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Rotation of Meiotic Spindle Is Controlled by Microfilaments in Mouse Oocytes[1]

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ABSTRACT

The completion of meiosis requires the spatial and temporal coordination of cytokinesis and karyokinesis. During meiotic maturation, many events, such as formation, location, and rotation of the meiotic spindle as well as chromosomal movement, polar body extrusion, and pronuclear migration, are dependent on regulation of the cytoskeleton system. To study functions of microfilaments in meiosis, we induced metaphase II (MII) mouse oocytes to resume meiosis by in vitro fertilization or parthenogenetic activation, and we treated such oocytes with cytochalasin B (CB). The changes of the meiotic spindle, as visualized in preparations stained for β-tubulin and chromatin, were observed by fluorescent confocal microscopy. The meiotic spindle of MII oocytes was observed to be parallel to the plasmalemma. After meiosis had resumed, the spindle rotated to the vertical position so that the second polar body could be extruded into the perivitelline space. When meiosis resumed and oocytes were treated with 10 μg/ml of CB, the spindle rotation was inhibited. Consequently, the oocyte formed an extra pronucleus instead of extruding a second polar body. These results indicate that spindle rotation is essential for polar body extrusion; it is the microfilaments that play a crucial role in regulating rotation of the meiotic spindle.

gamete biology, in vitro fertilization, meiosis, oocyte development, ovum

INTRODUCTION

Completion of the first meiosis results in formation of the first polar body, and in almost all mammals, oocytes are arrested at metaphase II (MII). After fertilization or parthenogenetic activation, MII-arrested oocytes enter into anaphase, followed by completion of the second meiosis and formation of the second polar body. Meiosis (as well as mitosis) requires the spatial and temporal coordination of cytokinesis and karyokinesis [1]. The cytoskeleton system is important for cytokinesis in most mammalian cells [2].

The completion of two meiotic divisions is a result of the coordination of meiotic spindle assembly and function with meiotic cell-cycle progression [3]. The dynamic changes of assembly and disassembly of microtubules (MTs) and microfilaments (MFs) in meiosis have been studied in *Xenopus* [4, 5], *Drosophila* [6], yeast [7], and many mammals [8–14]. Nevertheless, little is known about MT and MF functions or about their interaction in meiotic spindle formation and rotation, chromosomal movement, and polar body extrusion.

Polymerization of G-actin into F-actin results in MF assembly. Cytochalasin B (CB), an inhibitor of MF polymerization, is widely used in animal cloning [15, 16] and polyploid embryo or cell induction [17–19]. It is generally accepted that CB inhibits the polymerization of MFs by blocking monomer addition at the fast-growing end of F-actin.

In the present study, we observed meiotic progress in oocytes treated with CB to block F-actin polymerization during in vitro fertilization (IVF) or parthenogenetic activation to analyze the role of MFs in processes accompanying meiosis.

MATERIALS AND METHODS

Superovulation and Collection of Mouse Oocytes

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethical committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Female Kunming White mice (age, 6–8 wk) were injected with 7.5 IU of eCG (Hua Fu Biotechnology Company, Tianjin, China), followed 48 h later by 7.5 IU of hCG (Institute of Zoology, Chinese Academy of Sciences, Beijing). The females were killed 14–17 h post-hCG administration, and the ovulated oocytes were released from the ampullae of oviducts into pre-equilibrated M2 medium (Sigma, St. Louis, MO). Cumulus-oocyte complexes (COCs) were used directly for IVF. Alternatively, the cumulus cells removed by brief exposure to M2 containing 300 IU/ml of hyaluronidase (Sigma), and then the cumulus-free, MII-stage oocytes were used for parthenogenetic activation after being washed at least three times with M2.

In Vitro Fertilization

Cauda epididymides from male Kunming mice (age, 8–12 wk) were immersed in a 200-μl drop of M16 medium (Sigma). Sperm were released by cutting cauda epididymides with a pair of scissors. For capacitation, the sperm were cultured for 1–1.5 h in M16 medium under liquid paraffin in a humidified atmosphere of 5% CO_2 at 37°C. Five 10-μl drops of sperm suspension was added to a 50-μl drop of M16 medium containing COCs, giving a final concentration of 10^{6-7} sperm/ml. After coculturing sperm and oocytes for 6 h, sperm were removed by washing oocytes in M2 medium. The fertilized oocytes were further cultured until they were used.

Parthenogenetic Activation

Just before use, $SrCl_2$ was added to Ca^{2+}-free M16 medium. Cumulus-free MII oocytes were incubated in M16 medium containing 10 mM $SrCl_2$

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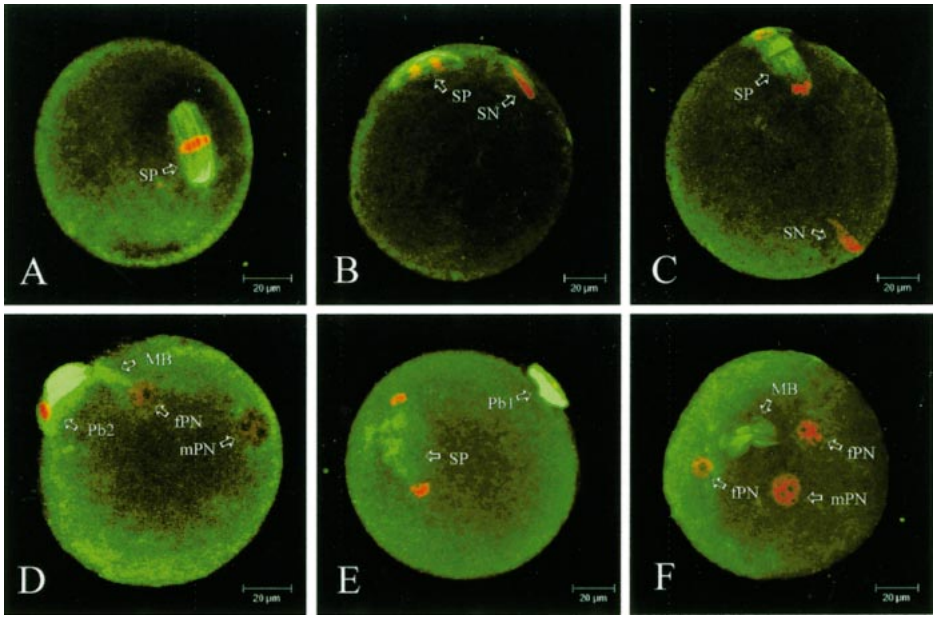

FIG. 1. Resumption of meiosis induced by IVF and influence of CB on spindle rotation. Green indicates microtubules; red indicates chromatin. **A**) At 0 h postinsemination, MII spindle (SP) was parallel to plasmalemma, and chromosomes were arranged in the equatorial plate of spindle. **B**) At 1–2 h, meiosis resumed as a result of sperm penetration. Daughter chromosomes separated toward the two spindle poles. SN, Expanded sperm head. **C**) At 1–3 h, the spindle rotated so that its long axis became oriented perpendicular to the oocyte's surface from parallel to vertical with respect to the plasmalemma. **D**) At 3–6 h (i.e., telophase II), the second polar body (Pb2) was extruded, while female and male pronuclei (fPN and mPN, respectively) formed. **E**) At 1–4 h, CB-treated oocytes initiated nuclear division, but the spindle failed to rotate. **F**) At 4–6 h, because spindle rotation was inhibited by CB, the second polar body failed to form and both groups of maternal chromosomes that remained within the oocyte formed two female pronuclei connected by a midbody (MB).

for 4–6 h. After washing with M2, the oocytes were further cultured in CZB (Chatot-Ziomek-Bavister) medium.

Treatment with CB

Cytochalasin B (Sigma) was dissolved as a stock solution (1 mg/ml) in dimethyl sulfoxide (DMSO; Sigma) and stored at −20°C. It was later diluted to a final concentration of 10 μg/ml in insemination medium or activation medium. An equivalent dilution of DMSO was used for controls. The IVF oocytes were treated with CB for 6 h, and the parthenogenetic oocytes were treated for 4 h. Both the IVF oocytes and the parthenogenetic oocytes were removed from the medium containing CB, washed at least three times in M2, and then cultured in CB-free medium. In addition, the effects of diverse CB-treatment periods (0–5 h) on polar body extrusion of parthenogenetic oocytes were analyzed. Data were analyzed using the chi-square test, with significance determined at $P < 0.05$.

Immunofluorescent Staining and Confocal Microscopy

Samples were taken at 1-h intervals until clear pronuclei were observed. Oocytes were fixed with 3.7% (w/v) paraformaldehyde in PBS for 40 min at room temperature. Fixed oocytes were stored in PBS containing 0.3% (w/v) BSA for up to 1 wk at 4°C. Fixed oocytes were permeabilized by transferring into PBS containing 0.1% (w/v) Triton X-100 and 0.3% BSA and then incubating them for 30–40 min at 37°C. After washing twice with PBS containing 0.01% Triton X-100, oocytes were incubated in block solution (PBS containing 150 mM glycine and 0.3% BSA) for 30 min at 37°C. The MTs were localized with a mouse monoclonal antibody against β-tubulin (Sigma), which was diluted in the blocking solution (1:160 [w/v]) before use. Oocytes were incubated for 30–40 min at 37°C or overnight at 4°C, followed by three washes of 5 min each. Oocytes were incubated with fluorescein isothiocyanate-labeled goat-anti-mouse IgG (Sigma) at 1:80 (w/v) final dilution for 30–40 min at 37°C, followed by three washes of 5 min each. Chromatin was stained with 10 μg/ml of propidium iodide (Sigma). Finally, oocytes were mounted on slides with antifluorescence-fade medium (DABCO). The samples were examined with a laser-scanning confocal microscope (Leica TCS-4D, Bensheim, Germany). Images were processed with Photoshop 6.0 software (Adobe Systems, Inc., San Jose, CA).

RESULTS

Dynamic Changes of Spindle During IVF-Induced Meiosis

By immunofluorescent staining, MTs were found mainly in the well-organized spindle in MII oocytes. The spindle was symmetrical, bipolar, barrel-shaped, and located near the cortex of the oocyte (Fig. 1A). From 1 to 3 h postinsemination, a period during which oocytes were at the anaphase of meiosis II, the spindle migrated further into the cortex, and daughter chromatids separated toward the two poles, followed by the spindle rotating from parallel to vertical with respect to the surface of the oocyte (Fig. 1, B and C). From 3 to 6 h (i.e., at the telophase stage), the spindle was oriented vertically with respect to the oocyte's surface. It then elongated and formed the second polar body, which was extruded into the perivitelline space. A female pronucleus and a male pronucleus were observed in the cytoplasm before long (Fig. 1D).

Effect of CB on Spindle During IVF-Induced Meiosis

Sperm and oocytes were cocultured in the insemination medium containing 10 μg/ml of CB for 6 h. The oocytes were washed to remove sperm and transferred into a CB-free M16 medium. Neither the resumption of meiosis nor the separation of daughter chromatids (i.e., nuclear division) was disturbed. However, in CB-treated IVF oocytes, spindle rotation was inhibited. At 1 to 4 h post-CB treatment, 35 of 56 spindles (62.5%) of fertilized oocytes were still parallel to the plasmalemma, whereas only 7 of 56 spindles (12.5%) were vertical with the oocyte's surface (Fig. 1E). At 4–6 h, meiosis was terminated. Chromosomes that had reached spindle poles decondensed and, therefore, formed two female pronuclei that were connected by a mid-

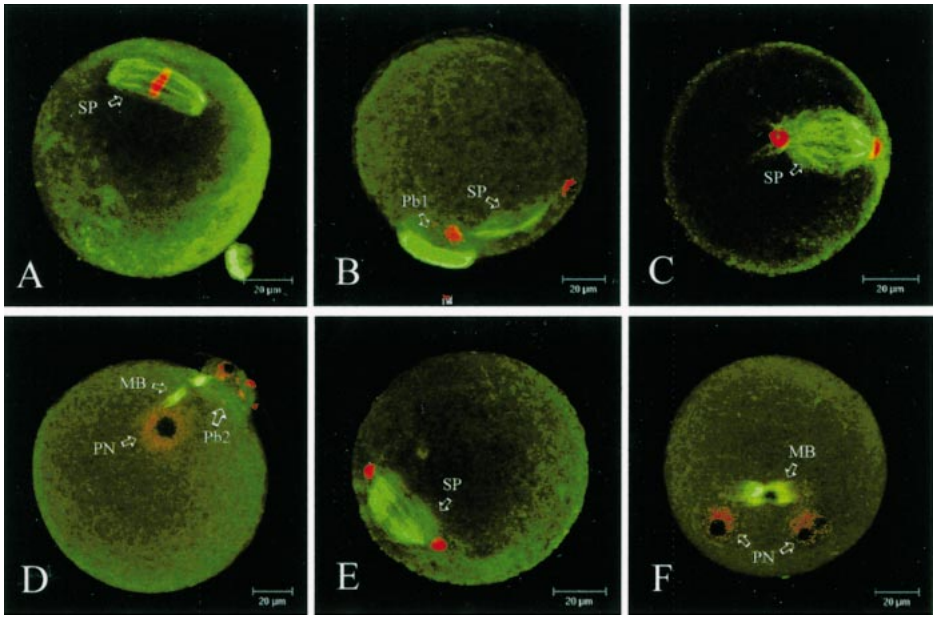

FIG. 2. The influence of CB on spindle rotation in parthenogenetically activated oocytes. Green indicates microtubule; red indicates chromatin. **A**) At 0 h postactivation, MII spindle (SP) was parallel to the oocyte's surface, and chromosomes were arranged on the equatorial plate. **B**) At 1 h, meiosis was resumed, and anaphase II was entered. **C**) At 2 h, the spindle rotated from parallel to vertical respective to the plasmalemma. **D**) At 2–6 h (i.e., telophase II), the second polar body (Pb2) extruded, followed by female pronucleus formation. **E**) At 2–3 h, CB-treated oocytes failed to undergo spindle rotation. **F**) At 4–6 h, because spindle rotation was inhibited by CB, Pb2 was released into the cytoplasm, where two female pronuclei were connected by a midbody (MB).

body. As a consequence of this treatment, these fertilized oocytes were triploid, containing one male pronucleus and two female pronuclei (Fig. 1F).

Dynamic Changes of Spindle During Meiosis Induced by Parthenogenetic Activation

Dynamic changes of spindle during meiosis induced by parthenogenetic activation were similar to those of IVF-induced meiosis. Spindle rotation and second polar body extrusion occurred mainly within 2 and 3 h, respectively, which was slightly earlier than in IVF oocytes. However, a female pronucleus was formed at 4–6 h, which was not earlier than in IVF oocytes (Fig. 2, A–D).

Effect of CB on Spindle During Parthenogenetic Activation of Oocytes

Addition of CB to the activation medium inhibited spindle rotation, but nuclear division did not appear to be disturbed. Fifty-one of 75 spindles (68%) of activated oocytes were still parallel to the plasmalemma, whereas only 8 of 75 (10.7%) were vertical to the oocyte's surface (Fig. 2E). The maternal chromatin normally released into the forming second polar body remained within the oocyte's cytoplasm. Therefore, two haploid female pronuclei connected by a midbody were observed in the cytoplasm (Fig. 2F).

Influence of CB Treatment Time on Second Polar Body Extrusion

Oocytes were treated with CB for various times (0–5 h) during activation. As shown in Table 1, 88.7% of activated oocytes in the control group extruded the second polar body at 3 h postactivation. When the CB treatment time was less than 2 h, polar body extrusion and spindle rotation occurred on time. When the CB treatment time was 3 h, 55.9% of activated oocytes failed to extrude the second polar body, which transformed into an extra female pronucleus that remained within the cytoplasm. The rates of second polar body extrusion were 19.8% and 7.6% when oocytes were treated for 4 and 5 h, respectively.

DISCUSSION

We observed the dynamic changes of meiotic spindles in both IVF oocytes and parthenogenetically activated oocytes. The results were consistent with previous reports in mouse [8, 9], and the dynamics of the spindle in mouse oocytes were similar to those observed in porcine [10, 11], horse [12], bovine [13], and human [14] oocytes. In most mammalian species, including human, assembly/disassembly of the meiotic spindle and its dynamics during meiosis are similar, although species-specific properties, such as spindle shape and size, have been found [20]. The formation of metaphase spindle, both in meiosis I and II, is es-

TABLE 1. Influence of CB treatment time on second polar body extrusion.

Time of CB treatment	No. of oocytes	No (%) of activated oocytes	No. (%) of second polar body extruded/no. activated oocytes	No. (%) of oocytes containing two pronuclei/ no activated oocytes	No. (%) of second polar body extruded 3 h post-CB/no activated oocytes
0	129	97 (75.2)	94 (96.9)[a]	3 (3.1)	86 (88.7)
1	98	86 (87.6)	87 (97.6)[a]	2 (2.4)	79 (91.9)
2	127	109 (85.8)	98 (89.9)[a]	11 (10.1)	89 (81.7)
3	132	111 (84.1)	49 (44.1)[b]	62 (55.9)	9 (8.1)
4	121	91 (75.2)	18 (19.8)[b]	73 (80.2)	10 (11.0)
5	98	79 (80.6)	6 (7.6)[b]	73 (92.4)	5 (6.3)

[a,b] Values with different superscripts within the same column were significantly different ($P < 0.001$; χ^2 test).

sential to nuclear division. At metaphase, MTs distribute mainly in the bipolar spindle, which is located near the cortex region and is parallel to the plasmalemma. At anaphase and telophase, homologous chromosomes or daughter chromatids are drawn to the spindle poles, followed by spindle rotation. Finally, the first or second polar body is extruded.

Cytochalasin B, an MF depolymerization drug widely used in animal cloning [15, 16] and polyploid induction [17–19], can inhibit polar body extrusion and cytokinesis, but its mechanism has not been clearly understood. Longo and Chen [21] reported that germinal vesicle breakdown could occur normally when germinal vesicle-stage oocytes were treated with CB but that spindles could not migrate to cortex region, so that the extrusion of the first polar body was inhibited. Our results showed that CB did not affect chromosomal movement and nuclear division but did inhibit spindle rotation and, thus, cytokinesis. As a result, the chromatin normally partitioned to the second polar body remained within the oocyte cytoplasm and was transformed into an extra pronucleus. These results illustrate that the spindle rotation is essential for polar body extrusion, and MFs are instrumental in controlling rotation of the meiotic spindle.

Changes in oocyte cytoplasmic organization are executed with great temporal and spatial precision to ensure that peri- and postfertilization events of embryogenesis proceed on schedule and without error [22]. When the time of CB treatment was beyond 3 h during activation, irreversible inhibition of spindle rotation was observed. This is probably because CB depolymerizes MFs during the key period of spindle rotation and polar body extrusion. Following washing to remove CB, both spindle rotation and polar body extrusion failed to occur, indicating that temporal and spatial factors associated with these procedures were no longer satisfied. We also showed that 2-h CB treatment did not inhibit second polar body extrusion, which is consistent with previous reports [1, 8].

Recent studies have demonstrated that jasplakinolide, a drug promoting MF polymerization and stabilization, in contrast to CB, also inhibited oocyte maturation and polar body extrusion [23]. It is suggested that the dynamic balance between assembly and disassembly of MFs is disordered by the drug either promoting or inhibiting MF polymerization and, thus, inhibiting spindle rotation and polar body extrusion.

In conclusion, the present results indicate that MFs play a crucial role in controlling spindle rotation in mouse oocytes.

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