

1 **Title: Rotation of Meiotic Spindle is Controlled by Microfilaments in Mouse**

2 **Oocytes***

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4 **Short title: Spindle is controlled by microfilaments in mouse**

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6 **Key words: in vitro fertilization, meiosis, oocyte development, ovum, gamete**

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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 the regulation of the cytoskeleton system. In order to study functions of microfilaments (MF) in meiosis, we induced MII mouse oocytes to resume meiosis by in vitro fertilization (IVF) or parthenogenetic activation, and 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 stage 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 μ g/ml 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 the rotation of meiotic spindle.

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Introduction

Completion of the first meiosis results in the formation of the first polar body and in almost all mammals, oocytes are arrested at metaphase II. After fertilization or parthenogenetic activation, MII-arrested oocytes enter into anaphase followed by the completion of the second meiosis and the formation of the second polar body. Meiosis, as well as mitosis, requires the spatial and temporal coordination of cytokinesis and karyokinesis [1]. 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 (MT) and microfilaments (MF) 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 and interaction in the formation and rotation of the meiotic spindle, chromosomal movement and polar body extrusion.

Polymerization of G-actin into F-actin results in microfilament 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 MF by blocking monomer addition at the fast-growing end of F-actin.

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

1 *Parthenogenetic activation*

2 SrCl₂ was added to Ca²⁺-free M16 just before use. Cumulus-free MII oocytes were incubated in
3 M16 medium containing 10mM SrCl₂ for 4-6h. After washing with M2, the oocytes were further
4 cultured in CZB medium.

5 *Treatment with CB*

6 CB (Sigma) was dissolved as a stock solution (1mg/ml) in DMSO (Sigma) and stored at -20°C. CB
7 was diluted to a final concentration of 10µg/ml in insemination medium or activation medium. An
8 equivalent dilution of DMSO was used for controls. IVF oocytes were treated with CB for 6h and
9 parthenogenetic oocytes were treated for 4h. Both the IVF oocytes and parthenogenetic oocytes were
10 removed from the medium containing CB, washed at least 3 times in M2 and then cultured in
11 CB-free medium. In addition, the effects of diverse CB-treatment periods (0~5h) on polar body
12 extrusion of parthenogenetic oocytes were analyzed. Data were analyzed using the X² test with
13 significance determined at P<0.05.

14 *Immunofluorescent staining and confocal microscopy*

15 Samples were taken at 1h intervals until clear pronuclei were observed. Oocytes were fixed with
16 3.7% paraformaldehyde in PBS for 40min at room temperature. Fixed oocytes were stored in PBS
17 containing 0.3% BSA for only up to one week at 4°C. Fixed oocytes were permeabilized by
18 transferring into PBS containing 0.1% Triton X-100 and 0.3% BSA and incubating them for 30-40
19 min at 37°C. After washing twice with PBS containing 0.01% Triton X-100, oocytes were incubated
20 in block solution (PBS containing 150mM glycine and 0.3% BSA) for 30min at 37°C. MT were

1 localized with a mouse monoclonal antibody against β -tubulin (Sigma), which was diluted in the
2 blocking solution (1:160) before use. Oocytes were incubated for 30-40min at 37°C or overnight at 4
3 °C followed by 3 washs 5min each. Oocytes were incubated with FITC-labeled goat-anti-mouse IgG
4 (Sigma) at 1:80 final dilution for 30-40min at 37°C, and washed 3 times for 5min each. Chromatin
5 was stained with 10 μ g/ml propidium iodide (Sigma). Finally, oocytes were mounted on slides with
6 anti-fluorescence-fade medium (DABCO). The samples were examined with a laser scanning
7 confocal microscope (Leica TCS-4D). Images were processed with Photoshop 6.0 software.

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Results

10 *Dynamic changes of spindle during IVF-induced meiosis*

11 By immunofluorescent staining, microtubules were found mainly in the well-organized spindle in
12 MII-stage oocytes. The spindle was symmetrical, bipolar, and barrel-shaped, and was located near
13 the cortex of oocyte (Fig. 1A). From 1 to 3h post-insemination, a period during which oocytes were
14 at the anaphase of meiosis II, the spindle migrated further into the cortex, and daughter chromatids
15 separated toward the two poles, followed by the spindle rotated from parallel to vertical with respect
16 to the surface of the oocyte (Fig. 1B, C). From 3 to 6h, that is, at the telophase stage, the spindle was
17 oriented vertically with respect to the oocyte's surface. It then elongated and formed the second polar
18 body (Pb2) which was extruded into the perivitelline space. A female pronucleus and a male
19 pronucleus were observed in the cytoplasm before long (Fig.1D).

1 *Effect of Cytochalasin B on spindle during IVF-induced meiosis*

2 Sperm and oocytes were co-cultured in the insemination medium containing 10 μ g/ml CB for 6h.
3 The oocytes were washed to remove sperm and transferred into a CB-free M16 medium. Neither the
4 resumption of meiosis nor daughter chromatids separation, namely nuclear division, was disturbed.
5 However, in CB-treated IVF oocytes, spindle rotation was inhibited. At 1 to 4h post-CB treatment,
6 most (62.5%, 35/56) spindles of fertilized oocytes were still paralleled to the plasmalemma, while
7 only 12.5%(7/56) of spindles were vertical with the oocyte's surface (Fig. 1E). At 4-6h, meiosis was
8 terminated. Chromosomes which had reached spindle poles, decondensed and therefore formed two
9 female pronuclei which were connected by a midbody. As a consequence of this treatment, these
10 fertilized oocytes were triploid, containing one male pronucleus and two female pronuclei (Fig.1F).

11 *Dynamic changes of spindle during meiosis induced by parthenogenetic activation*

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

16 *Effect of CB on spindle during parthenogenetic activation of oocytes*

17 Addition of CB to the activation medium inhibited spindle rotation, but nuclear division did not
18 appear to be disturbed. Sixty-eight percent (51/75) of spindles of activated oocytes were still
19 paralleled to the plasmalemma, while only 10.7%(8/75) were vertical to the oocyte's surface (Fig.
20 2E). The maternal chromatin normally released into the forming second polar body remained within

1 the oocyte's cytoplasm. Therefore, two haploid female pronuclei connected by a midbody were
2 observed in the cytoplasm (Fig. 2F).

3 *The influence of CB treatment time on the 2nd polar body extrusion*

4 Oocytes were treated with CB for various times (0-5h) during activation. As shown in Table 1,
5 88.7% of activated oocytes in control group extruded the 2nd polar body at 3h post-activation. When
6 the CB treatment time was less than 2 hours, polar body extrusion and the rotation of spindles
7 occurred on time. When treated with CB for 3 hours, 55.9% of activated oocytes failed to extrude the
8 Pb2, which transformed into an extra fPN remained within the cytoplasm. The rates of Pb2 extrusion
9 were 19.8% and 7.6%, respectively, when oocytes were treated for 4h and 5h.

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Discussion

12 We observed the dynamic changes of meiotic spindles in both IVF oocytes and
13 parthenogenetically activated oocytes. The results were consistent with the previous report in mouse
14 [8,9]; the dynamics of spindle in mouse oocytes was similar to those observed in porcine [10,11],
15 horse [12], bovine [13] and human [14] oocytes. In most mammalian species, including human,
16 assembly/disassembly of the meiotic spindle and its dynamics during meiosis are similar, although
17 there are specie-specific properties, such as spindle shape and size [20]. The formation of metaphase
18 spindle, both in meiosis I and II, is essential to nuclear division. At metaphase, microtubules
19 distribute mainly in the bipolar spindle, which is located near the cortex region, and is parallel to the
20 plasmalemma. At anaphase and telophase, homologous chromosomes or daughter chromatids are

1 drawn to the spindle poles followed by spindle rotation. Finally, the first or second polar body is
2 extruded.

3 It has been known that cytochalasin B, a microfilament depolymerization drug widely used in
4 animal cloning [15,16] and polyploid induction [17~19], can inhibit the polar body extrusion and
5 cytokinesis, but its mechanism has not been clearly understood. Longo and Chen(1985) reported that
6 GVBD could occur normally when GV-stage oocytes were treated with CB, but spindles could not
7 migrate to cortex region so that the extrusion of Pb1 was inhibited [21]. Our results showed that CB
8 did not affect chromosomal movement and nuclear division, but it inhibited spindle rotation, and thus
9 cytokinesis. As a result, the chromatin normally partitioned to the second polar body remained within
10 the oocyte cytoplasm and was transformed into an extra pronucleus. These results illustrate that the
11 spindle rotation is essential for the polar body extrusion, and microfilaments are instrumental in
12 controlling the rotation of meiotic spindle.

13 Changes in oocyte cytoplasmic organization are executed with great temporal and spatial precision
14 to ensure that peri- and post-fertilization events of embryogenesis proceed on schedule and without
15 error [22]. When the time of CB treatment is beyond 3 hours during activation, irreversible inhibition
16 of spindle rotation was observed. This is probably due to the fact that CB depolymerizes
17 microfilaments during the key period of spindle rotation and polar body extrusion. Following
18 washing to remove CB, both spindle rotation and polar body extrusion failed to occur, indicating that
19 temporal and spatial factors associated with these procedures are no longer satisfied. We also
20 showed that 2-hour CB treatment did not inhibit Pb2 extrusion, consistent with the previous reports
21 [1,8].

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

6 In conclusion, the results presented here indicate that microfilaments play a crucial role in
7 controlling spindle rotation in mouse oocytes.

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1 Figure legends:

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3 Fig.1. Resumption of meiosis induced by IVF and influence of CB on spindle rotation. Green: microtubules; Red:

4 chromatin. (A) 0h post-insemination, MII-stage spindle(SP) was parallel to plasmalemma, and chromosomes were

5 arranged in the equatorial plate of spindle. (B) At 1-2h, meiosis resumed as a result of sperm penetration. Daughter

6 chromosomes separated toward the two spindle poles. SN, expanded sperm head. (C) At 1-3h, the spindle rotated so

7 that its long axis became oriented perpendicular to the oocyte's surface from parallel to vertical with respect to the

8 plasmalemma. (D) At 3-6h, namely telophaseII, the second polar body (Pb2) was extruded while female and male

9 pronuclei (fPN, mPN) formed. (E) At 1-4h, CB-treated oocytes initiated nuclear division, but the spindle failed to

10 rotate. (F) At 4-6h, as spindle rotation was inhibited by CB, the second polar body failed to form and both groups of

11 maternal chromosomes that remained within the oocyte formed two female pronuclei connected by a midbody (MB).

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13 Fig.2. The influence of CB on spindle rotation in parthenogenetically activated oocytes. Green: microtubule; red:

14 chromatin. (A) At 0h post-activation, MII-stage spindle(SP) was parallel to the oocyte's surface, and chromosomes

15 were arranged on the equatorial plate. (B) At 1h, meiosis was resumed and entered anaphase II. (C) At 2h, spindle

16 rotated from parallel to vertical against plasmalemma. (D) At 2-6h, namely telophaseII, the second polar body (Pb2)

17 extruded followed by female pronucleus formation. (E) At 2-3h, CB-treated oocytes failed to undergo spindle rotation.

18 (F) At 4-6h, as spindle rotation was inhibited by CB, Pb2 was released into the cytoplasm where two female pronuclei

19 connected by midbody (MB) .

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1 Table 1. The influence of CB treatment time on the 2nd polar body extrusion

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Time(h) of CB treatment	No. of oocytes	No. of activated oocytes(%)	No. of Pb2 extruded/ No. activated oocytes(%)	No. of oocytes containing 2 PN/ No. activated oocytes(%)	No. of Pb2 extruded 3h 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)	84(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)

3 ^{ab}Values with different superscripts within the same column were significantly different ($P < 0.001$; χ^2
4 test).



