again (34/44, 77%, Fig. 11-e). On the other hand, 49% (20/41) of growing oocytes injected with nucleoli from full-grown oocytes underwent GVBD (Fig. 11-f). These results show that growing oocyte nucleolus inhibits GVBD

Table 4. Maturation stage of enucleolated growing pig oocytes

Treatment	Total No. of oocytes examined*	No. (%) of		No. (%) of oocytes at the stage** of				
		degenerated oocytes	surviving oocytes	GV	LD	MI	MII	
Control	54	7 (13) ^a	47 (87) ^a	41 (87) ^a	5 (11) ^a	1 (2)	0 (0)	
AD	79	11 (14) ^a	68 (86) ^a	60 (88) ^a	5 (7) ^a	2 (3)	1 (2)	
AD ENL	84	38 (45) ^b	46 (55) ^b	25 (54) ^b	18 (39) ^b	2 (5)	1 (2)	
AD ENL+gNL	89	45 (51) ^b	44 (49) ^b	34 (77) ^a	7 (16) ^{ac}	3 (7)	0 (0)	
AD ENL+fNL	100	59 (59) ^b	41 (41) ^b	21 (51) ^b	14 (34) ^{bc}	5 (12)	1 (3)	

* Growing oocytes were cultured without (Control) or with actinomycin D (AD) for 24 hr. Some AD-treated oocytes were enucleolated (AD ENL) and injected with the nucleolus from growing oocytes (ENL+gNL) or full-grown oocytes (ENL+fNL). All of the oocytes were then cultured for maturation for 30 hr.

** After maturation culture, surviving oocytes were stained with aceto-orcein and examined the maturation stage. GV: germinal vesicle stage, LD: late diakinesis stage, MI: metaphase I, and MII: metaphase II.

^{a-c} Values with different superscripts in the same column differ significantly (P<0.05).

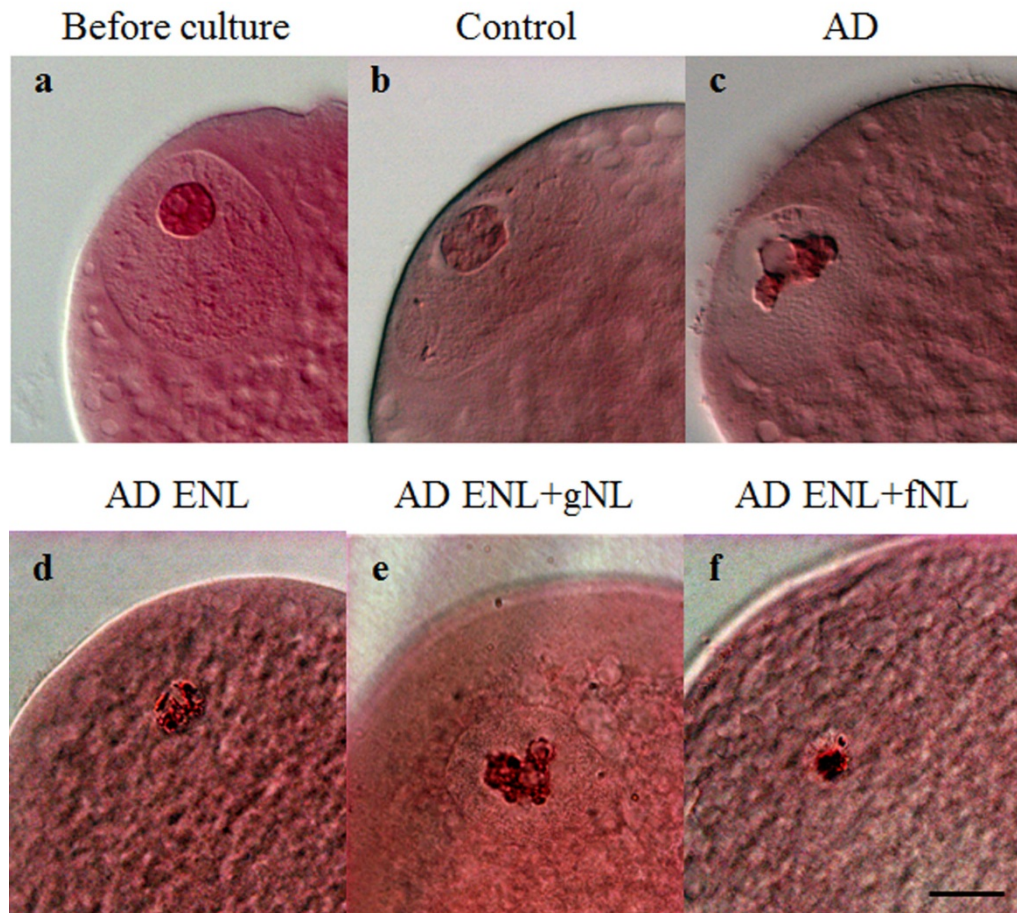


Fig. 11 Differential interference contrast images of growing pig oocytes before (Before culture, a) and after (Control, b) maturation culture for a total of 54 hr. The oocytes were whole-mounted and stained with aceto-orcein. A group of growing oocytes were treated with 5 μ g/ml actinomycin D (AD) for 24 hr and cultured for maturation for 30 hr (c). Other growing oocytes were enucleolated after actinomycin D treatment (AD ENL, d), injected with a nucleolus from growing (AD ENL+gNL, e) or full-grown oocytes (AD ENL+fNL, f), and further cultured for maturation for 30 hr. Scale bar shows 10 μ m.

and this ability is not present in the full-grown oocyte nucleolus.

Maturation of Full-grown Oocytes Injected with Nucleoli from Growing Oocytes

Effect of growing oocyte nucleolus on the maturation of full-grown oocytes was examined. Nucleoli from growing oocytes were transferred to intact full-grown oocytes. The oocytes were cultured for 30 hr and examined the maturation stage (Table 5). Intact oocytes (Control) matured to MII at 78%. Full-grown oocytes injected with nucleoli from full-grown oocytes matured to MII at 60%. However, the maturation rate of full-grown oocytes that were injected with nucleoli from growing oocytes significantly decreased to 41%, and 44% (18/41) of oocytes stopped at the LD or MI stage.

Maturation of Enucleolated Full-grown Oocytes Injected with Nucleoli from Growing Oocytes

The above experiment suggests that the nucleoli from growing oocytes have an inhibitory effect on oocyte maturation. To confirm it, full-grown oocytes were enucleolated and then they were injected with nucleoli from full-grown or growing oocytes. First, nucleoli from growing or full-grown oocytes were injected into the cytoplasm of enucleolated full-grown oocytes at the GV stage, and the oocytes were

Table 5. Maturation stage of full-grown pig oocytes after cytoplasmic injection of an additional nucleolus from growing oocytes

Treatment	Total No. of oocytes examined*	No. (%) of		No. (%) of surviving oocytes	No. (%) of oocytes at the stage** of		
		degenerated oocytes	oocytes		GV	LD	MI
Control	49	4 (8) ^a	45 (92) ^a	3 (7)	2 (4)	5 (11)	35 (78) ^a
+fNL	56	16 (29) ^b	40 (71) ^b	6 (15)	6 (15)	4 (10)	24 (60) ^{ab}
+gNL	50	9 (18) ^{ab}	41 (82) ^{ab}	6 (15)	7 (17)	11 (27)	17 (41) ^b

* Intact full-grown oocytes (Control) were injected with an additional nucleolus from full-grown oocytes (+fNL) or growing oocytes (+gNL) and cultured for maturation for 30 hr.

** After maturation culture, surviving oocytes were stained with aceto-orcein and examined the maturation stage.

GV: germinal vesicle stage, LD: late diakinesis stage, MI: metaphase I, and MII: metaphase II.

^{a,b} Values with different superscripts in the same column differ significantly (P<0.05).

cultured for 4 hr with dbcAMP and examined possible reassembly of the nucleolus in the GV (Table 6). Intact oocytes (Control) had a nucleolus in the GV and the chromatin surrounded the nucleolus (30/30, Fig. 12-a–c). No nucleoli were formed in the GV of enucleolated oocytes and their chromatin formed some clumps in the GV (31/31, Fig. 12-d–f). However, when enucleolated full-grown oocytes were injected with nucleoli from full-grown oocytes, they formed nucleoli in the GV and their chromatin surrounded the nucleolus again (22/30, Fig. 12-g–i). Furthermore, nucleoli were formed in enucleolated full-grown oocytes after injection of nucleoli from growing oocytes (18/30, Fig. 12-j–l). Some of the oocytes formed multiple nucleoli in the GV (ENL+fNL: 32%, ENL+gNL: 27%).

Next, enucleolated full-grown oocytes cultured with dbcAMP for 4 hr after nucleolus injection were matured for 30 hr (Table 7). Enucleolated oocytes (ENL) matured to MII (73%) which percentage was similar to that of control oocytes (Control). Enucleolated oocytes injected with nucleoli from full-grown oocytes (ENL+fNL) matured to MII (56%). The value was lower than that of the control oocytes but not significantly different from that of enucleolated full-grown oocytes. However, maturation rate of enucleolated oocytes injected with nucleoli from growing oocytes (ENL+gNL) was significantly low (21%). Half of the oocytes stopped at the LD stage

Table 6. Nucleolus assembly in the GV of enucleolated full-grown pig oocytes after cytoplasmic injection of a nucleolus from full-grown or growing oocytes

Treatment	Total No. of oocytes examined*	No. (%) of oocytes			
		nucleolus		nucleolus number	
		+	-	single	multi
Control	30	30 (100)	0 (0)	30 (100)	0 (0)
ENL	31	0 (0)	31 (100)	0 (0)	0 (0)
ENL+fNL	30	22 (73)	8 (27)	15 (50)	7 (23)
ENL+gNL	30	18 (60)	12 (40)	15 (50)	3 (10)

*Enucleolated full-grown oocytes (ENL) were injected with a nucleolus from full-grown oocytes (ENL+fNL) or growing oocytes (ENL+gNL). They were culture for 4 hr after nucleolus injection, and formation of the nucleolus in the GV was examined by the DIC microscope.

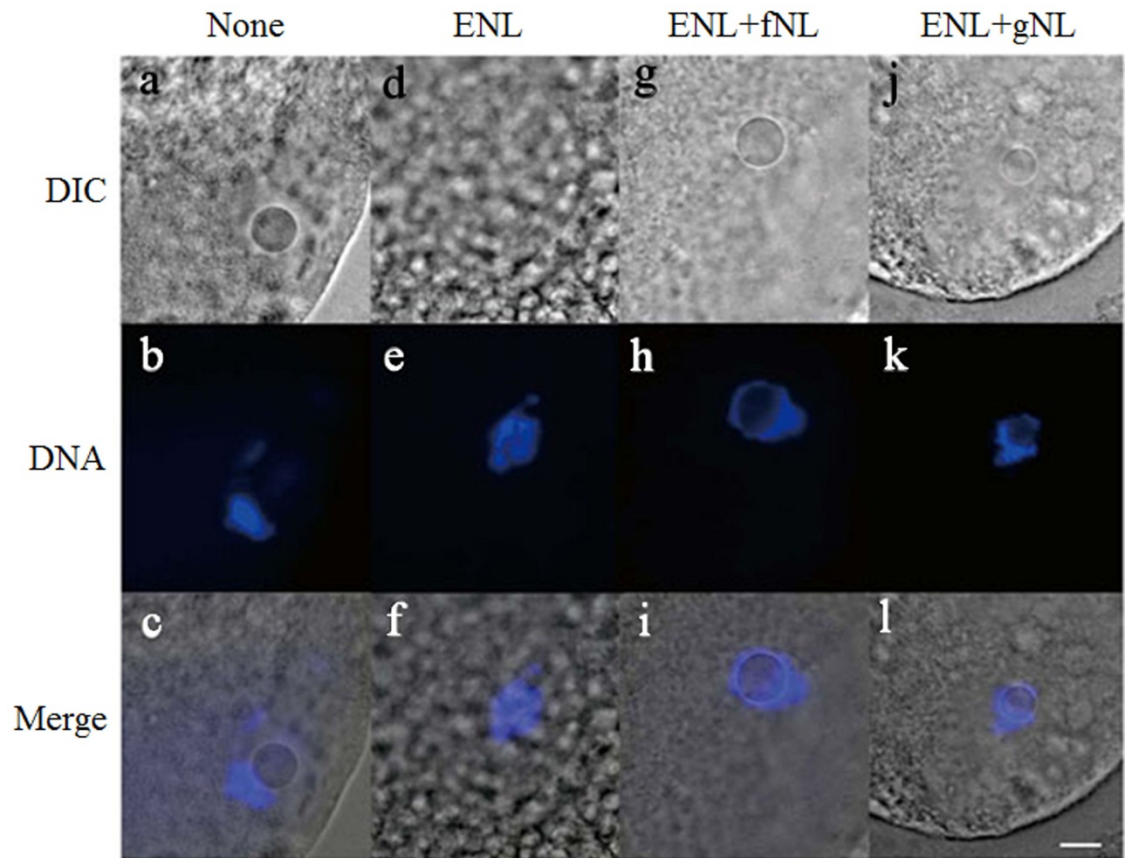


Fig. 12 Nucleoli in the GV of intact pig oocytes (Control, a–c) and enucleolated full-grown oocytes after cytoplasmic injection of a nucleolus from full-grown (ENL+fNL, g–i) or growing oocytes (ENL+gNL, j–l). No nucleolus was found in the enucleolated oocytes (ENL, d–f). DAPI staining marks chromatin in blue. Scale bar shows 10 μm .

Table 7. Maturation stage of enucleolated full-grown pig oocytes after cytoplasmic injection of growing oocyte nucleoli

Treatment	Total No. of oocytes examined*	No. (%) of degenerated oocytes	No. (%) of surviving oocytes	No. (%) of oocytes at the stage** of			
				GV	LD	MI	MII
Control	54	3 (6) ^a	51 (94) ^a	1 (2) ^a	6 (11) ^a	1 (2) ^a	43 (84) ^a
ENL	60	7 (12) ^a	53 (88) ^a	2 (4) ^a	3 (6) ^a	9 (17) ^b	39 (73) ^{ab}
ENL+fNL	87	38 (44) ^b	49 (56) ^b	5 (10) ^{ab}	8 (16) ^a	9 (18) ^b	27 (56) ^b
ENL+gNL	88	40 (45) ^b	48 (55) ^b	10 (21) ^b	23 (48) ^b	5 (10) ^{ab}	10 (21) ^c

* Intact full-grown oocytes (Control) were enucleolated (ENL) and injected with the nucleolus from full-grown oocytes (ENL+fNL) or growing oocytes (ENL+gNL). All of the oocytes were then cultured for maturation for 30 hr.

** After maturation culture, surviving oocytes were stained with aceto-orcin and examined the maturation stage.

GV: germinal vesicle stage, LD: late diakinesis stage, MI: metaphase I, and MII: metaphase II.

^{a-c} Values with different superscripts in the same column differ significantly (P<0.05).

(48%). These results show that the nucleoli from growing oocytes have the inhibitory effect on maturation of full-grown oocytes.

Discussion

The nucleoli of growing pig oocytes, which were treated with actinomycin D, supported embryonic development to a similar extent as the nucleoli in full-grown oocytes in Chapter II. Chapter II also showed that growing oocyte nucleoli were able to be collected, when oocytes were treated with actinomycin D. Using this enucleolation method for growing oocytes, the role of the growing oocyte nucleolus in oocyte maturation was examined in this chapter.

In the ovary, the increase in oocyte volume and morphological changes in the chromatin and nucleolus progress in a synchronous manner so that the oocytes develop the ability to mature, to be fertilized and to develop. Thus the oocyte size (Kanayama *et al.* 2002; Motlik *et al.* 1984a), nucleolus morphology (Maddox-Hyttel *et al.* 2007) and chromatin morphology (Hirao *et al.* 1995; Inoue *et al.* 2008) are thought to be important for the development of embryos and often served as the markers of the ability of oocyte maturation and early embryonic development. Pig oocytes acquire the ability to resume meiosis when they approach the end of the growth phase in antral follicles larger than 0.8 mm in diameter. They acquire the capacity to complete meiotic maturation to MII at a follicular diameter of larger than 2 mm (Motlik *et al.* 1984a; Tsafiriri and Channing

1975). This acquisition of full meiotic competence coincides with the morphological change in the nucleolus. In the present experiment, growing pig oocytes collected from 0.6–1.0 mm follicles were around 100 μm in diameter (Chapter II). Those oocytes do not have the ability to support oocyte maturation and development (Kanayama *et al.* 2002). The limited meiotic competence in the growing oocytes has been thought to be due to insufficient amount of Cdc2 (Hampl and Eppig 1995; Hirao *et al.* 1995), inability to activate Cdc2 kinase, incomplete MEK-MAP-kinase pathway (Kanayama *et al.* 2002), and continuous activation of Wee1 B (Nishimura *et al.* 2009).

In the first experiment in this chapter, enucleolated growing pig oocytes underwent GVBD. This finding confirms the results of previous report showing growing oocyte nucleoli have an inhibitory effect of GVBD (Fulka *et al.* 2003), and may indicate that the nucleoli play roles in cell cycle regulation and in meiotic arrest of growing oocytes. In the present study, growing oocytes were enucleolated followed by actinomycin D treatment, whereas enucleolation was performed without any treatment in previous study (Fulka *et al.* 2003). When the enucleolated growing oocytes were injected with the nucleolus from growing oocytes, they restored the ability to maintain the GV stage. On the other hand, enucleolated growing oocytes injected with the nucleolus from full-grown oocytes underwent GVBD. This result indicates that the

full-grown oocyte nucleolus has lost the inhibitory ability on the meiotic resumption of oocytes.

In the next step, intact and enucleolated full-grown oocytes were injected with the nucleolus from growing oocytes. Growing oocyte nucleoli inhibited the maturation of full-grown oocytes, and the inhibitory effect was more substantial in enucleolated full-grown oocytes than that in intact full-grown oocytes. However, enucleolated full-grown oocytes injected with the nucleolus from full-grown oocytes matured to MII. These results indicate again that growing oocyte nucleolus has an inhibitory effect on oocyte maturation, and that the full-grown oocyte nucleolus has lost the ability.

The role of the nucleolus in the cell cycle regulation has been documented in studies in yeasts (Buonomo *et al.* 2003; Cockell and Gasser 1999; Visintin and Amon 2000; Visintin *et al.* 1999) and somatic cells (Hudson *et al.* 2001). In these cells, cell cycle regulatory molecules, such as CDC14, Mdm2 and Pch2 are present in the nucleolus. Although the roles of these molecules have not been determined in oocyte maturation in mammals, there is a report showing that CDC14A regulates oocyte maturation and have function to promote the meiosis I to meiosis II transition (Schindler and Schultz 2009a). However, it is not localized in the oocyte nucleolus (Schindler and Schultz 2009b). Cell-cycle regulators of meiotic resumption or progression in the

nucleolus of mammalian oocytes have not been understood well.

When the nucleoli from full-grown and growing oocytes were injected into the cytoplasm of enucleolated full-grown oocytes, these nucleoli disappeared and was reassembled in the GV of enucleolated oocytes. In the GV, oocyte chromatin contacted with the reassembled nucleolus. Nucleoli were formed in pronuclei of activated enucleolated-matured oocytes after reinjection with nucleoli at MII stage (Ogushi *et al.* 2008). Since the oocyte nucleoli in the GV disappear during oocyte maturation and reappear in the pronucleus of zygotes, it is reasonable that injected nucleoli disappear in oocyte cytoplasm at MII stage. However, the nucleolus disappeared in oocyte cytoplasm at the GV stage in the present study. After injection, the nucleolus couldn't be observed under the light microscope. The nucleolus might dissolve in cytoplasm and move to inside of the GV, then reassemble in the GV. One previous report with electron microscope observations indicates that small nucleoli (called tertiary nucleolus) were extruded from pronuclei in rat zygotes (Szollosi 1965). The mechanism of disappearing, moving and reassembly of the nucleolus in GV oocytes is remained to be revealed.

In summary, in this chapter, nucleoli from growing oocytes inhibit the maturation of enucleolated full-grown oocytes. During oocyte growth, the growing oocyte nucleolus may relate to the meiotic arrest of the oocytes in the GV, and the

full-grown oocyte nucleolus has lost this ability.

Chapter IV

Nucleoli from 2-cell Embryos Support the Development of Enucleolated

Full-grown Oocytes

Introduction

In mammalian oocytes, the germinal vesicle (GV) contains the specific nucleolus that exhibits distinct structural and functional differences from the nucleolus of somatic cells. After GVBD, the nucleolus disappears in the cytoplasm of oocytes, and upon fertilization, nucleoli are formed in male and female pronuclei. Subsequently, embryonic nucleogenesis occurs in parallel with the activation of embryonic rRNA synthesis. Until the 2-cell stage, some components of the nucleolus are similar to the nucleolus of GV oocytes (Hyttel 2011), and no transcriptional activity is detected in the nuclei (Tomanek *et al.* 1989). After the 2-cell stage, the nucleoli become somatic cell-type by the morula stage (Tomanek *et al.* 1989).

Based on the morphological similarities between nucleoli in GV oocytes and 2-cell embryos, nucleolus replacement experiments were conducted in this chapter in order to determine the ability of nucleoli from 2-cell embryos to substitute for nucleoli

of GV oocytes in terms of supporting embryonic development. In addition, I report here the fusion of multiple nucleoli in 2-cell embryos by centrifugation of the embryos.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Collection and Culture of Oocytes

The collection methods of large antral follicles (4.0–5.0 mm in diameter) from pig ovaries were described in Chapter II. After the follicles were opened in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical Co. Ltd.) containing 0.1% PVA, 0.85 mg/ml sodium bicarbonate (Wako Pure Chemical Industries), and 0.08 mg/ml kanamycin sulphate, cumulus-oocyte complexes (COCs) were isolated from the follicles. The cumulus cells were then completely removed from GV oocytes by pipetting to obtain denuded oocytes.

Some denuded oocytes were enucleolated as described below and cultured for maturation in 0.5 ml of culture medium in a 4-well dish (4-Well Multidish, Thermo Fisher Scientific K.K.) at 38.5°C under an atmosphere of 5% CO₂ in air for 30 hr. The culture medium was bicarbonate-buffered medium 199 supplemented with 10% FBS (ICN Biomedicals, Inc.), 0.1 mg/ml sodium pyruvate (Nakalai Tesque), 0.08 mg/ml

kanamycin sulphate, and 2.2 mg/ml sodium bicarbonate.

Electro-stimulation and Development Culture

The enucleolated and intact oocytes with the first polar body after maturation culture were washed three times with 0.3 M mannitol solution containing 0.1 mM MgSO₄, 0.05 mM CaCl₂ and 0.01% PVA. They were transferred to the same solution between parallel electrodes, separated by 2 mm in a chamber (FTC-03; Shimadzu Co. Ltd.), and subjected to a single square pulse of direct current for 100 µsec at 1,500 V/cm from an electric cell fuser (LF101; BEX Co.). After electro-stimulation, the oocytes were washed and cultured for 4 hr in PZM3 (Yoshioka *et al.* 2002) containing 0.5 mg/ml cytochalasin B to prevent the emission of the second polar body. After being washed in PZM3, the oocytes were further cultured for 7 days in PZM3 at 38.5°C under an atmosphere of 5% CO₂ in air.

Experimental Design

Figure 13 shows the experiments that were conducted to elucidate the ability of nucleoli from 2-cell embryos (2-cell nucleolus) to substitute for the nucleoli of GV oocytes (GV nucleolus) in terms of supporting embryonic development.

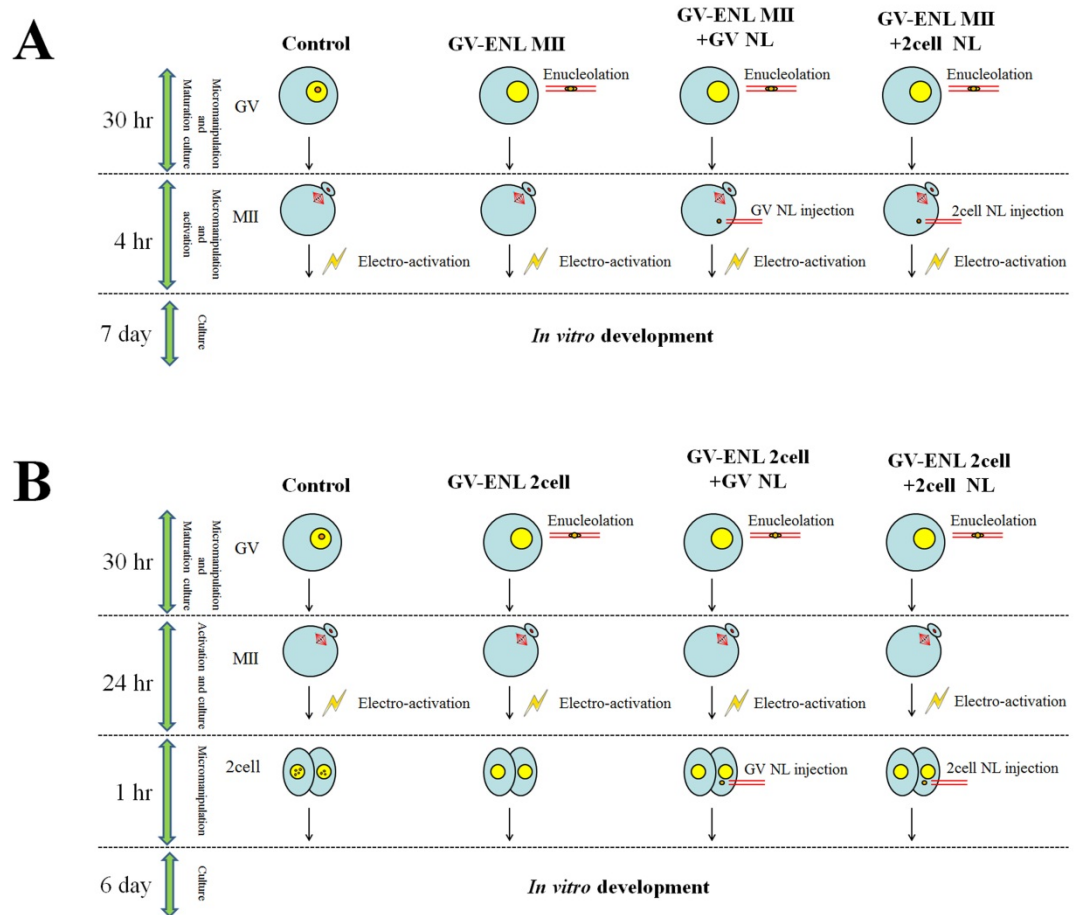


Fig. 13 Experimental schemes of nucleolus injection into GV-enucleolated oocytes at the MII (A) and 2-cell (B) stages. A: Recipient MII oocytes were prepared by enucleolation at the GV stage and maturation culture. An isolated nucleolus from a GV oocyte or a 2-cell embryo was injected into the cytoplasm of each recipient MII oocyte (GV-ENL MII+GV NL and GV-ENL MII+2cell NL, respectively). B: Recipient 2-cell embryos were prepared by enucleolation at the GV stage (GV-ENL 2cell), maturation, and electro-stimulation followed by culture in PZM3 for 24 hr. An isolated nucleolus from a GV oocyte or a 2-cell embryo was injected into the cytoplasm of a unilateral blastomere in each recipient 2-cell embryo (GV-ENL 2cell+GV NL and GV-ENL 2cell+2cell NL, respectively).

i) Development of Enucleolated GV-oocytes Injected with 2-cell Nucleoli at the MII Stage

After the denudation of the GV oocytes, they were enucleolated and cultured for maturation for 30 hr (Fig. 13-A). The matured oocytes were injected with GV or 2-cell nucleoli. Then the oocytes were activated by electro-stimulation and cultured in PZM3 for 7 days.

ii) Development of Enucleolated GV-oocytes Injected with 2-cell Nucleoli at the 2-cell Stage

After cumulus cells were removed from GV oocytes, they were enucleolated and cultured for maturation for 30 hr (Fig. 13-B). Then enucleolated-matured oocytes were activated by electro-stimulation and cultured for 24 hr to develop them to the 2-cell stage. The 2-cell embryos were injected with GV or 2-cell nucleoli into the unilateral blastomere. After the injection of nucleoli, 2-cell embryos were cultured for 6 days in PZM3. The enucleolation of GV oocytes or 2-cell embryos and the nucleolus injection methods are described below.

Enucleolation of GV Oocytes and 2-cell Embryos

GV oocytes were enucleolated as described in Chapter II. The nucleoli of 2-cell

embryos were aspirated using the same method used for the GV oocytes (Fig. 14). Briefly, GV oocytes or 2-cell embryos were transferred into HEPES-199 containing 12.5 $\mu\text{g/ml}$ cytochalasin B and 0.01 $\mu\text{g/ml}$ demecolcine for 10 min. Then they were centrifuged at 4,000 g for 10 min to move the lipid droplets to one side of the oocytes and embryos. They were enucleolated in a plastic dish (60 $\times$ 15 mm, BD Falcon) used as a micromanipulation chamber, which was put on a warmed stage (38.5°C) in an inverted microscope (Olympus Co.).

Each oocyte or embryo was held by a holding pipette (inner diameter. 10 μm , angle 30°) at the 9 o'clock position, and then the oocyte or embryo was rotated until the GV or a nucleus of 2-cell blastomere was at the 3 o'clock position. The zona pellucida was punctured with a square-ended injection pipette (inner diameter. GV: 8–10 μm , 2-cell: 6–8 μm) and piezo pulses using a micromanipulator (Narishige Group) equipped with a PIEZO drive (PNAS-CT150; Prime Tech). After penetration through the zona pellucida, the injection pipette was positioned against the membrane of the GV or the nucleus of the 2-cell blastomere.

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 cytoplasm. When the pipette opening with the captured nucleolus was just

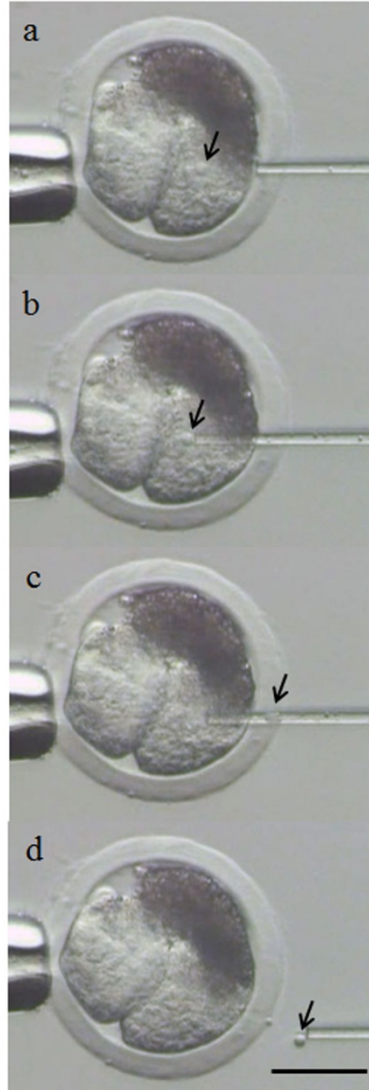


Fig. 14 Enucleation of pig 2-cell embryos. Arrows indicate the nucleoli. An injection pipette penetrates through the zona pellucida and its tip is then pushed against the nuclear membrane. With mild suction, the nucleolus is preferentially aspirated into the injection pipette. The nucleolus can be then collected from the embryos. Scale bar shows 50 μm .

outside the cell membrane, additional suction was applied. The nucleolus thereafter penetrated the membrane of the GV or 2-cell nucleus, leaving the entire nuclear content of the GV or 2-cell nucleus within the nuclear envelope. After enucleolation, the oocytes were washed 9 times in HEPES-199 and cultured for maturation for 25–26 hr (total 30 hr) as described above. Collected nucleoli from GV oocytes and 2-cell embryos were used for the nucleolus injection experiments.

Nucleolus Injection

Collected nucleoli from GV oocytes or 2-cell embryos were injected into some of the MII oocytes and 2-cell embryos derived from enucleolated GV oocytes. Enucleolated MII oocytes that showed polar body extrusion were selected after 30 hr (GV-ENL MII) of maturation culture (Fig. 13-A), and recipient 2-cell embryos were collected after 24 hr (GV-ENL 2cell) of electro-stimulation of enucleolated MII oocytes (Fig. 13-B). Nucleoli collected from intact GV oocytes or intact 2-cell embryos were injected into enucleolated MII oocytes or the unilateral blastomere in 2-cell embryos. The injection of nucleoli was performed as described in Chapter II. Each oocyte or embryo was held by a holding pipette (inner diameter 10 μm , angle 30°) at the 9 o'clock position. The zona pellucida was punctured by several

applications of piezo-pulse. Next, one nucleolus from a GV oocyte or from a blastomere of a 2-cell embryo, which had been aspirated into a glass micropipette (inner diameter. 6–8 μm), was ejected into the cytoplasm of the oocyte or embryo. The injection pipette was then slowly withdrawn. The MII recipient oocytes were cultured for 4 hr before electro-stimulation. When the nucleolus was injected into a 2-cell recipient embryo, it was injected into one blastomere, and the other blastomere was injected with a small volume of medium as a sham operation.

Fluorescence Microscopy

For immunofluorescence staining, 2-cell embryos were washed two times in PBS-PVA and stained as follows. The embryos were fixed and permeabilized in 4% paraformaldehyde in PBS-PVA containing 0.2% Triton X-100 (Nacalai Tesque, Inc.) for 40 min. The embryos were washed two times 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% Triton X-100 at 4°C overnight. The embryos were incubated with one of the primary antibodies diluted with PBS-PVA-BSA at 4°C overnight. The primary antibodies were mouse monoclonal anti-nucleolin antibody (1:200, C23, sc-8031) or mouse monoclonal anti-lamin A/C antibody (1:200,

sc-7292).

After being washed three times in PBS-PVA-BSA for 15 min each, the embryos were reacted with Alexa Fluor 488-labeled donkey anti-mouse IgG (1:400, A21202) or Alexa Fluor 568-labeled goat anti-mouse IgG (1:400, A11004) for 40 min at room temperature. The embryos were washed three times in PBS-PVA-BSA before being mounted on glass slides with ProLong Gold Antifade Regent with DAPI and observed under a confocal laser scanning microscope (FV1000-KDM, Olympus Co.). Some 2-cell embryos were collected before and after centrifugation. After staining, the diameters of all nucleoli were measured by software (FV10-ASW 3.1 Viewer, Olympus Co.) and acquired the total volume of nucleoli.

Statistical Analysis

The frequencies of oocytes and embryos at each stage of development were analyzed using the chi-square test. Differences in the mean ($\pm$ SEM) volumes of nucleoli were analyzed by the Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results

Enucleolation of 2-cell Embryos

Fig 2-cell embryos contained abundant lipid droplets in the cytoplasm and multiple nucleoli in each nucleus. For the enucleolation of 2-cell embryos, they were centrifuged to move the lipid droplets to one side of the blastomeres. Incidentally, I discovered that centrifugation reduced the nucleolus number in 2-cell blastomeres. The nucleolus number and volume were examined before and after centrifugation by immunostaining with anti-C23 antibody (Ogushi *et al.* 2008) (Table 8). Before centrifugation (Intact), most of the nuclei contained multiple nucleoli (82%, Fig. 15-a–c). However, after centrifugation (Centrifuged), all of the nuclei had a single nucleolus (Fig. 15-d–f). The total volume of the nucleoli per blastomere after centrifugation was similar to that before centrifugation, and about one-half of the nucleolus volume in GV oocytes (Table 8).

Based on this result, 2-cell embryos were centrifuged and their nucleoli were aspirated by micromanipulation. Nucleolus removal was checked immunocytologically. The nuclei of sham-operated blastomeres from which a small volume of nucleoplasm was aspirated (the left-side blastomere in each panel of Fig. 15-g–l) contained a single

Table 8. Nucleolus number and volume per blastomere in pig 2-cell embryos after centrifugation

Type of embryos	Total No. of embryos examined*	Total No. of blastomeres examined	No. (%) of nucleoli					Volume ** (μm^3)
			1	2	3	4	5	
Intact	25	50	9 (18)	8 (16)	11 (22)	6 (12)	16 (32)	206.0 $\pm$ 6.8 ^a
Centrifuged	16	32	32 (100)	0 (0)	0 (0)	0 (0)	0 (0)	229.6 $\pm$ 8.1 ^a
GV	35	35	35 (100)	0 (0)	0 (0)	0 (0)	0 (0)	510.6 $\pm$ 14.5 ^b

* The nucleolus number of 2-cell embryos was counted before (Intact) and after centrifugation (Centrifuged) immunocytolegically. The total nucleolus volumes of 2-cell embryos before (Intact) and after centrifugation (Centrifuged), and GV oocytes were examined after immunostaining.

** Values are means $\pm$ SEM.

^{a,b} Values with different superscripts in the same column differ significantly ($P < 0.05$).

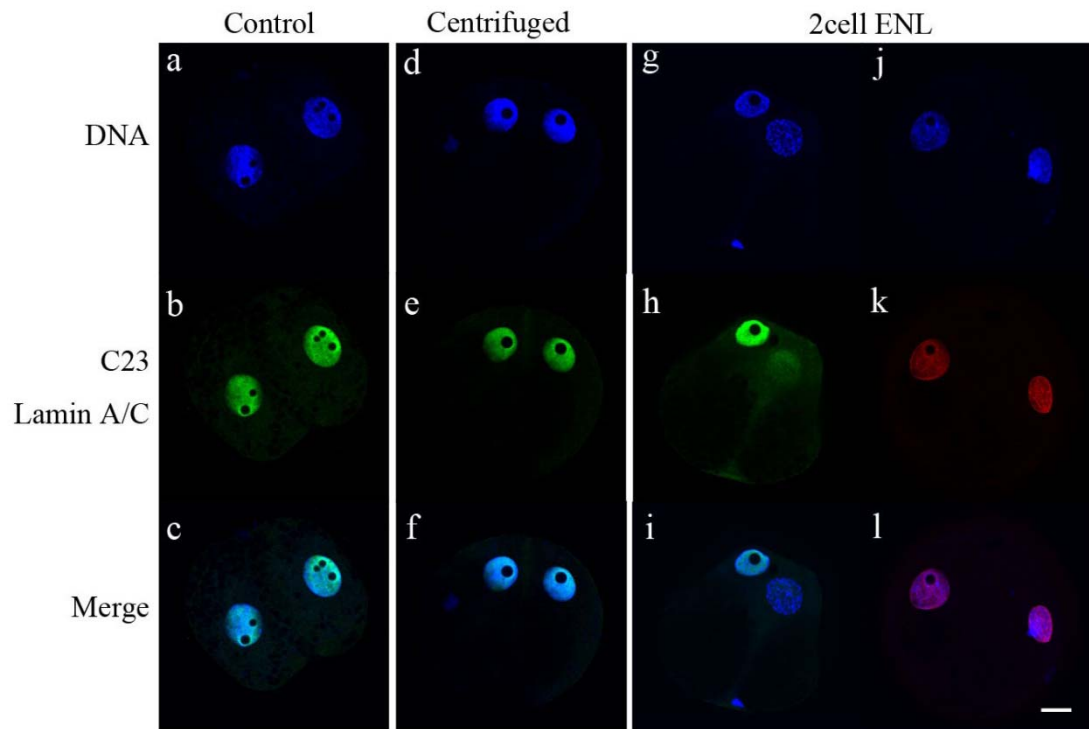


Fig. 15 Immunofluorescence labeling was performed with anti-C23 (in green) and anti-lamin A/C antibody (in red) on intact (Control, a–c) and centrifuged 2-cell embryos (Centrifuged, d–f). After centrifugation, 2-cell embryos whose nucleolus was aspirated from the unilateral blastomere (2cell ENL, g–l) were stained with anti-C23 (in green) and anti-lamin A/C antibody (in red). DAPI staining marks chromatin in blue. Scale bar shows 20 μm .

nucleolus and a nuclear envelope. In the enucleolated blastomeres (the right-side blastomere), chromatin remained in the nucleus, and the nucleolus was absent from the nucleus (Fig. 15-g–i). However, the blastomeres contained an intact nuclear envelope (Fig. 15-j–l).

Development of Enucleolated GV-oocytes Injected with 2-cell Nucleoli at the MII Stage

Table 9 shows the development of enucleolated GV-oocytes that were matured (GV-ENL MII), injected at the MII stage with GV nucleoli (GV-ENL MII+GV NL) or 2-cell nucleoli (GV-ENL MII+2cell NL), and electro-stimulated (Fig. 13-A). In all types of oocytes, the percentages of oocytes cleaved 2 days after electro-stimulation (Day 2) were similar (Control: 98%, GV-ENL MII: 86%, GV-ENL MII+GV NL: 87%, and GV-ENL MII+2cell NL: 92%). The percentage of control oocytes that developed to blastocysts at Day 7 was 73%. In contrast, only one in 41 enucleolated oocytes developed to the blastocyst stage (GV-ENL MII; 2%). The percentages of enucleolated oocytes injected with GV nucleoli (GV-ENL MII+GV NL) and 2-cell nucleoli (GV-ENL MII+2cell NL) that developed to blastocysts were 47% and 50%, respectively. The morphology of these blastocysts was similar to those of the control blastocysts.

Table 9. In vitro development of enucleolated-matured pig GV oocytes after injection of nucleoli from 2-cell embryos

Types of oocytes	Total No. of oocytes examined*	No. (%) of oocytes at the stage of						
		Day 2			Day 7			
		1 cell	2-3 cell	4 cell	5-8 cell	deg	morula	blastocyst deg
Control	41	1 (2)	9 (22) ^{ab}	31 (76) ^a	0 (0)	0 (0)	1 (2)	30 (73) ^a 6 (15)
GV-ENL MII	41	5 (12)	3 (7) ^a	30 (73) ^a	2 (5)	1 (2)	0 (0)	1 (2) ^c 11 (27)
GV-ENL MII+GV NL	45	5 (11)	10 (22) ^{ab}	29 (65) ^{ab}	0 (0)	1 (2)	1 (2)	21 (47) ^b 11 (24)
GV-ENL MII+2cell NL	40	1 (3)	11 (28) ^b	20 (50) ^b	6 (15)	2 (5)	4 (10)	20 (50) ^b 10 (25)

* Intact (Control) and enucleolated GV oocytes (GV-ENL) were cultured for 30 hr in the maturation medium. The oocytes which had a first polar body were selected, and some of the GV-ENL oocytes were injected with the nucleolus from GV oocytes (GV-ENL MII+GV NL) or 2cell embryos (GV-ENL MII+2cell NL). Then the oocytes were subjected to a single electric pulse (100 µsec, 1,500 V/cm) and further cultured for 7 days in PZM3 (Yoshioka *et al.* 2002). Developmental stages were examined 2 and 7 days after electro-stimulation. deg: degeneration.

^{a-c} Values with different superscripts in the same column differ significantly (P<0.05).

Development of Enucleolated GV-oocytes Injected with Nucleoli from 2-cell Embryos at the 2-cell Stage

It was examined in this experiment whether nucleoli from GV-oocytes or 2-cell embryos restored the developmental ability of 2-cell embryos derived from enucleolated GV-oocytes (Fig. 13-B). After maturation culture and subsequent electro-stimulation, the enucleolated GV-oocytes developed to the 2-cell stage (GV-ENL 2cell). Although the embryos had a nuclear envelope (Fig. 16-d–f), no nucleolus was formed in the nuclei (Fig. 16-a–c). The embryos were injected with GV nucleoli (GV-ENL 2cell+GV NL) or 2-cell nucleoli (GV-ENL 2cell+2cell NL), and cultured for 7 days post electro-stimulation (Table 10). The percentage of control oocytes that developed to blastocysts was 80%. In contrast, no enucleolated oocytes developed to blastocysts. The percentages of GV-enucleolated 2-cell embryos injected with GV nucleoli (GV-ENL 2cell+GV NL) and 2-cell nucleoli (GV-ENL 2cell+2cell NL) that developed to blastocysts were 3% and 6%, respectively.

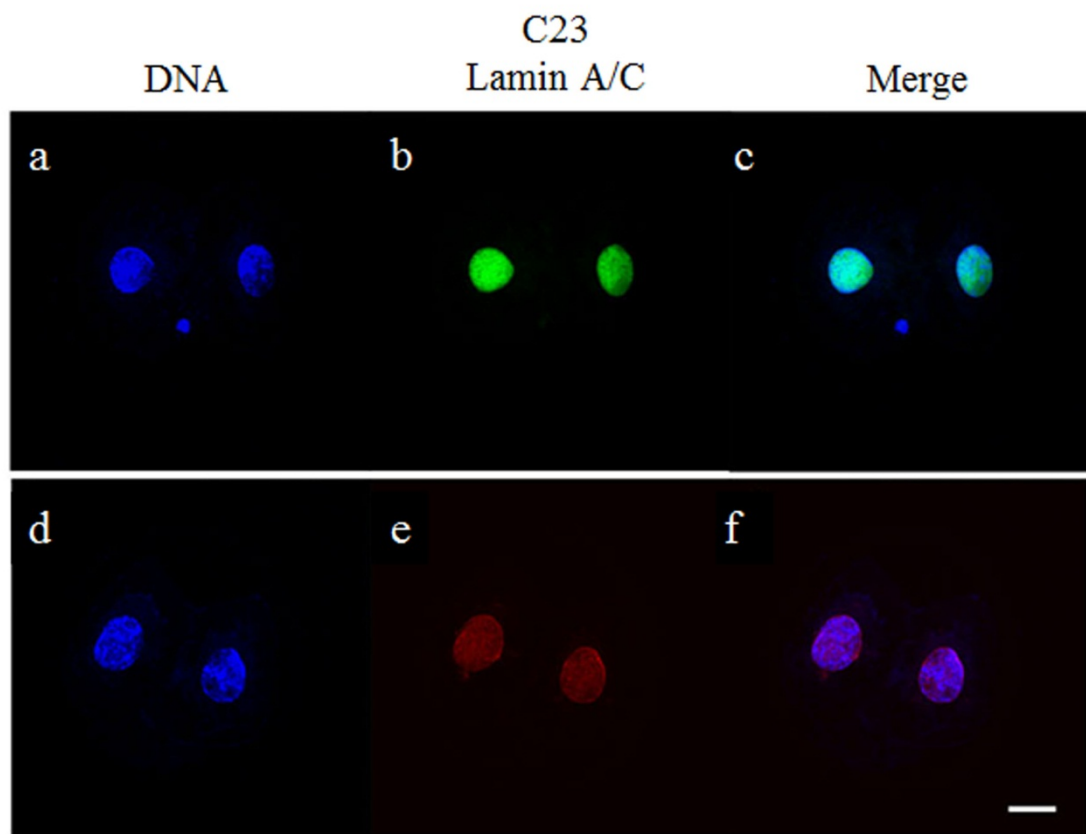


Fig. 16 Immunofluorescence labeling was performed with anti-C23 (in green) and anti-lamin A/C antibody (in red) on the 2-cell embryos derived from enucleolated GV oocytes (GV-ENL 2-cell, a–f). DAPI staining marks chromatin in blue. Scale bar shows 20 μm .

Table 10. In vitro development of pig 2-cell embryos derived from enucleolated GV oocytes after injection of nucleoli from GV oocytes or 2-cell embryos

Types of embryos	Total No. of embryos examined*	No. (%) of embryos at the stage of				
		2-3 cell	4 cell	5-16 cell	morula	blastocyst deg
Control	40	0 (0)	0 (0)	0 (0)	1 (3)	32 (80) ^a 7 (18) ^a
GV-ENL 2cell	39	9 (22)	21 (53) ^a	0 (0)	0 (0)	0 (0) 10 (25) ^{ab}
GV-ENL 2cell+ GV NL	32	5 (16)	11 (34) ^{ab}	1 (3)	0 (0)	1 (3) ^b 14 (44) ^b
GV-ENL 2cell+ 2cellNL	34	3 (9)	8 (23) ^b	4 (12)	1 (3)	2 (6) ^b 16 (47) ^b

* Intact (Control) and enucleolated GV oocytes (GV-ENL) were cultured for 30 hr in the maturation medium. The oocytes which had a first polar body were selected. Then the oocytes were subjected to a single electric pulse (100 μ sec, 1,500 V/cm) and further cultured for 24 hr in PZM3 (Yoshioka *et al.* 2002). After culture, normal morphological 2cell embryos derived from enucleolated GV oocytes were injected with the nucleolus from GV oocytes (GV-ENL 2cell+GV NL) or 2cell embryos (GV-ENL 2cell+2cell NL). Then the 2cell embryos were further cultured for 6 days in PZM3. Developmental stages were examined 7 days after electro-stimulation. deg: degeneration.

^{a,b} Values with different superscripts in the same column differ significantly ($P<0.05$).

Discussion

With centrifugation of 2-cell pig embryos, the multiple nucleoli in each nucleus fused into a single nucleolus. The average nucleolus volume after centrifugation was the same as the total volume of multiple nucleoli before centrifugation. Since the nucleolus is not a membrane-delimited structure and its active liquid-like behavior has been reported in *Xenopus* (Brangwynne *et al.* 2011) and mouse oocytes (Fulka *et al.* 2012), the centrifugation allowed the nucleoli to fuse in 2-cell embryos. The nucleolus volume of the 2-cell embryos was half as much as the volume of the GV oocyte nucleoli. Oocyte nucleoli disappear just before nuclear membrane breakdown during oocyte maturation, and nucleoli reappear during pronucleus formation. Then, the nucleoli disappear during the first cleavage of the embryos and reappear again in the nuclei of 2-cell embryos. Assuming that the total nucleolus volume per oocyte/embryo is maintained during these stages, it is reasonable that the nucleolus volume in a 2-cell blastomere is one-half the volume in a GV oocyte.

In this chapter, 2-cell enucleolation method was used to examine whether nucleoli from 2-cell embryos were able to substitute for the nucleoli of GV oocytes. No or few enucleolated oocytes developed to blastocysts, as described elsewhere (Ogushi *et*

al. 2008; Ogushi and Saitou 2010). However, when GV nucleoli or 2-cell nucleoli were injected into the cytoplasm of enucleolated-matured oocytes at the MII stage, both types of nucleoli supported development of the oocytes to blastocysts. This result indicates that the nucleoli from 2-cell embryos support embryonic development to a similar extent as the nucleoli from GV oocytes despite being half the volume.

It has been reported that some nucleolar components of 2-cell pig embryos are similar to those of GV oocyte nucleoli (Hyttel 2011). The results of Chapter II showed that nucleoli of GV oocytes are essential for early embryonic development, and the nucleoli of growing oocytes which were treated with actinomycin D to compact the nucleolus, can replace them to support the early embryonic development. The nucleolus treated with actinomycin D showed morphology similar to that of full-grown oocytes (Crozet 1983). Together with the results of this chapter, these findings indicate that the nucleoli of growing oocytes have accumulated the materials that are required for early embryonic development and the materials are maintained in nucleoli in full-grown GV oocytes and even in 2-cell embryos after disassembly and reassembly during oocyte maturation and first embryonic cleavage.

In the next experiment, it was examined whether injection of the nucleolus from GV oocytes or 2-cell embryos into the unilateral blastomere restored the

developmental ability of 2-cell embryos derived from enucleolated GV oocytes. I thought that unilateral injection was sufficient for the development, since single blastomeres derived from 2-cell pig embryos have been reported to develop to blastocysts (Dang-Nguyen *et al.* 2011). However, quite a few 2-cell embryos derived from enucleolated GV oocytes developed to blastocysts after the injection of nucleoli from GV oocytes or 2-cell embryos. Recent reports have shown that pig 1-cell embryos drive their development for one or several cell cycles using the stockpile of molecules originating from the oocytes, and that the nucleolus is essential for early embryonic development (Ogushi *et al.* 2008). Moreover, the critical point when the nucleolus is required for the progression of mouse embryonic development has been studied (Ogushi and Saitou 2010). In that experiment, when GV nucleoli were re-injected at the pronuclear stage, most of the reconstructed zygotes cleaved and formed nuclei with nucleoli at the 2-cell stage. However, the rate of blastocyst formation and the numbers of surviving pups were quite low compared to when the nucleolus was re-injected at the MII stage. Thus the critical time point when the nucleolus is required for the progression of embryonic development appears to be at the early step of pronucleus organization. Since the results of the present study confirmed that the nucleolus from GV oocytes is required for early embryonic development before the 2-cell stage in the

pig, it might be essential at the early stage of 1-cell embryos.

Moreover, the nucleoli from 2-cell stage embryos were not able to rescue the progression of embryonic development when the nucleoli were injected into embryos without nucleoli at the same 2-cell stage. These results indicate that the presence of the nucleolus before the 2-cell stage in the embryos is essential for embryonic development. Requiring the nucleolus during the first cell cycle of embryos might be related to the chromatin disorganization. During the first cell cycle of mouse embryos, maternal and paternal chromatin undergoes rapid remodeling and reaches a zygotic organization in which the pericentric satellites occupy restricted spatial domains surrounding the nucleolus (Martin *et al.* 2006; Probst *et al.* 2007). This organization is achieved in both normal and parthenogenetic embryos (Probst *et al.* 2007, 2010). The pig nucleolus might function similarly to the mouse nucleolus.

Nucleoplasmin 2 (NPM2) is a component of the nucleolus and suggested to be involved in the formation of nucleoli in mouse embryos (Burns *et al.* 2003; Inoue and Aoki 2010). NPM2 knockout females have fertility defects owing to failed early embryonic development (Burns *et al.* 2003). In the NPM2 knockout mice, nucleoli and heterochromatin formation surrounding nucleoli are absent in the 1-cell stage embryos. However, it has been suggested that potential compensatory mechanisms must exist,

because a few NPM2-null embryos developed to birth. The role of the nucleolus in the first cell cycle of embryos remains to be elucidated.

In summary, in this chapter, the results demonstrates that the nucleoli of 2-cell embryos support early embryonic development as nucleoli of GV oocytes, and that the presence of nucleoli is essential for embryos before the 2-cell stage in the pig.

Chapter V

General Summary

In the ovary, the increase in oocyte volume and morphological changes in the chromatin and nucleolus progress in a synchronous manner so that the oocytes develop the ability to mature, to be fertilized and to develop. Thus the oocyte size (Kanayama *et al.* 2002; Motlik *et al.* 1984a), nucleolus morphology (Maddox-Hyttel *et al.* 2007) and chromatin morphology (Hirao *et al.* 1995; Inoue *et al.* 2008) are thought to be important for the development of oocytes and embryos, and often served as the markers of oocyte maturation and development. Pig oocytes acquire the ability to resume meiosis when they approach the end of the growth phase in antral follicles larger than 0.8 mm in diameter, and acquire the capacity to complete meiotic maturation to MII at the follicular diameter of larger than 2 mm (Motlik *et al.* 1984b; Tsafiriri and Channing 1975). This acquisition of full meiotic competence coincides with the nucleolus morphological change. In this thesis, pig growing oocytes around 100 μm in diameter and full-grown oocytes around 120 μm were collected from ovaries and used for the experiments.

At the ultrastructural level, nucleoli of somatic cells and growing oocytes in mammals are composed of fibrillar centers (FCs), dense fibrillar components (DFCs), and granular components (GCs) (Olson *et al.* 2000; Crozet 1989). The nucleoli in growing oocytes and somatic cells are the site of active rRNA synthesis and ribosome production (Olson *et al.* 2000, Hyttel 2011). However, the rRNA synthesis decreases as oocytes approaching the final size (Crozet 1989). The decrease coincides with inactivation of the nucleolus, as visualized by the transformation of the fibrillogranular nucleolus to an electron-dense fibrillar sphere with remnants of the FCs at the surface (Bjerregaard *et al.* 2004). Consequently, the nucleoli of full-grown oocytes are compacted and separated from chromatin, and have no rRNA synthetic activity. During the subsequent oocyte maturation, the nucleolus disappears in cytoplasm of oocytes. However, nucleoli are formed again in pronuclei after fertilization, and after 2-cell stage, FCs, DFCs and GCs develop on the surface of nucleoli which are transformed to somatic cell-type nucleoli before the morula stage (Tomanek *et al.* 1989). Compacted nucleoli in full-grown oocytes and embryos before 2-cell stage do not contain chromatin and have no rRNA synthetic activity. Nucleoli of growing oocytes also become compacted morphologically similar to full-grown oocytes and 2-cell embryos after actinomycin D treatment (Fig. 17). However, function of these compacted nucleoli

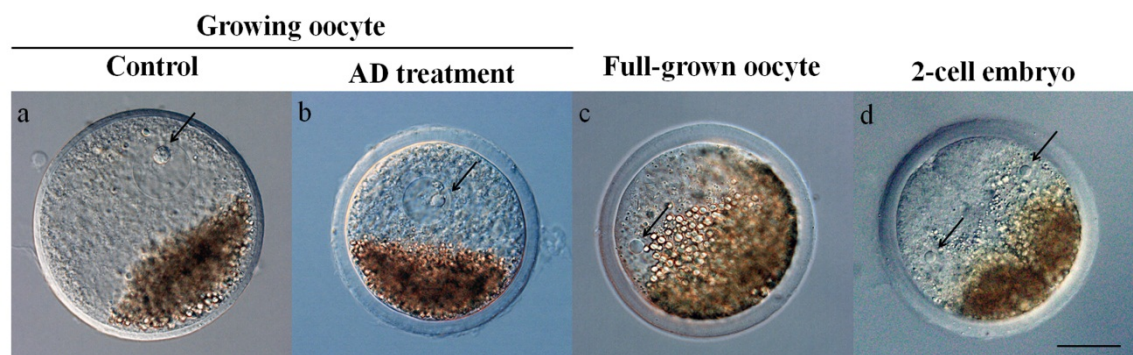


Fig. 17 Differential interference images of nucleolus morphology of growing oocytes before (Control, a) and after treatment with actinomycin D (AD treatment; b), full-grown oocyte (c), and 2-cell embryo (d). Arrows indicate the nucleoli. Scale bar shows 40 μ m.

and functional similarity between them have not been understood well.

Recent two reports have suggested new roles of the oocyte nucleolus other than rRNA synthesis. The first one suggests that nucleoli in growing oocytes have an inhibitory role in meiotic resumption of the oocytes (Fulka *et al.* 2003). The second one has suggested that the oocyte nucleolus is not necessary for oocyte maturation, but it is necessary for the formation of pronuclear nucleoli after fertilization or parthenogenetic activation and is essential for further embryonic development (Ogushi *et al.* 2008). In this thesis, the nucleoli of growing and full-grown oocytes and 2-cell embryos in the pig were removed and inter-replaced among oocytes/embryos using micromanipulation to determine the ability of each stage nucleoli to support embryonic development. In addition, the inhibitory role of growing oocyte nucleoli in meiotic resumption of oocytes was examined by inter-replacement of nucleoli.

In Chapter II, the ability of nucleoli from growing oocytes to substitute for nucleoli of full-grown oocytes in terms of supporting embryonic development was determined (Fig. 18). Nucleoli were aspirated from full-grown oocytes by micromanipulation, and the resulting enucleolated oocytes were matured to MII. Into the mature oocytes, nucleoli from full-grown and growing oocytes were injected. After being activated by electro-stimulation, sham-operated oocytes formed nucleoli in

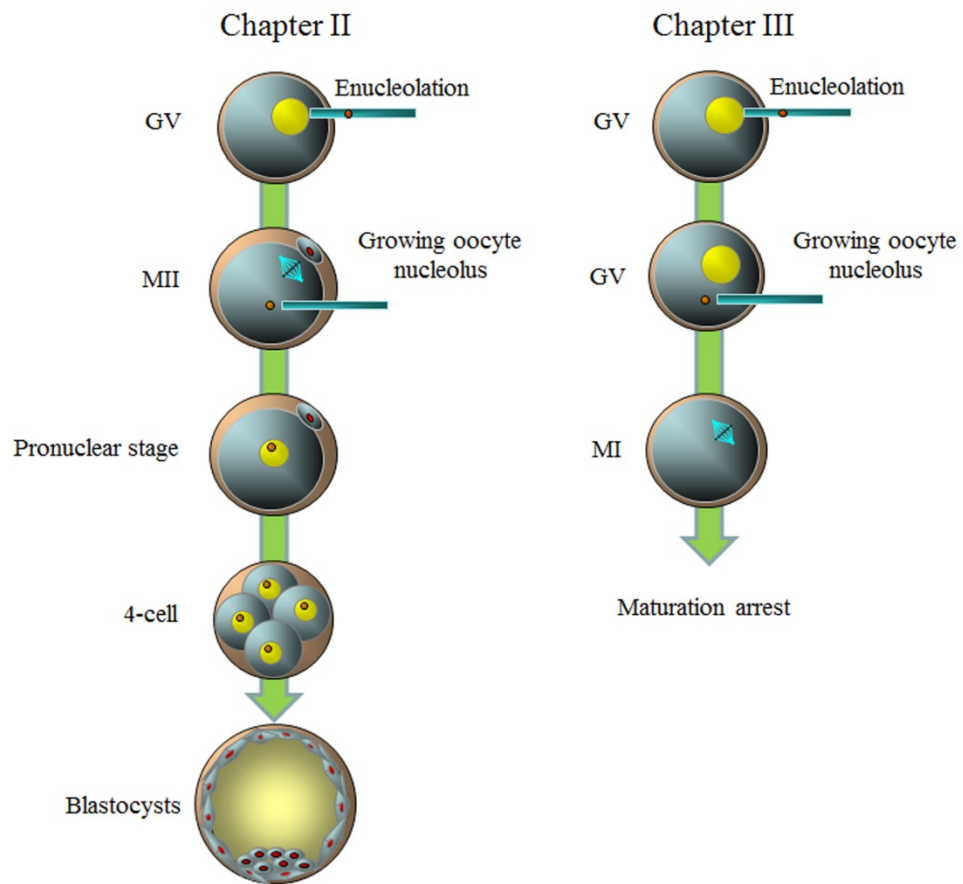


Fig. 18 Experimental summary of Chapter II and Chapter III. In Chapter II, full-grown oocytes were enucleolated and cultured for maturation. After maturation culture, MII oocytes were injected with growing oocyte nucleoli. The resulting oocytes formed nucleoli in pronuclei and developed to blastocysts. In Chapter III, full-grown oocytes were enucleolated and injected with growing oocyte nucleoli at the GV stage. After maturation culture, the resulting oocytes were arrested at MI stage.

pronuclei, and enucleolated oocytes that had been injected with nucleoli from either full-grown or growing oocytes formed nucleoli. However, enucleolated oocytes did not form any nucleoli in the pronuclei. None of the enucleolated oocytes developed to blastocysts, whereas enucleolated oocytes injected with nucleoli from full-grown oocytes (15%) or growing oocytes (18%) developed to blastocysts. These results indicate that the nucleoli from growing oocytes are able to substitute for nucleoli from full-grown oocytes for early embryonic development (Fig. 18).

In Chapter III, the effect of growing oocyte nucleoli on oocyte maturation was examined, because the growing oocytes had no ability to resume meiosis. Nucleoli were aspirated from growing or full-grown oocytes by micromanipulation and transferred to the cytoplasm of enucleolated growing or full-grown oocytes at the GV stage, and the oocytes cultured for maturation (Fig. 18). Enucleolated growing oocytes underwent germinal vesicle breakdown (GVBD; 46% vs. control 12%), while enucleolated growing oocytes injected with nucleoli from growing oocytes arrested at the GV stage again (77%). However, growing oocytes injected with nucleoli from full-grown oocytes underwent GVBD (49%). Enucleolated full-grown GV oocytes, which were injected with nucleoli from growing oocytes or full-grown oocytes, reassembled nucleolus in the GV (73% and 60%, respectively). After maturation culture, the enucleolated full-grown

oocytes injected with nucleoli from full-grown oocytes matured to MII (56%), whereas enucleolated full-grown oocytes injected with growing oocyte nucleoli had a reduced maturation rate (21%). These results suggest that growing oocyte nucleoli inhibit oocyte maturation (Fig. 18).

In Chapter IV, ability of nucleoli from 2-cell embryos to substitute for GV nucleoli in terms of supporting early embryonic development was examined (Fig. 19). Nucleoli of GV oocytes and 2-cell embryos were aspirated and transferred into MII oocytes or 2-cell embryos that were derived from oocytes enucleolated at the GV stage. When 2-cell embryos were centrifuged to move the lipid droplets to one side of the blastomere, multiple nucleoli in the nucleus fused into a single nucleolus. The nucleoli were then aspirated from the 2-cell embryos by micromanipulation. The injection of 2-cell nucleoli to GV enucleolated oocytes at the MII stage rescued the embryos from the early embryonic arrest, and the resulting oocytes developed to blastocysts. However, the injection of 2-cell and GV nucleoli to 2-cell embryos derived from GV enucleolated oocytes rarely restored the development to blastocysts. These results indicate that 2-cell nucleoli support early embryonic development as GV nucleoli if the nucleoli are injected to MII oocytes, and that the presence of nucleoli is essential for embryos before the 2-cell stage (Fig. 19).

Chapter IV

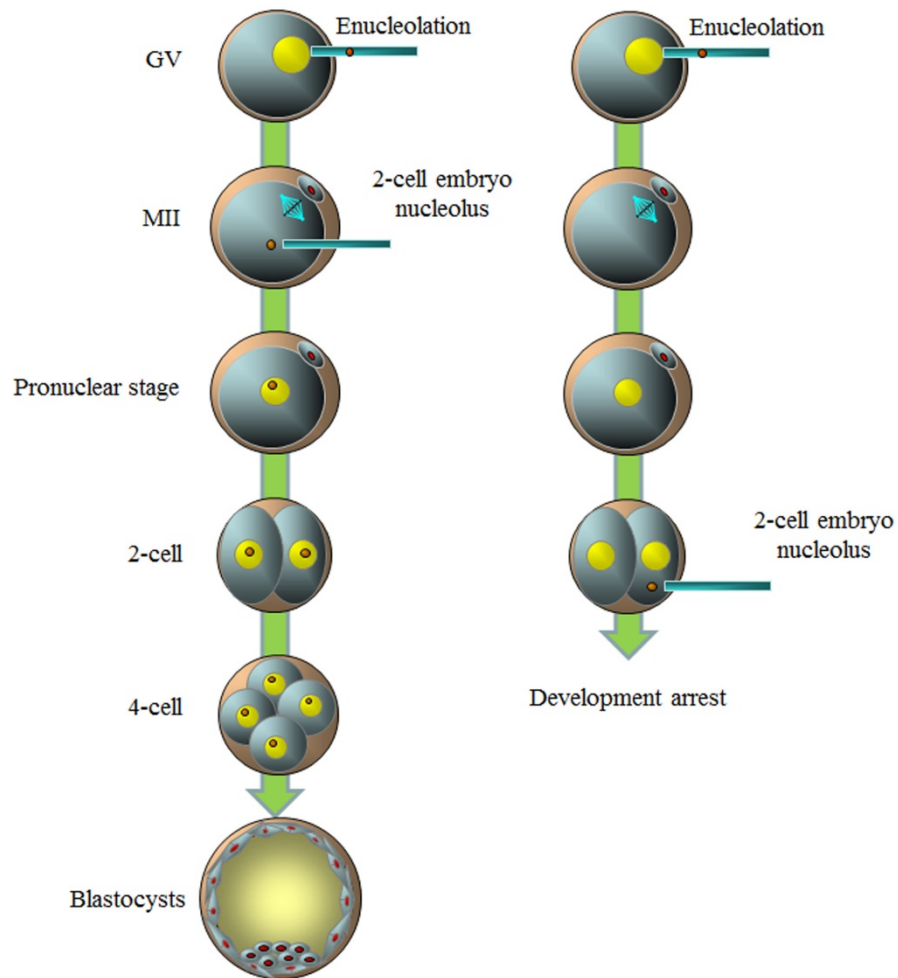


Fig. 19 Experimental summary of Chapter IV. In the first experiment, full-grown oocytes were enucleolated and cultured for maturation. After maturation culture, MII oocytes were injected with 2-cell embryo nucleoli. The resulting oocytes developed to blastocysts. In the second experiment, full-grown oocytes were enucleolated and cultured for maturation and development to 2-cell stage. The 2-cell stage embryos were injected with 2-cell embryo nucleoli. Development of the resulting embryos was arrested.

Growing oocyte nucleoli synthesize rRNA (Fig. 20). In addition, they have a role in inhibition of meiotic resumption of oocytes. It is possible that the nucleolus contains maturation inhibitory materials. Such inhibitory materials, however, disappear or are excluded from the nucleolus during the final growth of the oocyte, and full-grown oocyte nucleolus has eventually lost the inhibitory materials (Fig. 20). On the other hand, nucleoli of growing oocytes have accumulated the materials that are required for early embryonic development, and the materials are maintained in nucleoli in full-grown GV oocytes and even in 2-cell embryos after disassembly and reassembly during oocyte maturation and first embryonic cleavage (Fig. 20). However, it remains to be elucidated when the oocytes acquire the ability during the growth phase, and when the nucleolus loses the ability after 2-cell stage. Moreover, the materials responsible for maturation inhibition and developmental support in the oocyte nucleolus remain to be understood.

A recent report has shown that the critical time point when the nucleolus is required for the progression of embryonic development appears to be at the early step of pronucleus organization in the mouse (Ogushi and Saitou 2010). The results in Chapter IV confirm that the nucleolus from GV oocytes is required for early embryonic development before 2-cell stage in the pig. The presence of nucleoli may be essential at

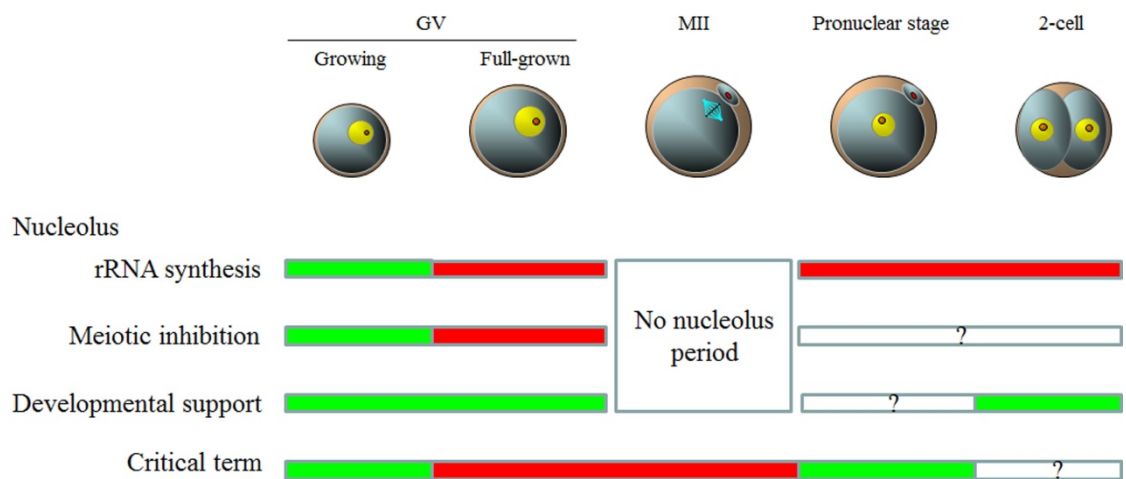


Fig. 20 Nucleolus function changes during oocyte growth and maturation, fertilization and initial embryonic development in the pig. The nucleoli in growing oocytes synthesize RNAs (green bar), while nucleoli in full-grown oocytes and 1-, 2-cell embryos show no RNA synthetic activity (red bar). During oocyte growth, oocyte nucleoli concern the meiotic arrest of the oocytes (green bar), although full-grown oocyte nucleoli have lost this ability (red bar). The nucleoli of growing oocytes have accumulated the materials that are required for early embryonic development, and the materials are maintained in nucleoli in full-grown GV oocytes and even in 2-cell embryos (green bar). During oocyte growth, growing oocytes synthesize RNAs, thus the nucleolus is essential for oocyte growth (green bar). However, the oocyte nucleolus is not necessary for oocyte maturation (red bar), but it is required for early embryonic development before the 2-cell stage (green bar).

the early stage of 1-cell embryos.

In conclusion, the oocyte nucleolus has specific two functions which are different from somatic cells. Firstly, the oocyte nucleolus supports early embryonic development not substituted by somatic cell nucleoli. Secondly, the oocyte nucleolus has the ability of meiotic arrest. Although those functions have been proposed by two papers, the results in this thesis confirm the ideas, and further indicate that the inhibitory functions undergo a significant change during oocyte growth and the supporting function is maintained up to 2-cell stage (Fig. 20). In other words, the oocyte nucleolus plays important roles in meiotic arrest of growing oocytes, while the compacted nucleolus in full-grown oocytes has lost the ability. Nucleoli of growing oocytes and 2-cell embryos support early embryonic development as nucleoli of full-grown GV oocytes. The oocyte nucleoli may prepare sufficient materials for early embryonic development before the completion of oocyte growth and the materials are maintained at least until 2-cell stage.

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