The Perivitelline Space-Forming Capacity of Mouse Oocytes is Associated with Meiotic Competence

Azusa INOUE1), Tomohiko AKIYAMA1), Masao NAGATA1) and Fugaku AOKI1)

1)Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Chiba 277-8562, Japan

Abstract. Although mouse oocytes progressively acquire meiotic competence during their growth in the ovaries, only half of full-grown oocytes can accomplish meiosis. Two types of full-grown oocytes have been reported on the basis of their chromatin configuration, the surrounded-nucleolus (SN) type and the non-surrounded-nucleolus (NSN) type. Therefore, full-grown oocytes collected from the ovaries of adult animals comprise a heterogeneous population; some oocytes are meiotically incompetent (NSN-type), and some are competent (SN-type). In the present study, we found that full-grown oocytes could be classified into two groups using the criterion of formation of the perivitelline space (PVS) after culture with 3-isobutyl-1-methylxanthine (IBMX) for 1 h. In oocytes with a PVS, actin-filled processes within zona pellucidae originating from cumulus cells were reduced, while they were rich in oocytes without a PVS, suggesting that a reduction in these processes contributes to PVS formation. PVS formation was highly correlated with meiotic competence and SN-type configuration. The results of this study demonstrate that PVS formation is a useful criterion for easily distinguishing between SN- and NSN-type oocytes, without injury to the cells.

Key words: Meiosis, Mouse, Oocyte, Perivitelline space (J. Reprod. Dev. 53: 1043–1052, 2007)

During oogenesis in the ovaries of female mouse pups after birth, oocytes that are arrested in the prophase of the first meiosis grow and increase in size. These oocytes have reached full size (70–85 µm in diameter) by about three weeks after birth, remain arrested at the prophase of first meiosis and contain a large nucleus known as the germinal vesicle (GV). In response to hormonal stimulation or upon removal from ovaries in vitro, full-grown oocytes resume meiosis; they undergo germinal vesicle breakdown (GVBD) and subsequent emission of the first polar body, and are then arrested at metaphase of meiosis II (MII) until fertilization. Although growing oocytes with a diameter <60 µm cannot resume meiosis [1, 2], they progressively acquire the ability to resume and complete meiosis (meiotic competence) as they continue to grow [3–5]. However, even when the oocytes are full-grown, some of them will not demonstrate meiotic competence [1, 6, 7]. Thus, the population of full-grown oocytes collected from the ovaries of adult animals is heterogeneous in that both meiotically competent and meiotically incompetent oocytes are obtained.

Meiotically competent and incompetent oocytes differ in several ways. In particular, they differ in their nuclear patterns of chromatin configuration [8–10]. The chromatin may be highly condensed and surround the nucleolus, forming the surrounded-nucleolus (SN) type of configuration, or it may be less condensed and not surround the nucleolus, forming the non-surrounded-nucleolus (NSN) type. All growing oocytes collected from mice less...
than 15 days old have NSN-type nuclei. Some of these will change into the SN-type, while others will remain as the NSN-type [11]. The percentage of SN-type oocytes increases with the age of the mice. The transformation from the NSN- to SN-type chromatin configuration has been suggested to be important in the acquisition of meiotic competence. The percentage of SN-type oocytes that complete meiotic maturation is much higher than that of NSN-type oocytes [7, 12, 13]. Various alterations also occur in the cytoplasm when the oocytes acquire meiotic competence, such as a decrease in, and shortening of, the cytoplasmic microtubules [1]; an increase in centrosome phosphorylation [14, 15]; an increase in density and shortening of the microvilli in the oolemma [16]; and localization of M-phase-specific phosphoproteins in the nucleus [1]. These phenomena appear to be associated with the G2/M transition, which would account for the ability to resume meiosis. Some studies have suggested that heterologous gap junctions between oocyte and cumulus cells are also required for acquisition of meiotic competence [17–19].

In the present report, we describe the classification of full-grown oocytes into two groups. The criterion used was formation of the perivitelline space (PVS) after culture with 3-isobutyl-1-methylxanthine (IBMX) for 1 h. Our results show that PVS formation highly correlates with the SN-type chromatin configuration and meiotic competence.

Materials and Methods

Collection, culture and observation of oocytes and embryos

Fully grown oocytes at the germinal vesicle (GV) stage were obtained from 8–11-week-old B6D2F1 mice (CLEA Japan, Inc., Tokyo, Japan). The ovaries were removed from the mice and transferred into α-MEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml epidermal growth factor (Sigma-Aldrich) and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich). In some cases, TYH [20], Waymouth’s [21], KSOM [22] or HTF [23] medium was used rather than α-MEM. The ovarian follicles were punctured with a 27-gauge needle, and the cumulus cells were gently removed from the cumulus-oocyte complexes using a narrow-bore glass pipette. Only the oocytes with a diameter of >70 μm were used in subsequent experiments. For in vitro maturation, the oocytes were washed in IBMX-free medium and incubated in a humidified atmosphere of 5% CO2/95% air at 38 C. The oocytes were observed under 90 x magnification using a stereoscopic microscope (Olympus, Tokyo, Japan).

For in vitro fertilization, MII-stage oocytes were transferred into HTF medium. Spermatozoa were obtained from the caudal epididymides of adult ICR male mice (SLC Japan, Shizuoka, Japan). The oocytes were inseminated with spermatozoa that had been capacitated by preincubation for 2 h in HTF medium. Five hours after insemination, the fertilized oocytes were washed and cultured with KSOM medium.

Measurement of oocyte diameter

Oocyte diameters were measured from images taken before and after incubation with IBMX. Each oocyte was cultured individually in a small droplet of medium to facilitate its identification after incubation. Since oocytes are approximately, but not exactly, globular in shape, diameter measurements for each oocyte were taken along both the shortest and longest axes and then averaged.

Fluorescence staining of actin filaments

The oocytes were fixed with 3.7% paraformaldehyde overnight at 4 C or for 1 h at room temperature. They were then permeabilized with 0.5% Triton X-100 for 15 min and incubated with 0.2 μg/ml fluorescein-phalloidin (Sigma-Aldrich) at 37 C for 90 min. DNA was visualized by counterstaining the oocytes with propidium iodide (Sigma-Aldrich). The oocytes were mounted onto glass slides in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA, USA). Fluorescence was detected using a laser-scanning confocal microscope (Carl Zeiss Micro Imaging GmbH, Oberkochen, Germany).

Results

Perivitelline space formation in oocytes during in vitro culture

The perivitelline space (PVS), the space between the surface of the oocyte and the zona pellucida (ZP), is formed during meiotic maturation in vivo.
To precisely determine when the PVS appears, fully grown, GV-stage oocytes were cultured in vitro. The percentage of oocytes exhibiting a visible PVS increased and nearly reached a plateau within 60 min after release from the ovary; the percentage then increased slightly until 120 min after release (Fig. 1A and B). The percentages of oocytes undergoing germinal vesicle breakdown (GVBD) at 30, 60 and 120 min were 0, 10 and 59%, respectively. Since PVS formation, but not GVBD, occurred within 30 min after release, the former process appeared to be independent of the latter. To confirm this interpretation, we examined the PVS of oocytes arrested at the GV-stage by IBMX treatment. Under this condition, the percentage of oocytes exhibiting a PVS increased at a rate similar to that in IBMX-free medium (Fig. 1B). This result confirms that PVS formation occurs independently of the progress of meiotic maturation during in vitro culture.

Reduction in actin-containing transzonal processes is associated with PVS formation

Formation of a PVS suggests that the volume of the oocyte decreases during in vitro culture. To examine this possible change in oocyte volume, we measured the diameters of GV-stage oocytes before and after culture with IBMX for 1 h. After isolation from ovaries, oocytes were placed individually into small drops, and their diameters were measured. After 1 h in culture, they were sorted into two groups according to the presence [PVS(+)] or absence [PVS(–)] of a visible PVS, and their diameters were measured again. Oocytes that could not be sorted because of PVS obscurity [PVS(±)] were excluded from further analysis. Of the 75 oocytes isolated, 52 (69%), 20 (27%) and 3 (4%) were PVS(+), PVS(–) and PVS(±), respectively. The average diameter of the PVS(+) oocytes decreased by 10.9% during the culture, a decrease comparable to that observed for the PVS(–) oocytes (10.4%). In contrast, the inner diameter of the zona pellucidae (ZP) of the PVS(+) oocytes barely decreased (by 2.4%), a significantly smaller decrease than that observed for the PVS(–) oocytes (P<0.001; Fig. 2A). Notably, the oocytes and their surrounding ZP reduced in size in a coordinated manner in PVS(–) oocytes, whereas only the oocytes shrank in PVS(+) oocytes, suggesting that the plasma membrane was bound to the ZP in the former but not in the latter. To confirm this supposition, the oocytes were transferred to PBS containing 1% glycerol. The PVS(+) oocytes shrank further, and their PVS became much larger, whereas the plasma membrane of the PVS(–) oocytes remained attached to the ZP, and no PVS

Fig. 1. Formation of the perivitelline space (PVS) in mouse oocytes during in vitro culture with IBMX. (A) Light micrographs of full-grown oocytes with a PVS. The oocytes were observed before (a, c) and after (b, d) culture with IBMX for 1 h. A PVS (arrowhead) was formed in the oocyte shown in panel b, but not in the one in panel d. Bar=30 µm. (B) Kinetics of PVS formation in full-grown oocytes during in vitro culture with or without 0.2 mM IBMX. In the culture without IBMX, the IBMX carrier solvent (DMSO) was added to a final concentration of 0.1%. This experiment was performed three times using 27–63 oocytes; the total numbers of oocytes examined were 99 (IBMX–) and 169 (IBMX+). Error bars represent ± SEM.
were formed (Fig. 2B).

The ZP of GV-stage oocytes contain actin-filled processes originating from cumulus cells [25, 26]. The termini of these processes intrude into the oocyte plasma membrane [27]. Since these processes remain within ZP after oocytes are isolated from cumulus cells [28], we hypothesized that the transzonal processes that attach the oocyte plasma membrane to the ZP would exist in larger numbers in the PVS(–) oocytes than in the PVS(+) oocytes. We therefore examined the transzonal processes using FITC-conjugated phalloidin to detect the actin filaments [29, 30]. Fewer transzonal processes were observed in the PVS(+) oocytes than in the PVS(–) oocytes (Fig. 3A), suggesting that loss of the transzonal processes is necessary for PVS formation.

To determine whether the reduction in actin-filled processes contributes to PVS formation, we culled the PVS(–) oocytes after 1 h in culture with IBMX and then cultured them further with IBMX in the presence of 10 µg/ml cytochalasin B (CB), which depolymerizes actin filaments. After 4 h in culture with CB, some oocytes formed a PVS. The percentage of oocytes that formed a PVS was significantly higher (P<0.01) in the CB-treated oocytes than in the non-CB-treated control oocytes (Fig. 3C). Fewer transzonal processes were observed in the CB-treated oocytes, whereas the control oocytes were still rich in transzonal processes (Fig. 3B). Thus, the loss of actin-containing transzonal processes was associated with PVS formation.

PVS formation reflects meiotic competence in full-grown, GV-stage oocytes

When GV-stage oocytes were cultured in IBMX-free medium, a PVS was observed in all of the oocytes that had undergone GVBD and in few oocytes that had not. Therefore, we hypothesized that oocytes with the capacity to form a visible PVS were meiotically competent and that those without this capacity were not. To examine this hypothesis, we compared the meiotic competence of PVS(+) and PVS(–) oocytes. After GV-stage oocytes were cultured for 1 h with IBMX, they were sorted into PVS(+) and PVS(–) groups and then cultured without IBMX. Of the 228 oocytes used in this experiment, 159 (70%), 63 (28%) and 6 (3%) were PVS(+), PVS(–) and PVS(±), respectively. After culture without IBMX, 91.0% of the PVS(+) oocytes underwent GVBD, and 80.1% of them emitted a first polar body. On the other hand, only 14.9% of PVS(–) oocytes underwent GVBD, and only 5.5% of them emitted a first polar body (Fig. 4). The difference in meiotic competence between the PVS(+) and PVS(–) oocytes was significant (P<0.001 for both GVBD and first polar body emission). Since
the treatment with IBMX for 1 h had no detrimental effect on accomplishment of meiosis or preimplantation development (Fig. 5), PVS formation should be a useful index for predicting the meiotic competence of GV-stage oocytes.

PVS formation is associated with chromatin configuration in full-grown, GV-stage oocytes

Chromatin configuration has been reported to be associated with the meiotic competence of GV-stage oocytes; SN-type oocytes complete meiotic maturation with high frequency, whereas NSN-type oocytes complete it with low frequency [7, 12].
Since we found that the PVS formation of GV-stage oocytes correlated with meiotic competence, as described above, we next examined the correlation between PVS formation and chromatin configuration. Of the 290 oocytes used in this experiment, 200 (69%), 84 (29%) and 6 (2%) were PVS(+), PVS(–) and PVS(±), respectively. Their chromatin configurations were evaluated under UV irradiation following staining of the DNA with Hoechst 33342. The SN-type oocyte was characterized by highly condensed chromatin concentrated in the area around the nucleolus (Fig. 6A). The chromatin of the NSN-type oocyte was uniformly distributed in the GV nucleus and did not surround the nucleolus. We also observed intermediate-type (M-type) oocytes at a low frequency. In these oocytes, the chromatin was diffused throughout the nucleus but partially or completely surrounded the nucleolus. In the PVS(+) group, most of the oocytes (91%) displayed the SN-type configuration, whereas only a small fraction (7%) displayed the NSN-type configuration (Fig. 6B). In the PVS(–) group, all of the oocytes displayed NSN-type configurations. Thus,

Fig. 4. Meiotic competence of PVS(+) and PVS(–) oocytes. Full-grown oocytes were sorted into two groups according to the presence [PVS(+)]) or absence [PVS(–)] of a visible PVS after culture with IBMX for 1 h. After sorting, the oocytes were cultured without IBMX. After 4 and 16–18 h, they were examined for the occurrence of germinal vesicle breakdown (GVBD) and emission of a first polar-body (1st Pb), respectively. This experiment was performed five times using 36–51 oocytes; 159 PVS(+) and 63 PVS(–) oocytes were examined in total. Error bars represent ± SEM.

Fig. 5. Effect of treatment with 3-isobutyl-1-methylxanthine (IBMX) on meiotic maturation and preimplantation development. (A) Full-grown oocytes were collected in medium with (IBMX-1 h) or without (IBMX-0 h) IBMX. In the IBMX-0 h group, oocytes were collected and cultured in IBMX-free medium, while the IBMX-1 h group oocytes were cultured with IBMX for 1 h and then washed and cultured without IBMX. Four and 16–18 h after culture without IBMX, they were observed for the occurrence of germinal vesicle breakdown (GVBD) and emission of a first polar-body (1st Pb), respectively. This experiment was performed four times using 32–50 oocytes for each case; 162 oocytes were examined in total for each group. Error bars represent ± SEM. (B) MII stage oocytes matured in vitro were inseminated and examined for the progress of their preimplantation development. Six hours after insemination, the embryos with two pronuclei were collected and further cultured. The numbers of embryos that developed to the 2-cell, 4-cell, morula and blastocyst stage were counted 24, 48, 72 and 96 h after insemination, respectively. This experiment was performed four times using 9–17 oocytes in each case; 42 and 50 oocytes were examined in total for the IBMX-0 h and IBMX-1 h groups, respectively. Error bars represent ± SEM.
PVS formation and chromatin configuration in GV-stage oocytes were highly correlated. This correlation was not specific to the mouse strain or the medium used in this experiment, since it was always observed when oocytes were collected from ddY mice or cultured in TYH, Waymouth’s, KSOM or HTF medium (data not shown). In addition, a high correlation between PVS formation and chromatin configuration was also observed in the GV-stage oocytes collected from PMSG-primed 3-week-old mice (data not shown).

Growing oocytes acquire PVS-forming capacity after establishing the SN-type chromatin configuration

The high correlation between PVS formation and chromatin configuration in the full-grown oocytes collected from adult mice led us to extend this investigation to growing oocytes. Oocytes were collected from 16-, 18-, 20-, 23- and 25-day-old mice that were not subjected to hormonal stimulation and were then sorted into two groups according to the presence or absence of a visible PVS. After sorting, the oocyte DNA was stained with Hoechst 33342 and the number of surrounded-nucleolus (SN) type oocytes was counted. This experiment was performed four times using 42-101 oocytes in each experiment. The total numbers of oocytes examined from the 16-, 18-, 20-, 23- and 25-day-old mice were 231, 287, 313, 342 and 232, respectively. Error bars represent ± SEM.

Fig. 6. Chromatin configuration of full-grown PVS(+) and PVS(−) oocytes. (A) Fluorescent images are shown for the surrounded-nucleolus (SN) type (left panel), intermediate (M) type (middle panel) and non-surrounded-nucleolus (NSN) type configurations (right panel). Full-grown oocytes were stained with Hoechst 33342 and observed under an epifluorescence microscope. (B) Full-grown oocytes were sorted into two groups according to the presence or absence of a visible PVS after culture with IBMX for 1 h. The numbers of PVS(+) and PVS(−) oocytes were 200 and 84, respectively. After sorting, the oocyte DNA was stained with Hoechst 33342, and the chromatin configuration was evaluated. Similar results were obtained in eight independent experiments, and the data were pooled.

Fig. 7. Correlation between chromatin configuration and PVS-forming capacity in growing oocytes. Oocytes collected from 16-, 18-, 20-, 23- and 25-day-old mice were cultured with IBMX for 1 h, and the number of surrounded-nucleolus (SN) type oocytes was counted. This experiment was performed four times using 42-101 oocytes in each experiment. The total numbers of oocytes examined from the 16-, 18-, 20-, 23- and 25-day-old mice were 231, 287, 313, 342 and 232, respectively. Error bars represent ± SEM.

Growing oocytes acquire PVS-forming capacity after establishing the SN-type chromatin configuration

The high correlation between PVS formation and chromatin configuration in the full-grown oocytes collected from adult mice led us to extend this investigation to growing oocytes. Oocytes were collected from 16- to 25-day-old mice, and the numbers of PVS(+) and SN-type oocytes were counted after culture with IBMX for 1 h. As the oocytes grew, the percentages of SN-type and PVS(+)-oocytes increased (Fig. 7), but the kinetics of the increases in the two indices differed. The SN-type oocytes appeared before the PVS(+) oocytes did. Mice less than 20 days old yielded a lower percentage of PVS(+) oocytes than of SN-type oocytes (significance at 20 days, P<0.01), whereas no difference was observed for mice more than 23 days old. Although a large fraction of the PVS(+) oocytes exhibited an SN-type configuration at all stages (100, 76, 73, 83 and 86% on day 16, 18, 20, 23 and 25, respectively), the reverse was not true; only 6, 44, 44, 82 and 97% of the SN-type oocytes were PVS(+) on day 16, 18, 20, 23 and 25, respectively. These results demonstrate that the SN-type configuration is established before the PVS formation potential develops. Thus, the capacity to form a PVS is not associated with chromatin configuration in grow-
ing oocytes.

Discussion

In the present study, we found that GV-stage oocytes formed a visible PVS during in vitro culture, independently of the process of meiotic maturation (Fig. 1). PVS formation was caused by dissociation of the ZP from the plasma membrane (Fig. 2), which appeared to result from the loss of actin-filled transzonal processes (Fig. 3). PVS formation correlated well with meiotic competence and with chromatin configuration in full-grown oocytes (Figs. 4 and 6).

Our data showed that PVS(–) oocytes, but not PVS(+) oocytes, were rich in actin-filled transzonal processes (Fig. 3), and this characteristic probably contributed to PVS formation. When GV-stage oocytes were observed shortly after isolation from follicles, most were rich in transzonal processes (data not shown). The processes remaining within the ZP upon isolation from follicles seemed to disappear during in vitro culture. When the oocytes were cultured in vitro, they began to shrink. Some of the oocytes that retained intact transzonal processes shrank with their ZP, whereas others, in which the processes were reduced, shrank separately from their ZP; since the transzonal processes intrude into the plasma membrane of the oocyte, they hinder its dissociation from the ZP. Therefore, oocytes retaining an appreciable number of transzonal processes during in vitro culture may not form a PVS; however, those in which most of the processes disappear may form a PVS.

The capacity to form a PVS correlated well with meiotic competence (Fig. 4) and chromatin configuration (Fig. 6). The transzonal processes are thought to be important in establishing and maintaining the structural integrity of cumulus cell-oocyte gap junctions [26, 27], which are required for acquisition of meiotic competence and establishment of the SN-type configuration [18]. Once oocytes have acquired these characteristics, they may no longer require the processes that maintain the gap junctions, resulting in loss of the processes. This hypothesis is supported by a report showing that the number of transzonal processes is reduced and cumulus expansion occurs after gonadotropin stimulation [31] which promotes acquisition of meiotic competence [32] and establishment of the SN-type configuration [11, 33, 34]. Furthermore, the microvilli of oocytes derived from antral follicles are longer than those of oocytes derived from preovulatory follicles [16]. Indeed, our results show that growing oocytes acquire PVS-forming capacity after the SN-type configuration is established (Fig. 7).

Since PVS formation correlated well with the chromatin configuration in GV-stage oocytes (Fig. 6), observation of PVS formation appears to be a useful way of distinguishing between SN- and NSN-type oocytes without Hoechst staining. To determine the chromatin configuration, the oocyte DNA must be stained with Hoechst and visualized under ultraviolet (UV) light, which is reported to decrease meiotic competence [6, 35, 36]. Previous studies using Hoechst staining to determine the chromatin configuration have shown that even low concentrations of Hoechst combined with short exposure to UV light decrease the competence of preimplantation development [7, 12, 37]. In contrast, the manipulation performed in this study, i.e., 1-h treatment with IBMX, had no detrimental effect on the accomplishment of meiosis or preimplantation development (Fig. 5). Therefore, observation of PVS formation after culture with IBMX for 1 h is a useful alternative method for discriminating SN-type oocytes from NSN-type oocytes.

PVS formation is also a useful criterion for judging the meiotic competence of full-grown oocytes. Some oocytes collected from adult animals cannot accomplish meiosis [1, 6, 7]. This mixture of meiotically incompetent and competent oocytes hampers precise analysis of the mechanism of meiotic competence. Therefore, culling the meiotically competent oocytes is important. Furthermore, the PVS formation criterion for meiotic competence will contribute to assisted reproductive technologies, if it is also associated with meiotic competence in human oocytes. Incomplete meiotic maturation is a reported cause of human infertility [38, 39]. Since this incomplete maturation appears to arise from inadequate cytoplasmic development, GV transplantation might be an effective treatment [40–42]. Given that the recipient oocyte of GV transfer must be of high quality, the PVS formation parameter might be useful in identifying appropriate recipient oocytes.
PERIVITELLINE SPACE FORMATION IN OOCYTES

References


