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Intra-oocyte Localization of MAD2 and Its Relationship with Kinetochores, Microtubules, and Chromosomes in Rat Oocytes During Meiosis[1]

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ABSTRACT

The present study was designed to investigate subcellular localization of MAD2 in rat oocytes during meiotic maturation and its relationship with kinetochores, chromosomes, and microtubules. Oocytes at germinal vesicle (GV), prometaphase I (ProM-I), metaphase I (M-I), anaphase I (A-I), telophase I (T-I), and metaphase II (M-II) were fixed and immunostained for MAD2, kinetochores, microtubules and chromosomes. The stained oocytes were examined by confocal microscopy. Some oocytes from GV to M-II stages were treated by a microtubule disassembly drug, nocodazole, or treated by a microtubule stabilizer, Taxol, before examination. Anti-MAD2 antibody was also injected into the oocytes at GV stage and the injected oocytes were cultured for 6 h for examination of chromosome alignment and spindle formation. It was found that MAD2 was at the kinetochores in the oocytes at GV and ProM-I stages. Once the oocytes reached M-I stage in which an intact spindle was formed and all chromosomes were aligned at the equator of the spindle, MAD2 disappeared. However, when oocytes from GV to M-II stages were treated by nocodazole, spindles were destroyed and MAD2 was observed in all treated oocytes. When nocodazole-treated oocytes at M-I and M-II stages were washed and cultured for spindle recovery, it was found that, once the relationship between microtubules and chromosomes was established, MAD2 disappeared in the oocytes even though some chromosomes were not aligned at the equator of the spindle. On the other hand, when oocytes were treated with Taxol, MAD2 localization was not changed and was the same as that in the control. However, immunoblotting of MAD2 indicated that MAD2 was present in the oocytes at all stages; nocodazole and Taxol treatment did not influence the quantity of MAD2 in the cytoplasm. Significantly higher proportions of anti-MAD2 antibody-injected oocytes proceeded to premature A-I stage and more oocytes had misaligned chromosomes in the spindles. The present study indicates that MAD2 is a spindle checkpoint protein in rat oocytes during meiosis. When the spindle was destroyed by nocodazole, MAD2 was reactivated in the oocytes to overlook the attachment between chromosomes and microtubules. However, in this case, MAD2 could not check unaligned chromosomes in the recovered spindles, suggesting that a normal chromosome alignment is maintained only in the oocytes without any microtubule damages during maturation.

gamete biology, gametogenesis, meiosis, oocyte development

INTRODUCTION

Segregation of sister chromatids or homologous chromosomes during anaphase is a key event in both mitosis and meiosis. Any error in this process may cause aneuploidy. To avoid these errors, both mitotic and meiotic cells have evolved intrinsic chromosome segregation machinery with high fidelity [1, 2]. Sister chromatids or homologous chromosomes are tightly held together within the cells until the onset of anaphase. Both establishment and maintenance of cohesion between sister chromatids or homologous chromosomes depend on a complex called cohesin [3–6]. It has been indicated that, in yeast, most cohesin remains associated with chromosomes until the metaphase-to-anaphase transition. During the metaphase-to-anaphase transition, some subunit of cohesin is cleaved by a cysteine protease, separase [3–6]. This event is believed to destroy the bridge between sister chromatids or homologous chromosomes and trigger segregation of sister chromatids or homologous chromosomes to opposite poles. It is believed that separase is inactive before anaphase onset and it is activated when all sister chromatids or homologous chromosomes have attached to microtubules in the bipolar spindle [3–6]. The attachment of sister chromatids or homologous chromosomes to microtubules is detected by a surveillance mechanism, the spindle checkpoint [1–6].

Kinetochores act as a central part in the spindle checkpoint [2, 7, 8]. The kinetochores are macromolecular structures associated with the centromeres of chromosomes and are responsible for establishing and maintaining the connection with the microtubules in the mitotic and meiotic spindles. The kinetochores are made up mainly of proteins. So far, two types of centromere proteins (CENPs) have been found in the kinetochores. One type represents constitutive proteins, such as CENP-A, CENP-B, CENP-C, and CENP-D. These CENPs are present at the centromeres throughout the cell cycle and can be detected by anticentromere antibody (ACA), autoimmune sera from patients with calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) [7, 9]. The other type represents transient proteins, such as CENP-E, CENP-F, MAD1, MAD2, BUB1, and BUB3. These proteins can be detected in the specific cell cycle, mostly in M-phase by specific antibodies [10–15]. When CREST was used to label the CENPs in the kinetochores, it was found that these CENPs were present throughout the meiosis of oocytes from germinal vesicle (GV) to metaphase II (M-II)

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stages in mammalian oocytes [16–18]. These results indicate that CREST can be used to study structural dynamics of constitutive CENPs in the kinetochores in mammalian oocytes. As kinetochore functions are largely dependent on the transient proteins, the studies of localization and function of the transient proteins have become more important. These transient proteins include mitotic arrest deficient 1 (MAD1), MAD2, MAD3 (BUBR1), budding uninhibited by benzimidazole 1 (BUB1), BUB3, and motor proteins, such as CENP-E, and cytoplasmic dynein. It has been found that most of these proteins are also located in the kinetochores and the activities of these proteins (kinases) are related to the chromosome movement and anaphase onset during mitosis [7, 10–15, 19–22]. MAD2 is one of the proteins widely studied in mitosis. It has been found that MAD2 senses unattached kinetochores and delays premature anaphase until all kinetochores are attached by microtubules [10, 19–21, 23, 24]. It is well known that MAD2 plays important roles in the spindle checkpoint. However, it appears that differences exist between mitosis and meiosis, between male meiosis and female meiosis, and also between species.

This spindle checkpoint is able to detect a single, unaligned chromosome in the spindle and arrest the cell cycle at metaphase to allow more time for all the chromosomes to move into the correct orientation at the spindle equator before the chromosomes separate [1, 2, 8]. Prior to spindle attachment, kinetochores generate a diffusible wait-anaphase signal and prevent the anaphase-promoting complex/cyclosome (APC/C) from associating with its activator (e.g., cdc20 in yeast, fizzy in *Drosophila,* or p55cdc in mammals), thus inhibit anaphase onset. Unattached chromosomes trigger the production and/or activation of some spindle checkpoint proteins, such as MAD1, MAD2, MAD3 [25], BUB1, BUB3 [26], and MPS1 [27]. Although the mechanism(s) by which cells ensure the sister chromatids or homologous chromosomes attach to the microtubules and the spindle checkpoint proteins control anaphase onset is not clear, it seems that MAD1, MAD2, MAD3, BUB1, and BUB3 lie in the same metaphase-to-anaphase-transition checkpoint pathway, in which MAD2 plays an essential role by associating with APC/C upon unattached kinetochores to delay anaphase initiation until all chromosomes are well aligned at metaphase plate [1, 7, 8, 19–21, 28].

MAD2 is one of the spindle checkpoint proteins that have been widely studied in mitosis. It has been found that MAD2 is a metaphase-to-anaphase-transition checkpoint protein. A study of green fluorescent protein (GFP)-transfected fission yeast indicated that the majority of MAD2 was localized to the nuclear periphery and chromatin domain in the interphase but mainly on the unattached kinetochores in the prometaphase-like stage (microtubules do not attach to kinetochores). Once the microtubule attachment was complete in metaphase stage, MAD2 was localized in the spindle fibers [29]. However, in living PtK1 cells injected with fluorescence-labeled Alexa 488-XMAD2 or transfected with GFP-human MAD2, it was found that MAD2 was spread throughout the cytoplasm in interphase and prophase stages, mainly in the kinetochores in late prophase and early prometaphase, and remained at the kinetochores until the chromosomes were aligned to the bipolar spindle during late prometaphase and early metaphase. Some MAD2 was localized to the spindle poles during prometaphase and early metaphase and was frequently observed along spindle microtubules; no MAD2 was found at the kinetochores, spindle microtubules, or spindle poles during late metaphase, anaphase, and telophase [30]. Studies by using anti-MAD2 immunofluorescence in PtK1 cells and other tissue culture lines also got similar results [10, 23, 24]. When the microtubules were depolymerized by colcemid, nocodazole, and vinblastine or stabilized by Taxol, it was found that localization of MAD2 to kinetochores depends on microtubule attachment, not tension [10, 23, 24, 30, 31].

Further evidence for function of MAD2 was from the studies on the effects of MAD2 inhibition and MAD2 gene deletion and mutation of the MAD2 gene. In PtK1 cells, microinjection of anti-MAD2 antibody in prophase or prometaphase caused premature initiation of chromatid separation before all chromosomes had congressed to the metaphase plate [23]. In MAD2 knockout mice, embryonic cells were unable to arrest after spindle disruption and some cells showed widespread chromosome missegregation and apoptosis [32]; Furthermore, $MAD2^{+/-}$ mice developed lung tumors at high rates after long latencies, suggesting that defects in the mitotic spindle checkpoint could induce tumorigenesis [33]; reduced MAD2 expression caused checkpoint instability, which could be an important factor in the development of ovarian cancer [34], breast cancer, and lung cancer [35] and nasopharyngeal carcinoma [36].

Recently, dynamics and function of MAD2 have also been examined in meiosis in mammals [37–39]. Kallio et al. found that MAD2 remained at most kinetochores throughout the first meiotic division and was lost only at metaphase of the second meiotic division in mouse spermatogenesis [38], which was the same as that in grasshopper [39]. Wassmann et al. also found that a functional MAD2-dependent spindle checkpoint exists in the mouse oocytes during meiosis I, and overexpression of MAD2 in the oocytes resulted in metaphase arrest by activating spindle checkpoint [37]. MAD2 may also participate in M-II arrest [40]. It has also been found that another mitotic spindle checkpoint protein, BUB1, is present at the kinetochores in mouse oocytes and participates in metaphase II arrest [41]. Put together, these studies indicate that mitotic spindle checkpoint proteins are also present in meiosis in mammals. So far, the localizations of MAD2 and BUB1 have been examined only in mice [37, 41]. To further understand the localization of these spindle checkpoint proteins, in the present study, we used rat as a model in mammals to examine the localization of MAD2 as a spindle checkpoint protein during meiosis and its relationship with kinetochores, microtubules, and chromosomes.

MATERIALS AND METHODS

SD rats were used in this study. 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.

Cell Collection and Culture

Female SD rats (age 4–6 wk) were used in the study. The animals were injected with 20 IU eCG to induce follicle growth. Prophase stage oocytes, also called immature oocytes at the germinal vesicle (GV) stage, were collected from ovaries at 48 h after eCG injection by puncturing follicles with a needle in M2 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 60 μg/ml penicillin and 50 μg/ml streptomycin. All oocytes were cultured in M2 medium at 37°C in a humidified atmosphere of 5% CO_2 until treatment and examination.

Nuclear Examination of Oocytes

At various time points of culture, oocytes were fixed for examination of nuclear maturation. Nuclear stages were categorized as GV, prometaphase I (ProM-I), metaphase I (M-I), anaphase I (A-I), telophase I (T-I), or metaphase II (M-II) according to the methods reported previously [42].

Nocodazole and Taxol Treatment of Oocytes

Oocytes at various stages were treated by nocodazole or Taxol. For nocodazole treatment, 10 mg/ml nocodazole in DMSO stock (Sigma) was diluted in M2 medium to give a final concentration of 20 μg/ml and oocytes were incubated for 10 min; for Taxol treatment, 5 mM Taxol (Sigma) in DMSO stock was diluted in M2 medium to give a final concentration of 10 μM and oocytes were incubated for 45 min. After treatment, oocytes were washed thoroughly and used for further experiments (fixed for immunofluorescency or cultured for spindle recovery). In control, oocytes were also treated in the medium with the same concentration of DMSO before examination.

Immunofluorescence Staining of MAD2, Kinetochores, Microtubules, and Nuclei

Rabbit anti-MAD2 antibody prepared against bacterially expressed *Xenopus* MAD2 was a kind gift from Dr. R.H. Chen [31]. Its specificity against rat MAD2 was examined previously in rat spermatocytes [38]. In a preliminary experiment, we used both rabbit serum and fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit IgG for the immunostaining and did not find positive MAD2 signal. Also, in an immunoblotting experiment, we did not find any band if rabbit serum and/or anti-rabbit serum were used. Therefore, we believe that this anti-MAD2 antibody also binds MAD2 in rat oocytes.

As we cannot image the oocytes using three-color staining by confocal microscopy, oocytes used in this study were double stained for MAD2 and nuclei; microtubules and nuclei; MAD2 and kinetochores; and MAD2 and microtubules. Immunofluorescence staining was based on the procedures reported previously [10, 23, 24, 38].

For MAD2 and nuclei staining, oocytes were fixed in 4% paraformaldehyde/PHEM (60 Mm Pipes, 25 mM Hepes at pH 6.9, 10 mM EGTA, 8 mM $MgSO_4$) for 20 min and washed three times in PBS with 0.05% polyvinyl pyrrolidone (PVP), then treated in 1% Triton X-100/PHEM for 10 min and washed rapidly three times in PBS with 0.05% PVP. After being blocked with 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, the oocytes were incubated with anti-MAD2 antibody (1:100 in 1% BSA/PHEM with 100 mM glycine) at 4°C overnight. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with FITC-conjugated goat-anti-rabbit IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min. Then the oocytes were further washed three times in PBS with 0.05% Tween 20 and stained with propidium iodide in PBS with 0.05% Tween 20 for 2–3 min. Finally the oocytes were mounted on glass slides and examined with a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany).

For kinetochore and nucleus staining, oocytes were treated in 0.5% Triton X-100/PHEM for 4–5 min and washed rapidly three times in PBS with 0.05% PVP, then fixed in 4% paraformaldehyde/PHEM for 20 min and washed three times in PBS with 0.05% PVP. After being blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, the oocytes were incubated in human ANA-Centromere Auto-antibody (CREST; 1:500 in 1% BSA/PHEM with 100 mM glycine; Cortex Biochem) overnight at 4°C. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with FITC-conjugated goat-anti-human IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min. Nuclear staining was the same as the methods mentioned above.

For microtubule and nucleus staining, oocytes were treated in 0.2% Triton X-100/PHEM for 4–5 min and washed rapidly three times in PBS with 0.05% PVP, then fixed in 4% paraformaldehyde/PHEM for 20 min, and washed three times in PBS with 0.05% PVP. After being blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, the oocytes were incubated in monoclonal anti-α-tubulin (1:16 000 in 1% BSA/PHEM with 100 mM glycine; Sigma) overnight at 4°C. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with FITC-conjugated goat-anti-mouse IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min before nuclear staining using the methods mentioned above.

For MAD2 and kinetochore staining, oocytes were treated in 0.5% Triton X-100/PHEM for 4–5 min and washed rapidly three times in PBS with 0.05% PVP, then fixed in 4% paraformaldehyde/PHEM for 20 min and washed three times in PBS with 0.05% PVP. After being blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, oocytes were incubated in anti-MAD2 antibody (1:100 in 1% BSA/PHEM with 100 mM glycine) at 4°C overnight. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with FITC-conjugated goat-anti-rabbit IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min; after three washes in PBS with 0.05% Tween 20, the oocytes were blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, then were stained as in the methods mentioned above except that the primary antibody was CREST (1:500 in 1% BSA/PHEM with 100 mM glycine) and the second antibody was Alexa-conjugated goat-anti-human IgG (1:200 in 1% BSA/PHEM with 100 mM glycine).

For MAD2 and microtubule staining, oocytes were fixed in 4% paraformaldehyde/PHEM for 20 min and washed three times in PBS with 0.05% PVP, then treated in 1% Triton X-100/PHEM for 10 min and washed rapidly three times in PBS with 0.05% PVP. After being blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, the oocytes were incubated in anti-MAD2 antibody at 4°C overnight. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with FITC-conjugated goat-anti-rabbit IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min. After three washes in PBS with 0.05% Tween 20, the oocytes were again blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, then were stained as in the method mentioned above except that the primary antibody was anti-α-tubulin antibody (1:16 000 in 1% BSA/PHEM with 100 mM glycine) and the second antibody was Tris-conjugated goat-anti-mouse IgG (1:200 in 1% BSA/PHEM with 100 mM glycine).

Immunoblotting of MAD2 and Tubulin

Immunoblotting (Western blotting) of MAD2 and tubulin was carried out according to the methods reported previously with some changes [37, 38]. Briefly, at 0, 3, 6, and 10 h after culture, 200 oocytes at each culture point were collected in SDS sample buffer and heated to 100°C for 4 min. After being cooled on ice and centrifuged at 12 000 × *g* for 4 min, samples were frozen at −20°C until use. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and a 12.5% separating gel for 2.5 h at 120 V and then electrophoretically transferred onto nitrocellulose membrane for 2.5 h at 200 mA at 4°C. After being washed three times in TBS (20 mM Tris, 137 mM NaCl, pH 7.4) for 30 min, the membrane was cut into upper half and lower half according to the molecular masses of tubulin (55 kDa) and MAD2 (24 kDa). The membrane for tubulin examination was blocked for 2.5 h in TBST buffer (TBS with 0.1% Tween20) containing 5% low-fat milk at room temperature, then incubated in mouse anti-alpha tubulin (1:1000 in TBST with 0.5% low-fat milk) for 2 h at 37°C. After being washed three times, each for 10 min in TBST, the membrane was treated in peroxidase-conjugated goat anti-mouse IgG (1:3000 in TBST with 0.25% low-fat milk) for 1 h at 37°C. The membrane was washed three times in TBST and processed using enhanced chemiluminescence. The membrane for MAD2 was blocked with 5% BSA in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 1.5 h, washed, and then incubated with rabbit anti-XMAD2 (100 ng in TBST) overnight. The washed membrane was incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG 1:25 000 in TBST at 37°C for 1 h. The protein was detected by an enhanced chemiluminescence detection system.

Microinjection of MAD2 Antibody into Immature Oocytes

Anti-MAD2 antibody (0.5 mg/ml in 0.1% BSA/PHEM, pH 7.2) was injected into the cytoplasm of fully grown oocytes at GV stage as previously reported [19–21]. Isobutylmethylxanthine was added to M2 medium as a final concentration of 0.2 μM to prevent GV breakdown during injection and treatment. A micro-injection volume of 7 pl per oocyte was used in all experiments. Rabbit IgG (0.5 mg/ml in 0.1% BSA/PHEM, pH 7.2) or culture medium was also injected into other oocytes for controls. After micro-injection, oocytes were washed and cultured in M2 medium until examination.

Statistical Analysis

All percentage data were subjected to arcsine transformation before statistical analysis. Data were analyzed by analysis of variance (ANOVA) of EXCEL. Differences at $P < 0.05$ were considered significant.

TABLE 1. Nuclear maturation of rat oocytes during culture.

Time of culture (h)	No. of oocytes examined	No. of oocytes at oocyte nuclear stages[a]				
		GV (%)[b]	Pro-MI (%)[b]	MI (%)[b]	AI–TI (%)[b]	MII (%)[b]
0	256	241 (94.1 ± 0.97)[c]	15 (5.9 ± 0.97)[c]	0[c]	0[c]	0[c]
2	187	55 (29.4 ± 0.37)[d]	132 (70.6 ± 0.37)[d]	0[c]	0[c]	0[c]
3	193	39 (20.2 ± 13.9)[c]	154 (79.8 ± 13.9)[c]	0[c]	0[c]	0[c]
6	238	22 (9.2 ± 0.61)[f]	17 (7.1 ± 0.05)[cf]	195 (81.9 ± 0.81)[d]	2 (0.8 ± 0.66)[d]	2 (0.8 ± 1.49)[d]
8	197	15 (7.6 ± 0.25)[g]	10 (5.1 ± 0.32)[c]	143 (72.6 ± 21.42)[d]	26 (13.2 ± 21.01)[e]	3 (1.5 ± 1.41)[d]
9	223	9 (4.0 ± 0.83)[h]	11 (4.9 ± 1.57)[c]	105 (47.1 ± 9.82)[e]	57 (25.6 ± 15.74)[f]	41 (18.4 ± 12.66)[e]
10	172	6 (3.5 ± 0.08)[h]	6 (3.5 ± 1.13)[cg]	42 (24.4 ± 11.27)	21 (12.2 ± 9.32)[e]	97 (56.4 ± 9.19)[f]

[a] Abbreviations of oocyte nuclear stages: GV, Germinal vesicle; ProM-I, prometaphase I; M-I, metaphase I;, A-I, Anaphase I; T-I, Telophase I; M-II, metaphase II.
[b] Average (mean ± SD) percentage of oocytes from three replications.
[c–h] Values with different superscripts within the same column are significantly different, $P < 0.05$.

RESULTS

Nuclear Maturation of Rat Oocyte in Culture

As shown in Table 1, at 0 h of culture, most oocytes (94.1%) were at the GV stage. By 2–3 h of culture, 70.6–79.8% of the oocytes underwent GV breakdown (GVBD) and proceeded to ProM-I. The proportions of oocytes at M-I were increased to 81.9% when the oocytes were examined at 6 h. After that, the proportions of oocytes at M-II were increased as culture time was increased, and accordingly, the proportions of oocytes at M-I stage were decreased. When oocytes were cultured for 10 h, 56.4% of oocytes reached M-II. Some oocytes (0.8–12.2%) were at the A-I to T-I stages at 6–10 h after culture.

Localization of MAD2 and Microtubules in Rat Oocyte During Meiosis

The experiments were repeated three times. For oocytes at GV, ProM-I, M-I, and M-II stages, 30 oocytes at each stage were examined; for oocytes at A-I and T-I stages, 10 oocytes at each stage were examined. Typical images at each stage of oocytes are showed in Fig. 1.

GV stage. Nuclei in prophase were enclosed by a nuclear membrane and no chromatids were observed (Fig. 1). Some microtubule staining was found around the nucleus (GV). A few kinetochore-staining spots (usually 6–10) were observed around the periphery of the GV. Also, 6–10 spots of MAD2 staining were present around the GV. Costaining of MAD2 and kinetochores (Fig. 2) showed their signals nearly overlapped, which indicated that MAD2 were localized on kinetochores at the GV stage.

ProM-I stage. Chromosomes are completely separated, but individual chromosomes cannot be imaged in the oocytes at the ProM-I stage. Microtubules started to organize into a spindle, but the chromosomes were still scattered in the spindle. At the same time, kinetochores could be observed on both sides of the chromosomes. Again, costaining results (Fig. 2) showed that MAD2 were at kinetochores.

M-I stage. A bipolar spindle (M-I spindle) was formed and all chromosomes had aligned at the equator of the spindles. Kinetochores were also aligned clearly on both sides of the chromosomes; thus, two rows of kinetochores were observed in all oocytes examined. No MAD2 staining was observed in the chromosomes at the M-I stage (Fig. 1).

A-I to T-I stage. As shown in Figure 1, in the oocytes at the A-I stage, chromosomes started to separate; two rows of chromosomes could be observed in A-I oocytes. Kinetochore staining was still observed on the chromosomes. Again, no MAD2 staining was observed. When the oocytes proceeded to T-I stage, chromosomes were completely separated and two compacted chromosome masses were observed on the spindle poles. Kinetochores were also separated but they were compacted, and it is difficult to identify individual kinetochore staining spots. No MAD2 staining was observed in the oocytes.

M-II stage. When the oocytes proceeded to M-II stage, the oocyte released the first polar body and a complete, typical M-II spindle was formed. The chromosomes were aligned at the equator of the spindle. Again, the kinetochores were realigned on both sides of the chromosomes and formed two rows, as that observed in M-I oocytes. No MAD2 staining was observed in the oocytes (Fig. 1).

Localization of MAD2 in Rat Oocytes Treated with Nocodazole and Taxol

The experiments were repeated three times. Thirty oocytes at each stage were examined. Typical images at each stage of oocytes are shown in Figure 3.

When oocytes from GV to M-II stages were treated with nocodazole, microtubules were completely disassembled and no spindles were observed in all treated oocytes (data and figures were not shown). However, as shown in Figure 3 (left column), MAD2 staining was observed in all oocytes from GV to M-II stages. MAD2 in the oocytes from GV to ProM-I stages was the same as those in the control oocytes (Fig. 1). However, two rows of strong MAD2 signals at all the chromosomes in all (30) oocytes at M-I stage were observed. When the oocytes proceeded to the A-I to T-I stage, MAD2 was localized at a plate corresponding to the spindle midzone plate instead of chromosomes (Fig. 3), while in the oocytes at the M-II stage, MAD2 localization was similar to that in the oocytes at the M-I stage, i.e., two rows of MAD2 signals were observed on both sides of the chromosomes. Costaining of MAD2 and kinetochores also showed that MAD2 was localized at kinetochores in the

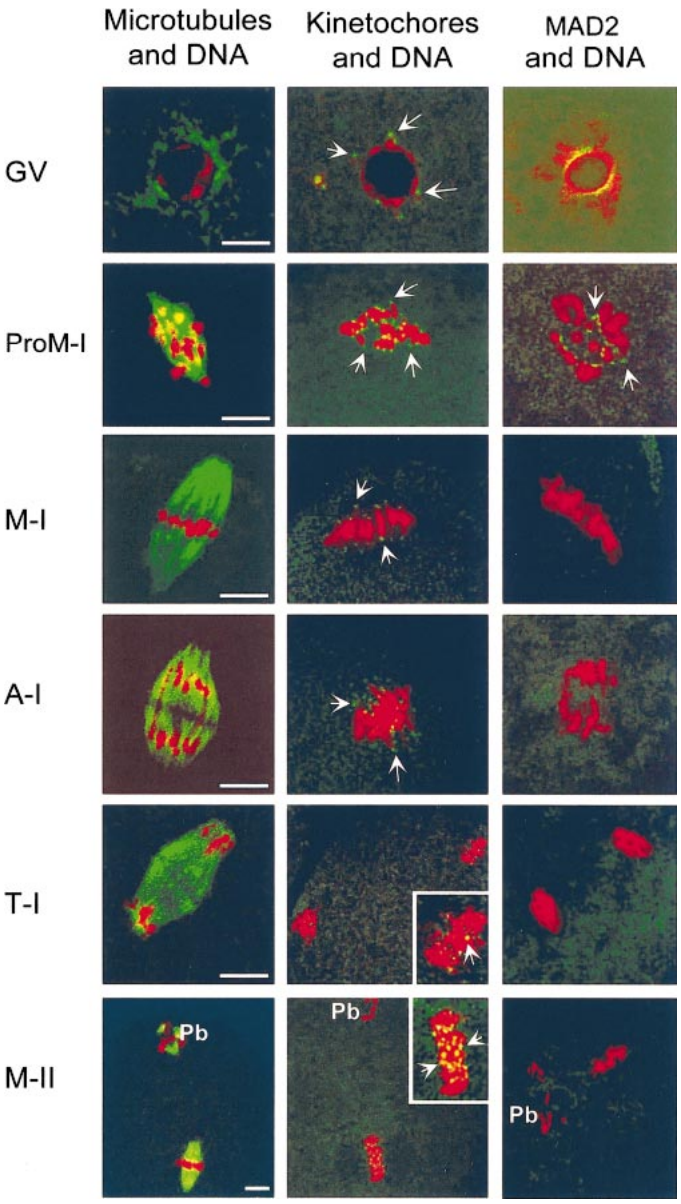

FIG. 1. Confocal micrographs of immunostaining of kinetochores, MAD2, microtubules, and DNA (nucleus) of rat oocytes during meiotic maturation. Oocytes at various stages were double stained: left column, microtubules (green) and DNA (red); middle column, kinetochores (green) and DNA (red); right column, MAD2 (green) and DNA (red). GV: Germinal vesicle; ProM-I, prometaphase I; M-I, metaphase I; A-I, anaphase I; T-I, telophase I; M-II, metaphase II. Arrows indicate kinetochores and MAD2. Scale bar = 5 μm.

oocytes at GV, ProM-I, M-I, and M-II stages treated by nocodazole (data and figures not shown).

On the other hand, when the oocytes were treated with Taxol, which stabilizes microtubules (Fig. 3, right column), the localization of MAD2, as shown in Figure 3 (middle column), was similar to that in the control oocytes (Fig. 1).

MAD2 Dynamics in Response to Repolymerization of Microtubules during Spindle Recovery in M-I and M-II Oocytes

To examine the relationship between microtubule polymerization, attachment to kinetochores, and MAD2 localization in the chromosomes after spindle destruction and recovery, oocytes at M-I and M-II stages were treated with

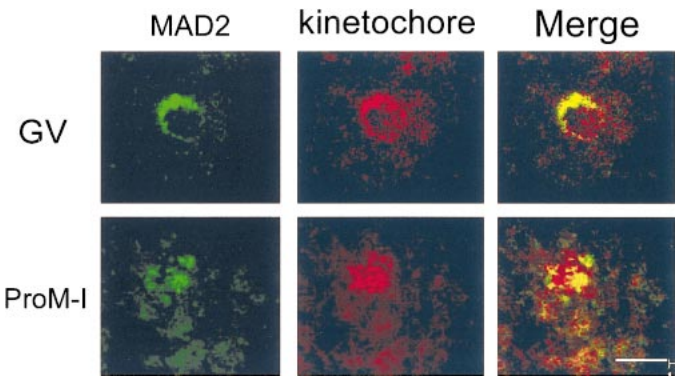

FIG. 2. Confocal micrographs of costaining of kinetochores and MAD2 of rat oocytes at GV and ProM-I stages. Left column, MAD2 (green); middle column, kinetochores (red); right column, merger of MAD2 and kinetochores. GV, Germinal vesicle; ProM-I, prometaphase I. Scale bar = 10 μm.

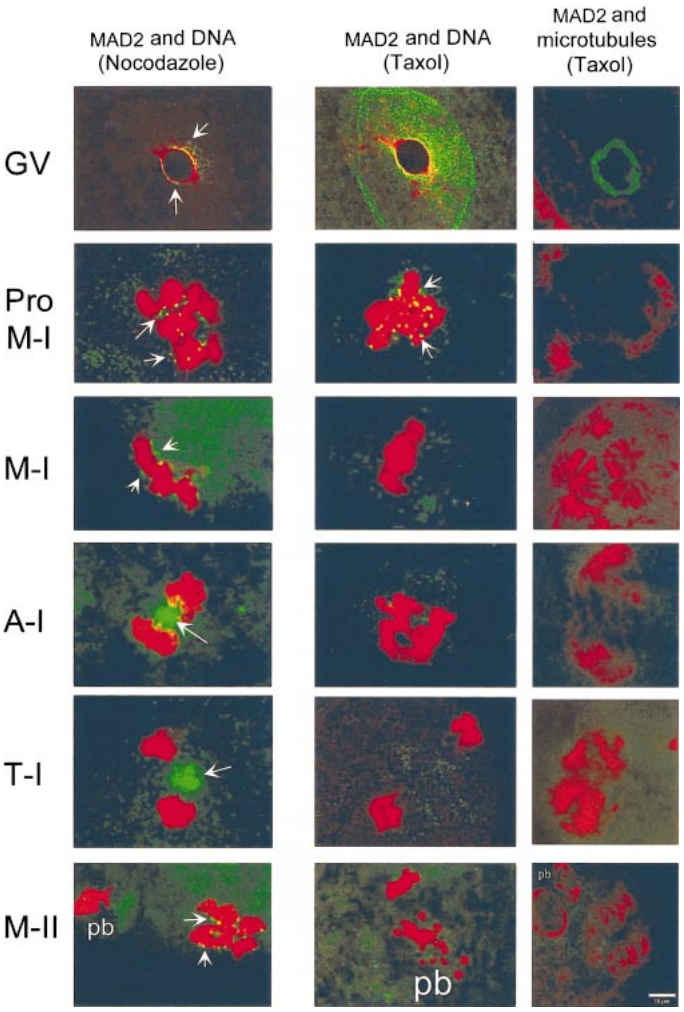

FIG. 3. Confocal micrographs of immunostaining of MAD2 and DNA (nucleus) or MAD2 and microtubules of rat oocytes treated with nocodazole or Taxol during meiotic maturation. Oocytes at various stages were double stained. Left column shows DNA (red) and MAD2 (green) of oocytes at various stages treated with nocodazole; middle column shows DNA (red) and MAD2 (green) of oocytes at various stages treated with Taxol; right column shows MAD2 (green) and microtubules (red) of oocytes at various stages treated with Taxol. GV, Germinal vesicle; ProM-I, prometaphase I; M-I, metaphase I; A-I, anaphase I; T-I, telophase I; M-II, metaphase II; pb, the first polar body. Arrows indicate MAD2. Scale bar = 10 μm.

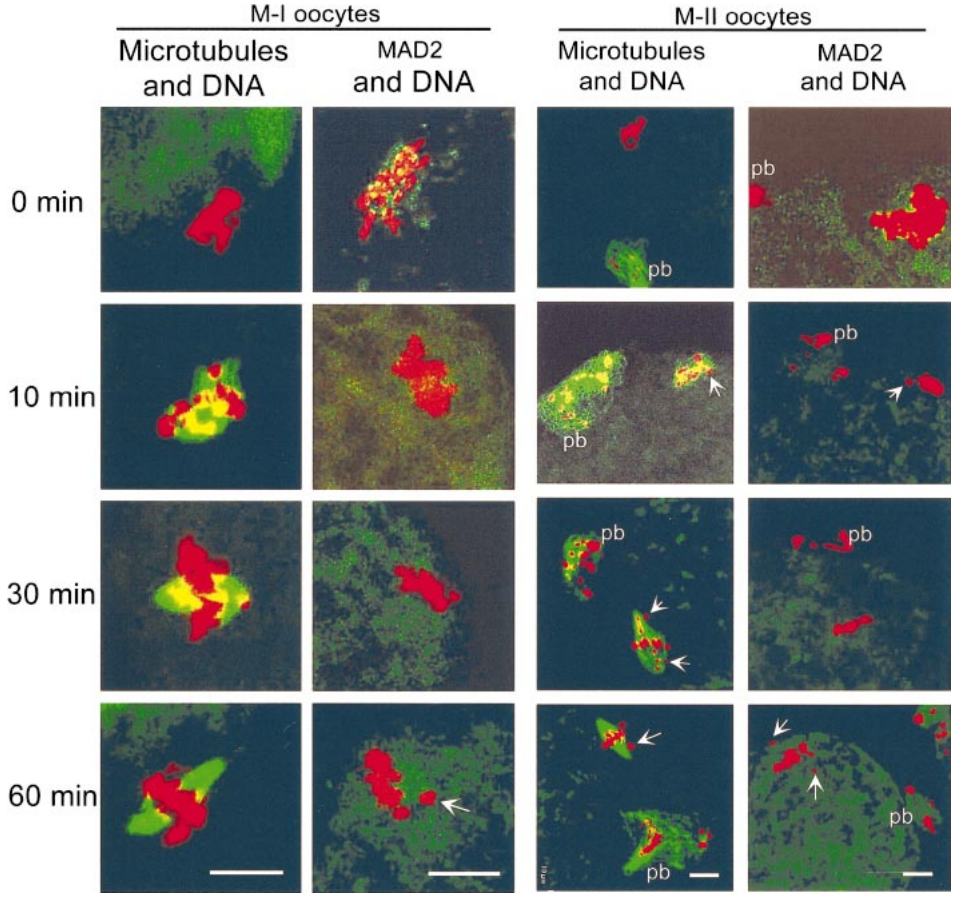

FIG. 4. Confocal micrographs of immunostaining of MAD2, microtubules, and DNA (nucleus) of rat oocytes treated with nocodazole at M-I and M-II stages and then cultured for spindle recovery. Left column, microtubules (green) and DNA (red); right column, MAD2 (green) and DNA (red). Treated oocytes were stained 0 min after treatment or stained after 10, 30, or 60 min of culture. pb, First polar body. Arrows indicate misaligned chromosomes. Scale bar = 10 μm.

nocodazole to depolymerize microtubules and then oocytes were washed completely to remove the drug and then cultured for 10, 30, or 60 min for spindle recovery. Experiments were repeated three times and 30 oocytes were examined at each oocyte stage and each examination point. Before treatment, there were intact spindles in both M-I and M-II oocytes with chromosomes being aligned at the metaphase plate and no MAD2 staining was observed in these oocytes (Fig. 1). As shown in Figure 4, when the oocytes were fixed soon after nocodazole treatment, it was found that no microtubules were present in the oocytes, indicating that microtubules were completely depolymerized. At the same time, MAD2 staining was observed in the oocytes, in which MAD2 was observed at both sides of the chromosomes. Ten minutes after culture, microtubules began to reassemble around the chromosomes and it would appear that all chromosomes were connected to the microtubules, but an intact spindle was not formed and chromosomes were scatted in the spindles. At the same time, MAD2 signals completely disappeared even though an intact spindle had not formed. At 30 min after culture, a new spindle was formed and several chromosomes remained near the spindle pole or were not aligned at the equator of the spindle, but MAD2 staining was not observed at the chromosomes, indicating that once the relationship between chromosomes and microtubules is established, MAD2 disappears even though some chromosomes are not aligned at the equator of the spindles. At 60 min of culture, the reconstructed spindle became more regular except a few chromosomes were still located away from the metaphase plate, but no MAD2 in the chromosomes was observed. All these findings were the same in the oocytes at both M-I and M-II stages.

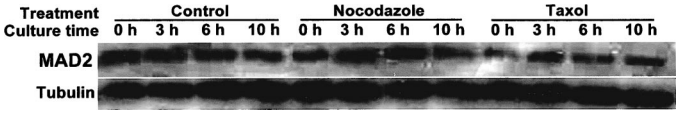

FIG. 5. Western blotting of MAD2 and α-tubulin in rat oocytes during meiotic maturation and after treatment with nocodazole and Taxol.

Immunoblotting of Tubulin and MAD2 in Rat Oocytes During Meiosis

As shown in Figure 5, tubulin and MAD2 were detected in the oocytes cultured for 0 (GV stage), 3 (ProM-I stage), 6 (M-I stage) and 10 h (M-II stage). Treatment of oocytes with nocodazole and Taxol did not affect the presence of MAD2 in the oocytes, indicating that MAD2 is present in the cytoplasm of oocytes at all stages of oocytes and its presence is not related to the presence of microtubules and/or intact spindles.

Microinjection of MAD2 Antibody into Immature Rat Oocytes Induced Premature Anaphase and Abnormal Chromosome Arrangement

As most (>80%) oocytes were at the M-I stage 6 h after culture and A-I oocytes were first observed at this time (Table 1), we chose this time point to examine whether microinjection of anti-MAD2 antibody could induce premature anaphase. We found that more oocytes showed characteristics of premature anaphase (51.2 ± 5.74, $P < 0.01$) in the antibody-injected group than in the IgG-injected control (26.6 ± 2.35) or medium-injected control (25.2 ± 0.05)

TABLE 2. Effect of anti-MAD2 antibody on chromosome alignment in rat oocytes.

Group	No. of oocytes examined[a]	No. of abnormal chromosome alignment (%)
MAD2 injected	87	45 (51.7 ± 5.74)[b]
IgG injected	91	24 (26.4 ± 2.35)[c]
Control	131	33 (25.2 ± 0.05)[c]

[a] Experiments were repeated three times.
[b,c] Values with different superscripts within the same column are significantly different, $P < 0.01$.

(Table 2). In abnormal M-I oocytes, some chromosomes were clearly not aligned at the equator of the spindle and some were still at the spindle poles (Fig. 6). In abnormal A-I oocytes, chromosomes began to separate before all chromosomes were well aligned at the metaphase plate (Fig. 6). In some A-I oocytes, chromosomes underwent unequal separation and the spindle fibers became loose or irregular, which might be caused by chromosome unequal separation (Fig. 6). Some chromosomes were also away from the spindles, suggesting that the connection between microtubules and chromosomes was not established.

DISCUSSION

In the present study, we found that MAD2 was localized only in the chromosomes before M-I stage and not in the chromosomes at M-I stage and beyond M-I stage in which all chromosomes had been aligned at the metaphase plate, indicating that MAD2 participates in delaying premature anaphase onset, but does not participate in anaphase onset if the chromosomes are well aligned in the metaphase plate [19–21]. These results also indicate that MAD2 is bound to unattached kinetochores in the early stages of oocytes (before M-I) and disappears when the oocytes reach M-I stage in which a strong attachment between microtubules and kinetochores has been established [37]. This conclusion is also supported by the results from nocodazole-treated oocytes and Taxol-treated oocytes. In nocodazole-treated oocytes, MAD2 reappeared as kinetochores lost the attachment to the microtubules. On the other hand, Taxol-treated oocytes showed similar MAD2 dynamics as control oocytes, suggesting that MAD2 did not regulate changes of tension at kinetochores in rat oocytes. Waters et al. showed that MAD2 was still localized to an average 2.6 out of the 22 kinetochores in Taxol-treated metaphase PtK1 cells and suggested that the presence of MAD2 on these kinetochores is probably the result of a decrease in their microtubule number [24]. However, we did not detect MAD2 in Taxol-treated oocytes from M-I to M-II stages; perhaps Taxol treatment did not significantly decrease the number of microtubules on kinetochores in rat oocytes.

In the present study, we did not detect any MAD2 in the kinetochores, spindle microtubules, or spindle poles in the oocytes at the stages from M-I to M-II. It would appear that different MAD2 localization is present not only between male and female meiosis, but also between rat and mouse female meiosis.

The present results on MAD2 localization in rat oocytes during meiotic maturation were consistent with those reported in the mouse oocytes [37] in which Wassmann et al. found that MAD2 was localized around chromosomes in the oocytes before ProM-I stages (3–4 h after GVBD) and disappeared in the oocytes at M-II stage. When we used immunoblotting in the present study, we found that MAD2 was present in the oocytes from the GV to the M-II stages. The different results between immunostaining and immunoblotting may indicate that MAD2 diffuses in the cytoplasm in the oocytes after the M-I stage. These results were also confirmed by immunostaining of oocytes at A-I and T-I stages treated by nocodazole in which MAD2 was not found in chromosomes, but was found in the cytoplasm around spindle midzone. As reported by Wassmann et al. in mouse oocytes, we found that nocodazole treatment reactivated MAD2 within the oocytes at M-I and M-II stages because MAD2 staining was observed in the oocytes. According to the study in the mouse, treatment of oocytes at M-I stage with nocodazole resulted in cell cycle arrest and these oocytes cannot proceed to M-II stage [37]. However, such an arrest is reversible [37]. It is clear that nocodazole treatment reactivates MAD2 due to unattachment of chromosomes to microtubules and reestablishment of connection between chromosomes and microtubules after removal of nocodazole, which deactivates the MAD2. These results suggest that MAD2 is a spindle checkpoint protein in both

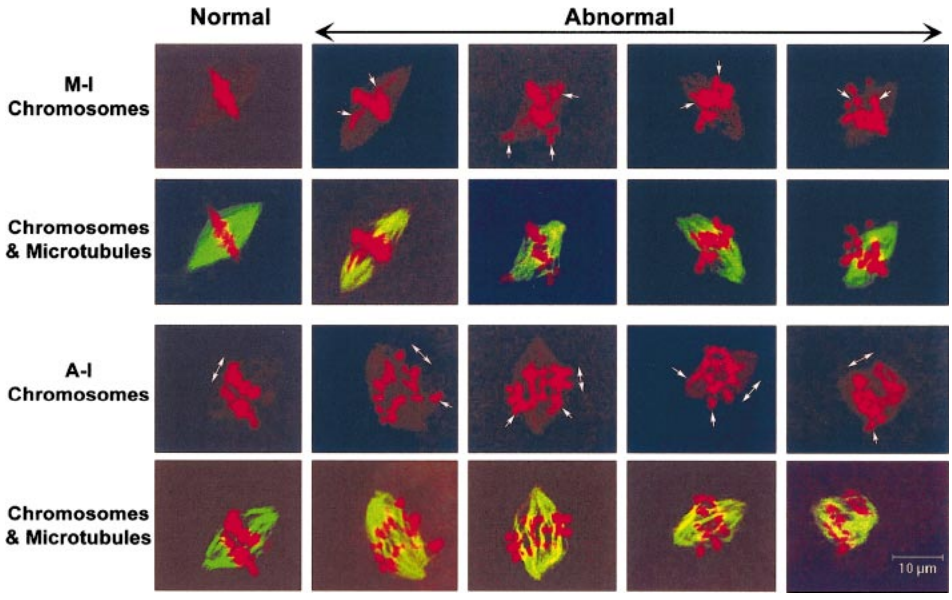

FIG. 6. Confocal micrographs of immunostaining of microtubules and DNA (nucleus) of rat oocytes injected with anti-MAD2 antibody into GV-stage oocytes and then cultured for 6 h for examination of chromosome alignment and spindle formation. Left column represents normal M-I and A-I stage oocytes, and others represent abnormal chromosome alignment in the injected oocytes. Red images represent DNA and green images represent microtubules. Arrows indicate misaligned chromosomes in the spindles. Scale bar = 10 μm.

mouse and rat oocytes. This conclusion is also supported by the study in the mouse oocytes that overexpression of MAD2 also leads to a M-I arrest [37].

When the microtubules were depolymerized by colcemid, nocodazole, and vinoblastine or stabilized by Taxol, it was found that localization of MAD2 to kinetochores depended on microtubules attachment, not tension [10, 23, 24, 30, 31]. In nocodazole-treated oocytes, we found that MAD2 recruited to chromosomes in the oocytes at M-I and M-II stages and its localization model was consistent with that of kinetochores. These results indicate that, once the attachment between kinetochores and microtubules is interrupted, MAD2 is activated; thus, the next step of the cell cycle (anaphase) is temporarily terminated. These results suggest that, before homologous chromosomes separate, MAD2 could check microtubule detachment from kinetochores and inhibit anaphase initiation. In nocodazole-treated M-II oocytes, MAD2 also recruits to chromosomes, suggesting MAD2 has the same functions in M-II oocytes, as in M-I oocytes. However, in nocodazole-treated A-I–T-I oocytes, unlike in M-I and M-II oocytes, MAD2 was localized at a place correspondent with the spindle midzone instead of at the chromosomes, suggesting that MAD2 was translocated to spindle fibers in A-I–T-I oocytes. Immunoblotting of MAD2 in the oocytes treated with or without nocodazole or Taxol indicated that MAD2 was present in the oocytes beyond the M-I stage; even immunostaining could not detect it in the oocyte. These interesting phenomena indicate that MAD2 is movable in the metaphase plate and in the cytoplasm, although it is mainly at (maybe close to) the kinetochore. However, such a relocation of MAD2 in microtubule-destroyed A-I–T-I oocytes was not observed in normal meiosis. When the nocodazole-treated oocytes were cultured for spindle recovery, we found that MAD2 localization was totally different from those in the normal spindle formation in the oocytes before the ProM-I stage. During normal spindle formation, MAD2 can check the chromosome misalignment before ProM-I and its activity disappeared only when all chromosomes had aligned at the equator of the spindle. However, during spindle recovery in nocodazole-treated oocytes, MAD2 can check unattachment of kinetochores to microtubules, but cannot check misaligned chromosomes, as most oocytes still had misaligned chromosomes in which MAD2 was not observed. These results indicate that microtubule disassembly can reactivate MAD2 at any stage of oocytes and the reactivated MAD2 in these oocytes can check chromosome attachment but cannot check unaligned chromosomes, which may explain aneuploidy in human oocytes from older women, in which high proportions of oocytes had abnormal spindles [43].

In anti-MAD2-injected oocytes, MAD2 lost its activity as a spindle checkpoint protein, so the oocytes entered a premature anaphase in which chromosomes started to separate before they were completely aligned at the metaphase plate, maybe starting from the prometaphase stage. In these abnormal oocytes, chromosomes underwent unequal alignment or separation and the spindle fibers became loose or irregular regardless of being at M-I- or A-I-like stages, which could induce unequal division of chromosomes in A-I; thus, aneuploidy could happen. These results were consistent with a recent study in mouse oocytes [37] and previous studies with other mitotic cells [23].

In summary, this is the first detailed examination of MAD2 localization and its function as a spindle checkpoint protein during rat female meiosis. Our results, together with those of Wassmann et al. [37] clearly indicate that mitotic spindle checkpoint protein MAD2 is also a meiotic spindle checkpoint protein in mammalian oocytes during meiosis. However, our finding suggests that MAD2 in rat oocytes just participates in the spindle checkpoint during normal meiosis and, when the normal meiosis is disrupted artificially at any time point of the cell cycle, MAD2 cannot accurately check the unattached chromosomes. A species-specific difference appears present among mammals and further studies are necessary to address the different MAD2 localization among mammals, especially in humans.

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