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Aurora-A Is a Critical Regulator of Microtubule Assembly and Nuclear Activity in Mouse Oocytes, Fertilized Eggs, and Early Embryos[1]

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ABSTRACT

Aurora-A is a serine/threonine protein kinase that plays a role in cell-cycle regulation. The activity of this kinase has been shown to be required for regulating multiple stages of mitotic progression in somatic cells. In this study, the changes in aurora-A expression were revealed in mouse oocytes using Western blotting. The subcellular localization of aurora-A during oocyte meiotic maturation, fertilization, and early cleavages as well as after antibody microinjection or microtubule assembly perturbance was studied with confocal microscopy. The quantity of aurora-A protein was high in the germinal vesicle (GV) and metaphase II (MII) oocytes and remained stable during other meiotic maturation stages. Aurora-A concentrated in the GV before meiosis resumption, in the pronuclei of fertilized eggs, and in the nuclei of early embryo blastomeres. Aurora-A was localized to the spindle poles of the meiotic spindle from the metaphase I (MI) stage to metaphase II stage. During early embryo development, aurora-A was found in association with the mitotic spindle poles. Aurora-A was not found in the spindle region when colchicine or staurosporine was used to inhibit microtubule organization, while it accumulated as several dots in the cytoplasm after taxol treatment. Aurora-A antibody microinjection decreased the rate of germinal vesicle breakdown (GVBD) and distorted MI spindle organization. Our results indicate that aurora-A is a critical regulator of cell-cycle progression and microtubule organization during mouse oocyte meiotic maturation, fertilization, and early embryo cleavage.

early development, fertilization, gamete biology, meiosis, ovum

INTRODUCTION

During the past several years, a growing number of serine-threonine kinases highly homologous to aurora kinases have been isolated in various organisms. Aurora kinases have been identified in many eukaryotes: IpLl in *Saccharomyces cerevisiae* [1], SpAIRK in *Schizosaccharmyces pombe* [2], two aurora kinases in *Drosophila* [3, 4] and at least three aurora kinases in mammals [5–7]. The three aurora kinases are referred to as aurora-A, aurora-B, and aurora-C, as recently suggested [8]. Recently, a number of studies have reported that aurora kinases play a role in crucial aspects of cell-cycle regulation, including spindle organization, metaphase entry and exit, centrosome separation and maturation, the metaphase-anaphase transition, chromosome segregation, and cytokinesis [9, 10].

The three mammalian kinases have 67–76% homology in their catalytic domain and are expressed at the G2/M phase transition [6, 11, 12]. The kinase activity of aurora-A subfamily peaks in G2 and in prophase, before the full activation of aurora-B and before the maximal activation of p34cdc2 [11]. Importantly, members of this family are overexpressed in a variety of cancers, implying their pivotal role in governing cell proliferation [11]. Studies on the intracellular localization of aurora kinases in mitotic cells have suggested their association with mitotic structures [13]. Aurora-A has been localized to centrosomes of interphase cells and to the spindle poles at metaphase [5, 9], and inhibition of its activity leads to formation of a monopolar spindle because its activity is necessary for centrosome separation [3, 14]. Aurora-B has been found at the midbody of anaphase cells and at the postmitotic bridge of telophase cells [11, 12], and inhibition of its activity leads to formation of multinucleated cells, which indicates that the kinase is involved in cytokinesis [15]. Aurora-C is localized in the centrosomes of anaphase cells [6] and its expression is testis specific [16].

Aside from the organization of the spindle, members of the aurora kinase family have been shown to be involved in mitotic phosphorylation of histone H3 at Ser-10 both in yeast and nematodes [17]. Crosio et al. [18] showed that the expression of aurora-A, aurora-B, and aurora-C was tightly coordinated with histone H3 phosphorylation during the G2/M transition in HeLa cells, while in NIH 3T3 cells, the accumulation of the aurora kinases preceded the phosphorylation of histone H3. Aurora-A appeared to be a more potent H3 kinase than aurora-B. This difference in kinase activity could be due to different signaling requirements for aurora-B. Indeed, while the recombinant aurora-A was efficiently autophosphorylated, aurora-B was much less active. In mammalian cells [19], Cdc20-associated aurora2/aurora-A activity peaks during G2 phase in somatic cells and is significantly lower in both unperturbed mitotic cells collected by mitotic shake-off and nocodazole-arrested M-phase cells, in which the APC/C activity is suppressed by checkpoint control mechanisms.

Although numerous studies have shown that aurora kinases are necessary for mitotic spindle organization, their functions in meiotic microtubule organization are not known. From the viewpoint of the cell cycle, meiosis differs from mitosis in several aspects. For example, oocytes

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undergo a period of extended meiotic arrest after entering the G2 phase, while quiescence in mitotic cells follows progression from G1 to a G0 phase of the cell cycle. Furthermore, meiosis lacks an intervening S phase between two meiotic divisions, while mitosis has an S phase and M phase alternating invariantly. Mammalian oocytes, which are ideal models for the study of the meiotic cell cycle, undergo considerable chromosomal and cytoplasmic changes during maturation and fertilization, including chromosome condensation, germinal vesicle breakdown (GVBD), spindle organization, polar body emission, pronuclear formation, and syngamy. The organization of microtubules is well known to be involved in the regulation of these dynamic events [20, 21]. Because aurora kinases are important for mitotic microtubule organization, their regulation of meiotic microtubules is highly possible. A study in *Xenopus* oocyte maturation shows that aurora-A is an early player in the cascade of reactions triggered by progesterone [22]. However, the exact role of aurora-A in meiotic maturation is still unclear, although the ectopic expression of the active kinase accelerates germinal-vesicle breakdown in *Xenopus* [23]. Furthermore, in the case of *Caenorhabditis elegans*, aurora-A is necessary for normal meiotic division. Whenever antisense mRNA neutralizes aurora-A during early development, abnormal meiosis occurs and polyploid embryos are the end result [24]. The dynamics of aurora-A during mammalian oocyte maturation, fertilization, and subsequent early development have not yet been studied.

In this study, we, for the first time, investigated 1) the dynamics and roles of aurora-A in cell-cycle progression and spindle organization during mouse oocyte meiosis, fertilization, and early embryonic mitosis and 2) the correlation of microtubule assembly and aurora-A localization in mouse oocytes after different treatments with microtubule regulators or after aurora-A antibody microinjection. All these experiments are aimed to elucidate the possible roles of this kinase in meiotic and mitotic microtubule assembly and its regulation in mouse eggs and early embryos.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned. Drugs (colchicine, staurosporine, taxol, isobutylmethylxanthine) were prepared as stock solutions dissolved in dimethyl sulfoxide (DMSO), hyaluronidase were prepared in M2 medium directly, and stored in a dark box at −20°C. The stock solutions were diluted with M2 medium before use.

Animals

Kunming White strain mice 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.

Oocyte Collection and Culture

Germinal vesicle (GV)-stage oocytes were collected from ovaries of 4- to 6-wk-old Kunming mice at 48 h after the females were injected with 10 IU eCG. Cumulus-free and GV-intact follicular oocytes were released from the large antral follicles by puncturing with a needle in M2 (Sigma) medium containing 60 μg/ml penicillin and 50 μg/ml streptomycin. All oocyte cultures were maintained in M2 medium at 37°C in a humidified atmosphere of 5% CO_2.

Cumulus cell-enclosed metaphase II-arrested eggs were obtained from mice of the same strain. Females were superovulated by i.p. injection with 10 IU of eCG, and 48 h later, they were injected with 10 IU of hCG. Mice were killed by cervical dislocation at 13–15 h post-hCG injection. The cumulus cell masses surrounding the eggs were removed by brief exposure to 300 IU/ml hyaluronidase in M2 medium.

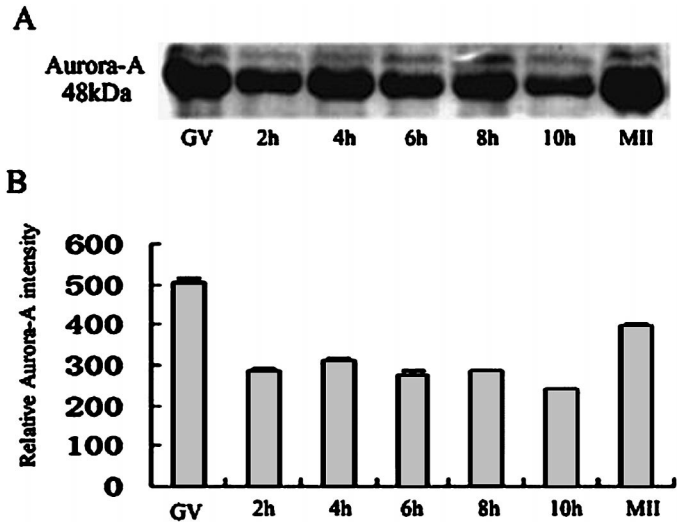

FIG. 1. Expression of aurora-A during mouse oocyte meiotic maturation. The GV oocytes were cultured in vitro for various times and collected for Western blot analysis. **A**) Western blot results. **B**) Relative aurora-A expression quantity was determined by densitometric scans. The value expression of each bar represents mean ± standard deviation (SD) (n = 3).

In Vivo and In Vitro Fertilization

In vivo-fertilized zygotes were collected 16 h post-hCG from the oviduct ampullae of superovulated females that had been mated with the same strain of males. After removing cumulus cells with 300 IU/ml hyaluronidase in M2 medium, zygotes were cultured in M16 (Sigma) medium until use. Two-cell embryos were flushed from the oviducts of copulated mice 44–46 h after hCG injection and cultured in M16 medium. Embryos at different stages of mitosis were collected for confocal microscopy.

In vitro fertilization was performed using 1×10^6/ml motile cauda epididymal sperm, which had been previously capacitated in M16 medium with 2.5 mM taurine for 1 h. Zona pellucida (ZP)-free eggs were used to achieve a more synchronous timing of fertilization within each stage group and to minimize the lag period of sperm-egg interaction. The emission of the second polar body and the formation of the pronuclei were observed with an inverted microscope. The eggs were collected at different stages for confocal microscopy.

Western Blot Analysis

Morphologically normal cells (200 oocytes/sample) were collected in SDS sample buffer at different time points, heated to 100°C for 4.5 min, and frozen at −80°C until use. The proteins were separated by SDS-PAGE with 4% stacking gel and a 10% separating gel at 90 V and 0.5 h and at 120 V and 2.0 h, respectively, and electrically transferred to polyvinylidene fluoride membrane (Sino-American Biotec, Beijing, China; pore size 0.45 μm) for 2 h, 200 mA, at 4°C. Following transfer, the membrane was immersed in methanol for 1 min and dried overnight at room temperature. The membrane was then incubated for 2 h at 37°C with polyclonal rabbit anti-aurora-A antibody (Cell Signaling Technology, Inc., Beverly, MA) diluted 1:500 in TBST (TBS containing 0.1% Tween-20) with 5% skimmed milk. After washing three times in TBST, 10 min each, the membrane was incubated for 1 h at 37°C with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000 in TBST. The membrane was washed three times in TBST, 10 min each, and processed by using the enhanced chemiluminescence detection system (Amersham International, Buckinghamshire, UK). All experiments were repeated at least three times.

Confocal Microscopy

Aurora-A detection was carried out using the procedures reported previously [25]. After removal of ZP in acidified Tyrode solution (pH 2.5), eggs were fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature and then incubated in incubation buffer (PBS

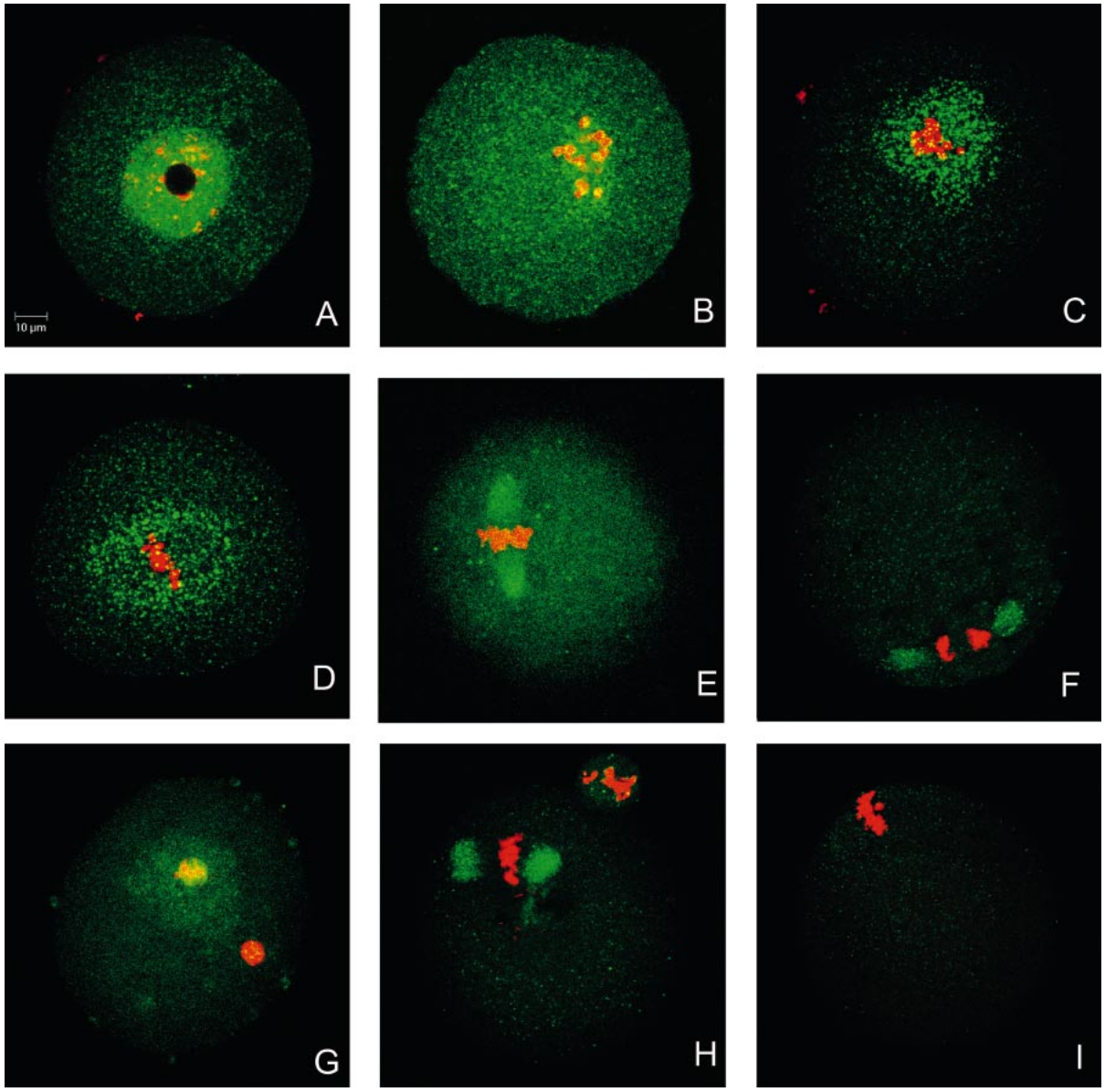

FIG. 2. Immunofluorescent localization of aurora-A during meiotic maturation. Green, aurora-A; red, chromatin; yellow, overlapping of green and red. Aurora-A distributed in the germinal vesicle in GV-stage oocytes (**A**). After GVBD, aurora-A concentrated around the condensed chromatin (**B**, **C**). And when the chromosomes concentrated to the equatorial plate, aurora-A migrated to the two poles of the spindle (**D**) and associated to spindle poles from MI (**E**) to anaphase I stages (**F**). After the first polar body extrusion, aurora-A still was localized to the poles of the spindle (**G**, **H**). An MI oocyte was used as a negative control for aurora-A confocal microscopy, in which no first antibody was used but the fluorescent second antibody was used just as the experimental group (**I**). Original magnification, ×630.

containing 0.5% Triton X-100) for 1 h at 37°C, followed by blocking in 1% BSA for 1 h at room temperature. The eggs were washed in PBS with 0.1% Tween-20 three times and then incubated with 1:200 polyclonal rabbit anti-aurora-A antibody for 1 h. The eggs were rinsed three times and incubated with 1:200 FITC-conjugated goat anti-rabbit IgG for 1 h, followed by staining with 10 μg/ml propidium iodide. Finally, the eggs were mounted on glass slides with DABCO and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany).

The spindle organization was determined by incubating the eggs in 1:100 diluted FITC-anti-α-tubulin for 1 h after blocking and permeabilization as described above.

Microinjection of Aurora-A Antibodies

Aurora-A antibody (0.5 mg/ml in PBS, pH 7.4) was microinjected into the cytoplasm of fully grown GV oocytes as described by Dai et al. [26]. The microinjection was repeated three times, and 50 oocytes were used each time. An Eppendorf microinjector (Hamburg, Germany) was used for these experiments. All microinjections were finished in 40 min. Isobutylmethylxanthine (IBMX; 0.2 μM) was added to the medium to prevent GVBD. A microinjection volume of about 7 pl per oocyte was used in all experiments. The same amount of rabbit IgG diluted in PBS was microinjected as control. After microinjection, eggs were washed thoroughly with M2 medium and cultured in the same medium. GV oocytes incubated in 0.2 μM IBMX for 30 min and then washed and cultured for the evaluation of GVBD and MI spindle formation were also used as control.

Experimental Design

Experiment 1. To detect the changes in aurora-A expression during meiotic maturation, the oocytes were collected at different stages for Western blot analysis.

Experiment 2. To investigate the possible roles of aurora-A in microtubule organization during meiotic maturation, fertilization, and early embryonic mitosis, eggs at different stages of meiosis, fertilization, or early embryos at first and second cleavages were collected for aurora-A localization with confocal microscopy.

Experiment 3. To further reveal the relationship between the spindle assembly/disassembly and the localization of aurora-A, MII oocytes were

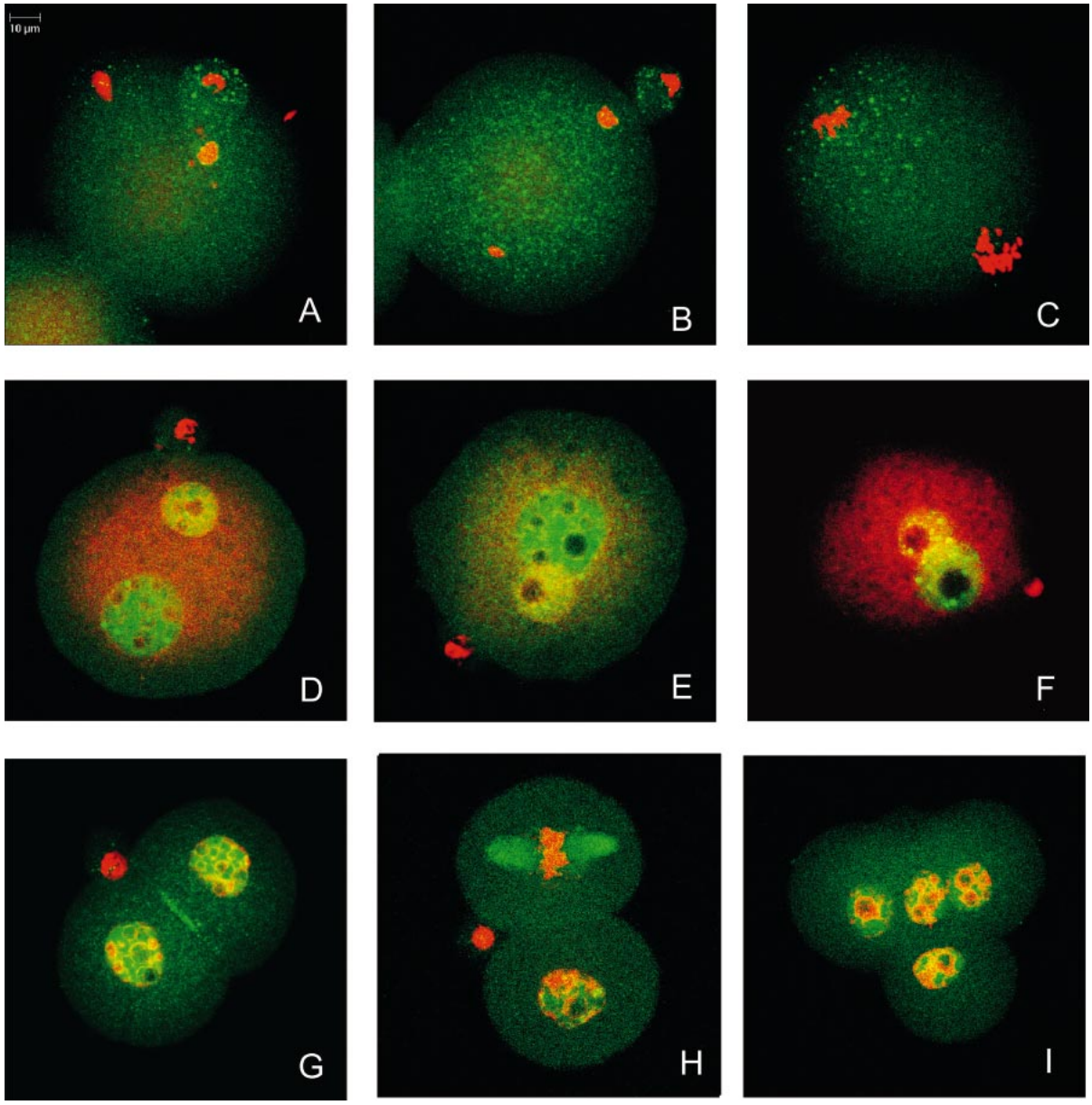

FIG. 3. Localization of aurora-A during fertilization and early embryonic mitosis. Aurora-A distributed around the oocyte chromatin 2 h after insemination (**A**). Then aurora-A distributed evenly in the cytoplasm (**B**), followed by the condensation of this protein around the oocyte chromosomes (**C**). After pronuclear formation, aurora-A was associated with the pronuclei (**D**, **E**). There were increases in aurora-A dots between the two pronuclei when they began to go through nuclear membrane breakdown (**F**). After the first cleavage, aurora-A was found in the nucleus and midbody (**G**, **I**). Aurora-A condensed at the poles of the mitotic spindle (**H**). Original magnification, ×630.

treated with 1 μM microtubule disassembly inhibitor taxol for 10 min or 10 μg/ml microtubule polymerization inhibitor colchicine for 1 h at 37°C. In another experiment, MII oocytes were treated with 30 μM protein kinase inhibitor staurosporine for 30 min to destruct the meiotic spindle. Some eggs treated with staurosporine were further exposed to 1 μM taxol for 10 min. After each treatment, oocytes were collected for confocal microscopy.

Experiment 4. The possible roles of aurora-A in the meiotic spindle organization were revealed by antibody microinjection. The aurora-A antibody was microinjected into GV-intact oocytes. The GVBD rate was recorded 2 and 4 h after culture. The oocytes undergoing GVBD within 4 h after culture were fixed at 8 h after microinjection, and the spindle structure was examined with confocal microscopy.

Statistical Analysis

All data on the GVBD rate of oocytes after antibody microinjection were evaluated by χ^2 analysis. Eggs showing degenerative signs were not included. The relative aurora-A quantity in different meiotic maturation stages was determined by the relative aurora-A intensity obtained by densitometric scan of the band.

RESULTS

Expression and Subcellular Localization of Aurora-A During Oocyte Maturation

Western blot analysis showed that aurora-A protein was highly expressed in mouse oocytes, and its quantity was high in the GV and MII oocytes and appeared unchanged during other stages of meiotic maturation (Fig. 1, A and B).

During oocyte maturation, the localization of aurora-A varied at different stages. In GV oocytes, aurora-A was concentrated in the germinal vesicle, while it was absent in

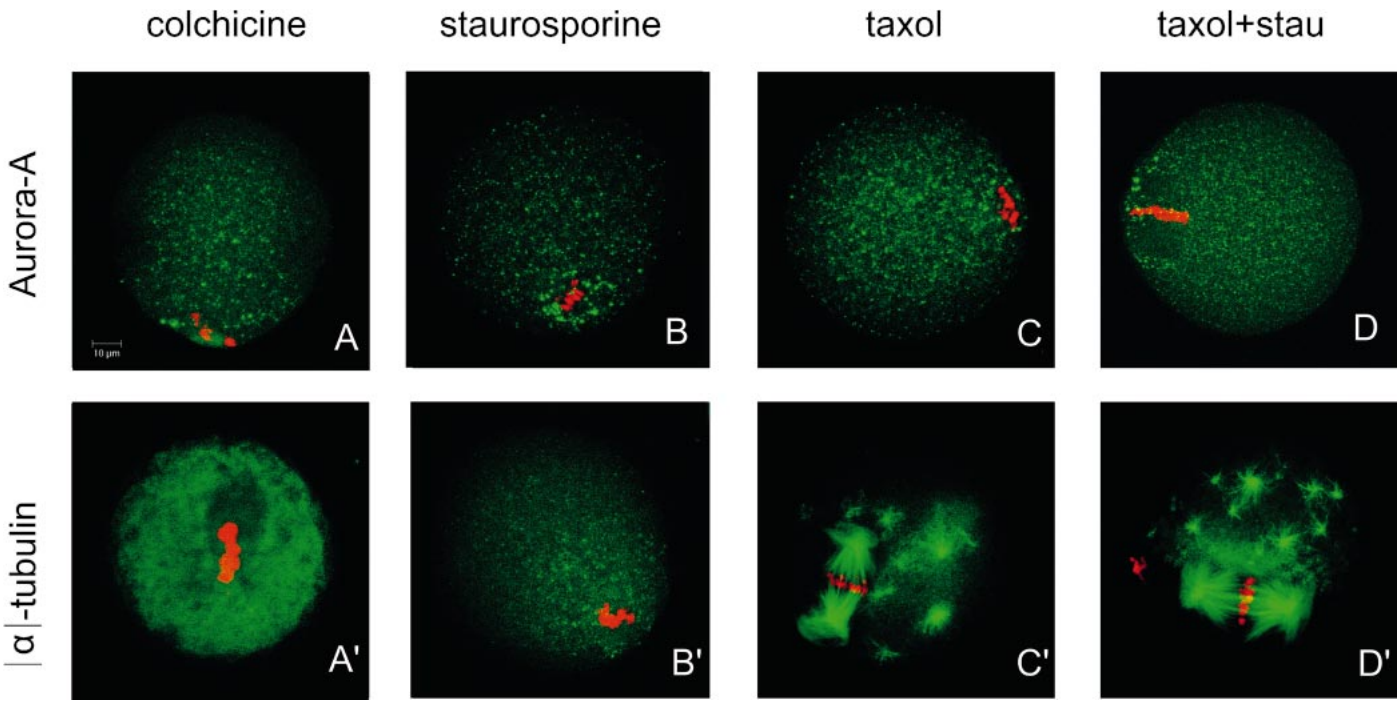

FIG. 4. Effect of cytoskeleton modulators and staurosporine on aurora-A (**A**–**D**) and α-tubulin (**A′**–**D′**) localization. When MII oocytes were cultured in medium containing colchicine, the spindle became disorganized (**A′**) and aurora-A (**A**) distributed as several dots in the cytoplasm. When MII oocytes were cultured in staurosporine-containing medium, spindles were also disorganized (**B′**) and aurora-A (**B**) distributed diffusely. When MII oocytes were treated with taxol, the spindle was abnormal, displaying more poles (**C′**), aurora-A was detected as several dots in the cytoplasm (**C**). When MII oocytes were treated with staurosporine + taxol, the spindle was enlarged and several cytastars were induced in the oocytes (**D′**), and aurora-A distributed diffusely around the chromosomes (**D**). Original magnification, ×630.

the nucleolus (Fig. 2A). There was an obvious difference in the green fluorescence intensity in the GV and the cytoplasm as judged by the TCS-NT system. Shortly after GVBD, aurora-A distributed around the condensed chromatin (Fig. 2, B and C) and then numerous aurora-A dots were observed near the chromosomes (Fig. 2D). With the organization of chromosomes to the equatorial plate, the dots of aurora-A became associated with the spindle poles until anaphase of the first meiosis (Fig. 2, E and F). Immediately after meiosis I, the kinase accumulated around the chromosomes. After formation of the second meiotic spindle at the early MII stage, aurora-A again appeared at the spindle poles (Fig. 2H). As a negative control, MI oocytes were not treated with the first antibody and showed no staining (Fig. 2I).

Localization of Aurora-A During Fertilization and Early Embryo Development

In our experiments, the eggs extruded their second polar body 2 h after insemination, and complete pronuclear formation was observed 8 h following insemination. As shown in Figure 3A, the chromosomes of oocytes moved to the spindle poles 2 h after insemination but the aurora-A dots were present around the chromosomes of the oocyte and not in the second polar body. With the extrusion of the second polar body, aurora-A distributed diffusely in the egg cytoplasm (Fig. 3B). Then aurora-A concentrated around the female chromosomes (Fig. 3C). After male and female pronuclear formation, aurora-A distributed in the two pronuclei (Fig. 3, C and D). When the chromatin began to condense, just before pronuclear membrane breakdown, most dots of aurora-A were detected between the male and female pronuclei (Fig. 3, E and F). After the first mitosis, aurora-A was also associated with the nucleus (Fig. 3, G and I). And during the second cleavage, aurora-A appeared at the spindle poles of the mitotic metaphase (Fig. 3H).

Localization of Aurora-A When Microtubule Organization Was Disturbed

After treatment of eggs with colchicine, a microtubule polymerization inhibitor, the meiotic spindle disappeared, and α-tubulin distributed evenly in the eggs. Aurora-A protein also diffused into the cytoplasm (Fig. 4, A and A′). When MII oocytes were treated with staurosporine, a broad-spectrum protein kinase inhibitor that perturbs the spindle as we reported before [27], the spindle was partially disorganized and α-tubulin could be found around the chromosomes. The localization pattern of aurora-A was the same as the α-tubulin distribution (Fig. 4, B and B′). When MII eggs were treated with taxol, the meiotic spindle expanded and several cytoplasmic asters were observed. Aurora-A disappeared from the spindle poles and distributed as numerous dots in the cytoplasm (Fig. 4, C and C′). In oocytes treated with taxol following pretreatment with staurosporine, multiple cytoplasmic asters could be found and the clusters of aurora-A distribution could also be detected. Overall, the distribution of α-tubulin and aurora-A in oocytes treated with taxol following pretreatment with staurosporine (Fig. 4, D and D′) were similar to those treated with taxol alone (Fig. 4, C and C′).

Microinjection of Aurora-A Antibody Affects GVBD and Spindle Assembly

The GVBD rates of oocytes at 2 or 4 h after antibody microinjection were 35.76% (49/137) and 45.52% (61/134),

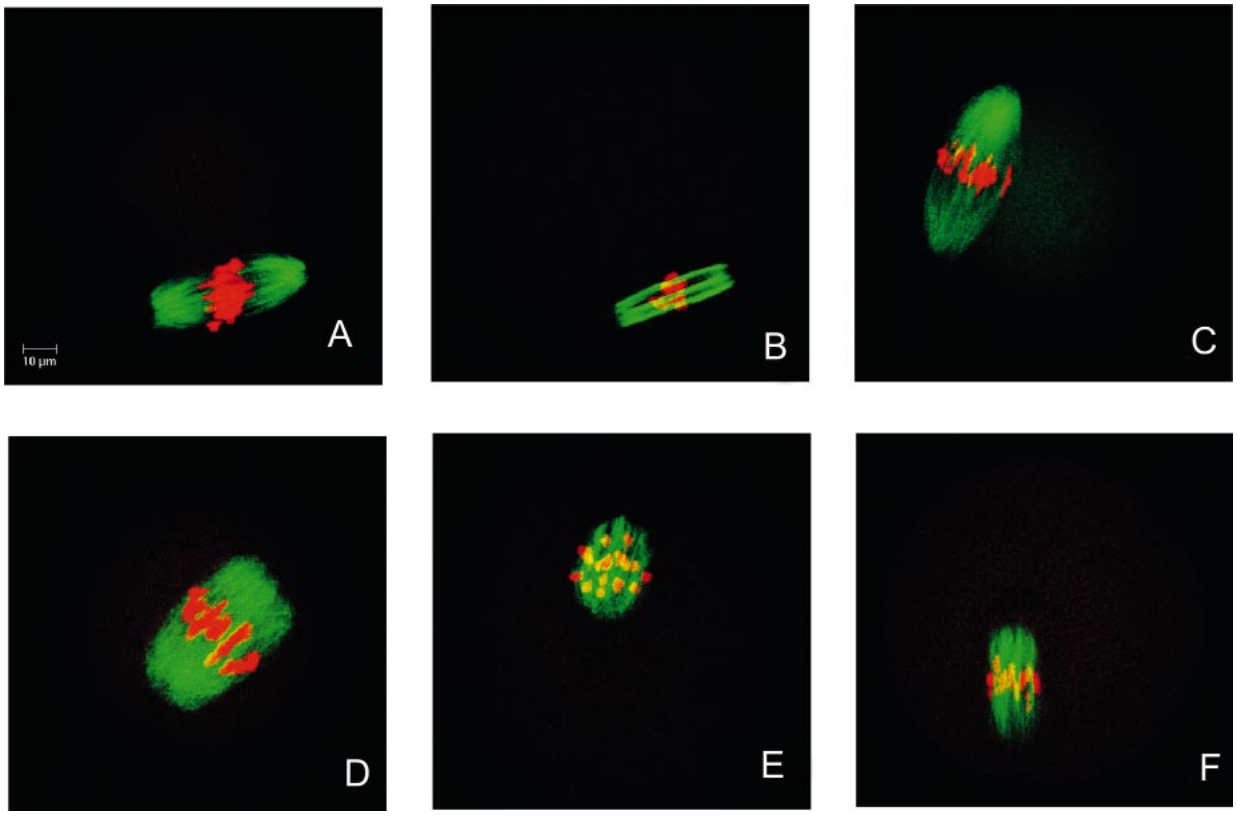

FIG. 5. Effect of aurora-A antibody microinjection on the spindle organization. A normal spindle formed in the control group microinjected with rabbit IgG (**A**). When the microinjected GV oocytes were cultured for 8 h, some formed thin spindles (**B**), some formed spindles with one pole (**C**), some formed broad spindles (**D**), and others did not form spindles (**E**, **F**). Original magnification, ×630.

respectively, significantly lower than that of the IgG microinjection control group (61.16% [36/57] and 70.91% [39/55], respectively). In control oocytes, a typical MI spindle was formed 8 h after culture (23/23) (Fig. 5A), while five types of microtubule organizations were observed in the oocytes microinjected with aurora-A antibody. Abnormal, thin spindles with two thin poles existed in 5 of the total 29 oocytes examined (Fig. 5B). One-pole spindles were found in 7 of 29 oocytes (Fig. 5C), and two broad spindle poles were observed in 4 of 29 oocytes (Fig. 5D). Abnormal spindles without poles were observed in most microinjected oocytes (13 of 29) (Fig. 5, E and F). Treatment of oocytes with IBMX for 30 min had no effect on GVBD (64.15% [34/53] 4 h after culture) and normal MI spindles formed after IBMX removal when compared with the nontreatment group (GVBD rate after 4 h culture, 69.33% [208/300] 4 h after culture).

DISCUSSION

We have shown in this study that aurora-A protein is present in mouse oocytes, and the quantity of this protein in GV and MII phases was higher than that in other meiotic maturation stages. This result was similar to that reported by Kimura et al. [9]. They reported that cell-cycle-dependent expression of Aik (aurora-A in *Homo sapiens*) was observed in HeLa cells by Northern and Western blotting. Its level is low in G1/S but high in G2/M. The kinase activity of Aik peaked at M phase. Because this activity appeared to be correlated with the protein levels, it is conceived that its activity at least partly depends on the changes in protein content.

Our study clearly showed that aurora-A is tightly associated with microtubule assembly during oocyte maturation. After GVBD, before the establishment of a bipolar spindle, aurora-A aggregated to form numerous condensed dots surrounding the chromosomes. At this stage of meiosis, several microtubule organizing centers (MTOCs) formed, and the multiarrayed microtubules were tightly associated with chromatin mass [28]. It is obvious that the aurora-A distribution was similar to that of the MTOC arrangement at this stage. At the MI stage, aurora-A concentrated to the spindle poles, the loci of MTOCs at metaphase. From metaphase to anaphase, aurora-A was still associated with the spindle poles. At metaphase II, aurora-A was concentrated to the poles of MII spindles. This phenomenon was also found in mitotic cells [9]. All these results indicate that aurora-A takes part in not only the initiation but also maintenance of meiotic spindle microtubule organization.

Various centrosomally localized protein kinases have been identified in vertebrate cells and oocytes, including Cdc2 [29], Plk1 [30–32], Fyn [33], and Src [34]. Some of these kinases were suggested to function as regulators of microtubule organization and spindle formation. In one study, one of the substrates of Cdc20 kinase was identified to be a kinesin-like motor protein and to regulate centrosomal localization [35]. Plk1 is phosphorylated and activated by Cdc2 kinase, and Cdc20 associated with Aik/aurora-A [19]; however, the Mos/MAPK pathway is not involved in aurora-A regulation and Cdc2 activation is necessary and sufficient for aurora-A activation [36], suggesting that the protein kinase cascade containing aurora-A may play a part in centrosomal function.

Because aurora-A accumulated at the spindle poles, we propose that this kinase is always localized to the subcellular area where microtubule organization takes place. During the early embryo development, aurora-A distributed to spindle poles, as is the case during somatic cell mitosis. After the spindle was disrupted by colchicine or staurosporine, aurora-A distributed evenly in the cytoplasm. When MII oocytes were treated with taxol, the MII spindle enlarged and microtubule asters formed in the oocytes. Correspondingly, aurora-A was found as several dots in the cytoplasm. This result further supports our suggestion that the distribution of aurora-A is associated with the organization of microtubules.

Aurora-A protein was found to accumulate in the germinal vesicle in the GV mouse oocytes, in the pronuclei in fertilized eggs, and in the nuclei of early embryos. This phenomenon indicates its possible roles in nuclear activity regulation. Indeed, the rate of GVBD was lower than normal when the aurora-A antibody was microinjected into the cytoplasm of GV oocytes. It is well known that the maturation-promoting factor (MPF) is activated at GVBD and its activation triggers chromatin condensation and nuclear envelope breakdown. Microinjection of aurora-A may disturb the activation of MPF and thus GVBD. We also found that, in the oocytes that went through GVBD, the assembly of meiotic spindles at the MI stage was disturbed. This result provides further evidence supporting the regulation of spindle microtubule assembly by aurora-A. Why and how Aurora-A regulates microtubule assembly is still under investigation. Proteins such as dynein, which is a microtubule minus-end directed motor, are good candidates for transporting the kinase to the centrosomes [37].

These results suggest that aurora-A may play important roles in the microtubule organization of centrosomes and cell-cycle progression in mouse oocytes, fertilized eggs, and early embryos. Further studies are necessary to determine the upstream kinases and the downstream substrates of the aurora family in mammalian meiosis.

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