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Characterization of Aurora-A in porcine oocytes and early embryos implies its functional roles in the regulation of meiotic maturation, fertilization and cleavage

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Summary

Aurora-A is a serine/threonine protein kinase that plays important regulatory roles during mitotic cell cycle progression. In this study, Aurora-A expression, subcellular localization, and possible functions during porcine oocyte meiotic maturation, fertilization and early embryonic cleavage were studied by using Western blot, confocal microscopy and drug treatments. The quantity of Aurora-A protein remained stable during porcine oocyte meiotic maturation. Confocal microscopy revealed that Aurora-A distributed abundantly in the nucleus at the germinal vesicle stage. After germinal vesicle breakdown, Aurora-A concentrated around the condensed chromosomes and the metaphase I spindle, and finally, Aurora-A was associated with spindle poles during the formation of the metaphase II spindle. Aurora-A concentrated in the pronuclei in fertilized eggs. 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. In conclusion, Aurora-A may be a multifunctional kinase that plays pivotal regulatory roles in microtubule assembly during porcine oocyte meiotic maturation, fertilization and early embryonic mitosis.

Keywords: Early development, Fertilization, Kinases, Meiosis, Oocyte

Introduction

Fully grown mammalian oocytes are arrested at the G2/M transition of the first meiotic division until gonadotropin stimulation or release from the follicular environment. The resumption of meiotic maturation is manifested by germinal vesicle breakdown, followed by chromatin condensation and microtubule reorganization. These transformations lead to the formation of the metaphase spindle and subsequent completion of the first meiotic division, after which oocytes become arrested in metaphase II again. This arrest is maintained until the fertilization or parthenogenetic activation of the oocytes (Fan *et al*., 2003).

Recently, the Aurora kinases emerged as a new family of mitotic serine/threonine kinases, conserved from yeast to humans (Schumacher *et al*., 1998; Roghi *et al*., 1998), that 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 (Kimura *et al*., 1997; Severson *et al*., 2000). The vertabrate Aurora family is composed of three members, termed Aurora-A, -B, and -C (Nigg, 2001). Aurora-A localizes to the centrosome and the spindle poles during mitosis (Roghi *et al*., 1998). Ablation or inactivation of Aurora-A in *Caenorhabditis elegans* embryos and mammalian culture cells prevents centrosome maturation and spindle assembly, showing a role of Aurora-A in centrosome separation and bipolar establishment of the

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spindle (Hannk *et al*., 2001; Dutertre *et al*., 2002). It has also been demonstrated that, in *Xenopus*, one substrate of Aurora-A could be the kinesin-related motor Eg5, involved in centrosome separation (Giet *et al*., 1999). Aurora-B has been found at the midbody of anaphase cells and at the post-mitotic bridge of telophase cells (Bischoff *et al*., 1998; Terada *et al*., 1998) and inhibition of its activity leads to formation of multinucleated cells, which indicates that the kinase is involved in cytokinesis (Tatsuka *et al*., 1998). Aurora-C is localized in the centrosomes of anaphase cells (Kimura *et al*., 1999), and its expression is testis specific (Tseng *et al*., 1998).

Subsequent studies (Frank-Vaillant *et al*., 2000; Mendez *et al*., 2000) showed that progesterone-induced Aurora-A hyperphosphorylation and activation occur at about the same time as MPF activation. Moreover, Aurora-A hyperphosphorylation and activation are abolished if MPF activity is inhibited by injection of $p21^{Cipl}$. These findings argue that Aurora-A hyperphosphorylation and activation are events downstream of MPF. Aurora-A involves a drop in cAMP, induced within minutes after progesterone addition, leading to inhibition of the cAMP-dependent protein kinase, protein kinase A (Maller *et al*., 1997). Protein kinase A downregulation could stimulate the synthesis of new protein, a process required to activate the Cdc2–cyclin B complex. The identification of the one or more proteins whose synthesis is needed for Cdc2 activation represents a major challenge. Mos kinase is synthesized and activated simultaneously with MPF activation and indirectly induces MAPK activation (Sagata *et al*., 1988). It has been proposed recently that Mos synthesis and MAPK activation are not required to induce Cdc2-cyclin B activation in *Xenopus* oocyte; however, they most probably facilitate this process (Fisher *et al*., 1999; Gross *et al*., 2000; Dupre *et al*., 2002). Thus, one or more other proteins, not identified yet, must be synthesized in response to progesterone to allow Cdc2-cyclin B activation.

Despite the numerous studies on Aurora-A in somatic cells and *Xenopus* oocytes, few reports concerning this enzyme's roles in mammalian meiotic cell cycles were available until now. Recently, the dynamics of localization and enzyme activity of Aurora-A was examined during mouse oocyte maturation, fertilization and early embryos mitosis (Yao *et al*., 2003). The quantity of Aurora-A protein was high in the germinal vesicle and metaphase II (MII) oocytes and remained stable during other meiotic maturation stages. Aurora-A concentrated in the germinal vesicle (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 meiotic spindle from the metaphase I (MI) stage to MII 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.

The meiotic cell cycle of porcine oocytes differs from that of the mouse oocyte in several ways. In order to investigate the role of Aurora-A in large mammals, we choose the pig as a model. In this study we aimed to evaluate: (1) the dynamics and roles of Aurora-A in cell cycle progression and spindle organization during porcine oocyte meiosis, fertilization and early embryonic mitosis; and (2) the correlatin of microtubule assembly and Aurora-A localization in porcine oocytes after different treatments with microtubule regulators. All these experiments aimed to elucidate the possible roles of this kinase in meiotic and mitotic microtubule assembly and its regulation in porcine eggs and early embryos.

Materials and methods

Chemicals

All chemicals used in this experiment were purchased from Sigma Chemical unless otherwise mentioned. Stock solutions of colchicine (10 mg/ml), taxol (1 mM) and staurosporine (Stau; 3 mM) were prepared with dimethyl sulfoxide and stored frozen at −20 °C in the dark. They were diluted with TCM-199 just prior to use.

Collection and culture of oocytes

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 1.5 h in 0.9% NaCl solution containing 75 μg/ml penicillin G and 50 μg/ml streptomycin sulphate at 37 °C. Oocytes were aspirated from antral follicles (2–6 mm in diameter) with an 18-gauge needle fixed to a 20 ml disposable syringe. After washing three times with maturation medium (see below), oocytes possessing a compact cumulus and evenly granulated ooplasm were selected for maturation culture. The medium used for maturation culture was improved TCM-199 (Gibco, Grand Island, NY) supplement with 0.1% polyvinyl alcohol (PVA), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μg/ml penincillin G, 50 μg/ml streptomycin sulphate, 0.57 mM cysteine, 0.5 μg/ml LH, 0.5 μg/ml FSH and 10 ng/ml epidermal growth factor (EGF). A group of 30–50 oocytes were cultured in a 300 μl drop of maturation medium for up to 44 h at 39 °C in an atmosphere of 5% CO_2 and saturated humidity.

After maturation culture, oocytes were freed of cumulus cells by treatment with 300 IU/ml hyaluronidase and repeated pipetting. The denuded eggs were then washed three times in TCM-199 and used for confocal microscopy, *in vitro* fertilization (IVF) or drug treatment.

Fertilization *in vitro*

The IVF was carried out by the method reported previously with minor modifications (Han *et al.*, 1999). Oocytes were inseminated in a 50 µl drop of modified Tris-buffered medium (mTBM) containing 0.4% bovine serum albumin (BSA; A-7888) and 2.5 mM caffeine with freshly ejaculated spermatozoa (5×10^5 cells/ml) that had been previously incubated for 2 h in the same medium. Six hours after insemination, eggs were removed from the fertilization drop and cultured in 500 µl North Carolina State University 23 (NCSU-23) medium containing 4 mg/ml BSA (A-8022, Sigma). Embryos at different stages of mitosis were collected for confocal microscopy.

Western blot analysis

A total of 100 oocytes at the GV stage or MI or MII stage were collected in SDS sample buffer and heated to 100 °C for 4 min. After cooling on ice and centrifuging at 12 000 *g* for 4 min, samples were frozen at −80 °C until use. The total proteins were separated by SDS-PSGE with a 4% stacking gel and a 10% separating gel at 90 V, 0.5 h and 120 V, 2.0 h, respectively, and electrically transferred to PDVF membrane (Sino-American Biotec, pore size 0.45 µm) for 2 h, at 4 °C, 200 mA. Following transfer, the membrane was immersed in methanol for 1 min, then dried overnight at room temperature. The membrane was incubated for 2 h at 37 °C with polyclonal rabbit anti-Aurora-A antibody (Cell Signaling Technology, 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, Santa Cruz, CA) diluted 1:1000 in TBST. The membrane was washed three times in TBST, 10 min each, and processed by using the ECL detection system (Amersham International, Buckinghamshire, U.K.). All experiments were repeated at least three times.

Confocal microscopy

After removing the zona pellucida in acidified Tyrode's solution (pH 2.5), eggs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for at least 30 min at room temperature. Cells were permeabilized with 1% Triton X-100 for 12 h at 38 °C, followed by blocking in 1% BSA for 1 h at room temperature, 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 fluorescein isothiocyanate (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.

Experimental design

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

Experiment 2
To investigate the possible roles of Aurora-A during *in vitro* meiotic maturation, fertilization and early embryonic mitosis, eggs at different stages were collected for Aurora-A detection with confocal microscopy.

Experiment 3
To further reveal the relationship between spindle assembly/disassembly and the localization of Aurora-A, MII oocytes were treatment with 1 µM of the microtubule disassembly inhibitor taxol for 10 min or 10 µg/ml of the microtubule polymerization inhibitor colchicines for 1 h at 37 °C. In another experiment, MII oocytes were treated with 30 µM of the protein kinase inhibitor staurosporine for 30 min to destroy 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.

Statistical analysis

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 oocytes maturation

Samples were collected from oocytes at the GV stage until 44 h after maturation *in vitro*. Western blot analysis showed that Aurora-A protein was expressed in

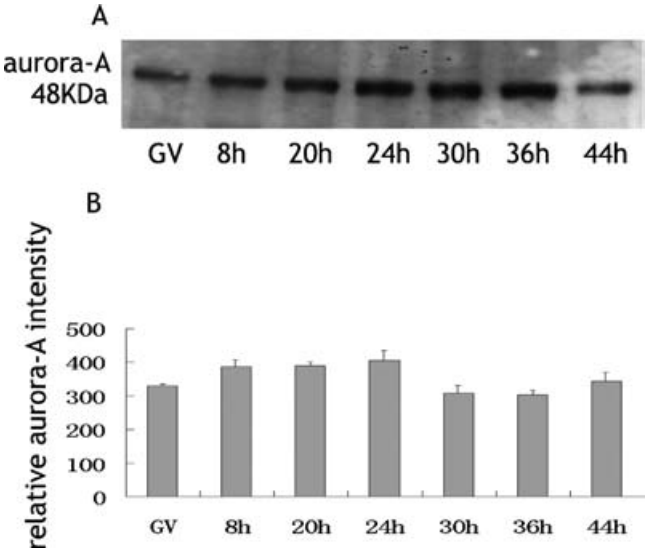

Figure 1 Expression of Aurora-A during porcine oocyte meiotic maturation. The GV oocytes were cultured *in vitro* for various times and collected for Western blot analysis. (*A*) Western blot results. (*B*) Relative Aurora-A expression quantity was determined by densitometric scans. The value by each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

porcine oocytes, and its quantity appeared unchanged during meiotic maturation (Fig. 1).

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 the nucleolus (Fig. 2A, B). There was an obvious difference in the green fluorescence intensity in the GV and the cytoplasm as judged by the TCS-NT system. After GVBD, Aurora-A distributed around the condensed chromatin (Fig. 2C) and then numerous Aurora-A dots were observed near the chromosomes (Fig. 2D). With the organization of chromosomes to the equatorial plate, most of the kinase dots associated with the spindle poles until anaphase of the first meiosis (Fig. 2E, F). During the first polar body emission, Aurora-A accumulated around the chromosomes (Fig. 2G). At MII, Aurora-A was localized at the spindle poles (Fig. 2H). As a negative control, GV 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, sperm penetrated the oocytes 6 h after *in vitro* fertilization, and complete pronuclear formation was observed 18 h following insemination. With the extrusion of the second polar body, Aurora-A dots distributed diffusely in the egg cytoplasm (Fig. 3A). Then Aurora-A concentrated around the female chromosomes (Fig. 3B). After male and female pronuclear formation, Aurora-A distributed in the two pronuclei (Fig. 3C). 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. 3D, E). After the first mitosis, Aurora-A was also associated with the nucleus (Fig. 3F).

Localization of Aurora-A when microtubule organization was disturbed

After treatment of eggs with colchicine, a protein microtubule polymerization inhibitor, the meiotic spindle disappeared, and α-tubulin distributed around the chromosomes as an abnormal spindle. Aurora-A protein diffused into the cytoplasm (Fig. 4A, A'). When MII oocytes were treated with staurosporine, a broad-spectrum protein kinase inhibitor that disturbs the spindle, the spindle was partially disorganized and α-tubulin could be found around the chromosomes. The localization pattern of Aurora-A was not the same as the α-tubulin distributions (Fig. 4B, 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. 4C, C'). In oocytes treatment with taxol following pretreatment with staurosporine, a cytoplasmic aster could be found around the chromosomes and the clusters of Aurora-A distribution could also be detected (Fig. 4D, D').

Discussion

We have shown in this study that Aurora-A protein is present in porcine oocytes, and the quantity of this kinase remains stable at different stages of meiosis. This result was different from that in previous studies (Kimura *et al*., 1997; Yao *et al*., 2003), which reported a high level in G/M and peak activity at M phase, and in the mouse oocyte maturation the quantity of this protein was higher in GV and MII phase. This inconsistency may be due to the different antibodies used by different authors or to the deference in experimental animal.

Our study clearly showed that Aurora-A is tightly associated with microtubule assembly during oocyte maturation. In porcine oocytes, Aurora-A was localized to different component of the spindle apparatus. Following GVBD, when the bipolar spindle formed, Aurora-A concentrated into several dots around the condensed chromosomes. These foci may be involved in the merging of the multiple cytoplasmic MTOCs that will form the two spindle poles (Yao *et al*., 2003). This

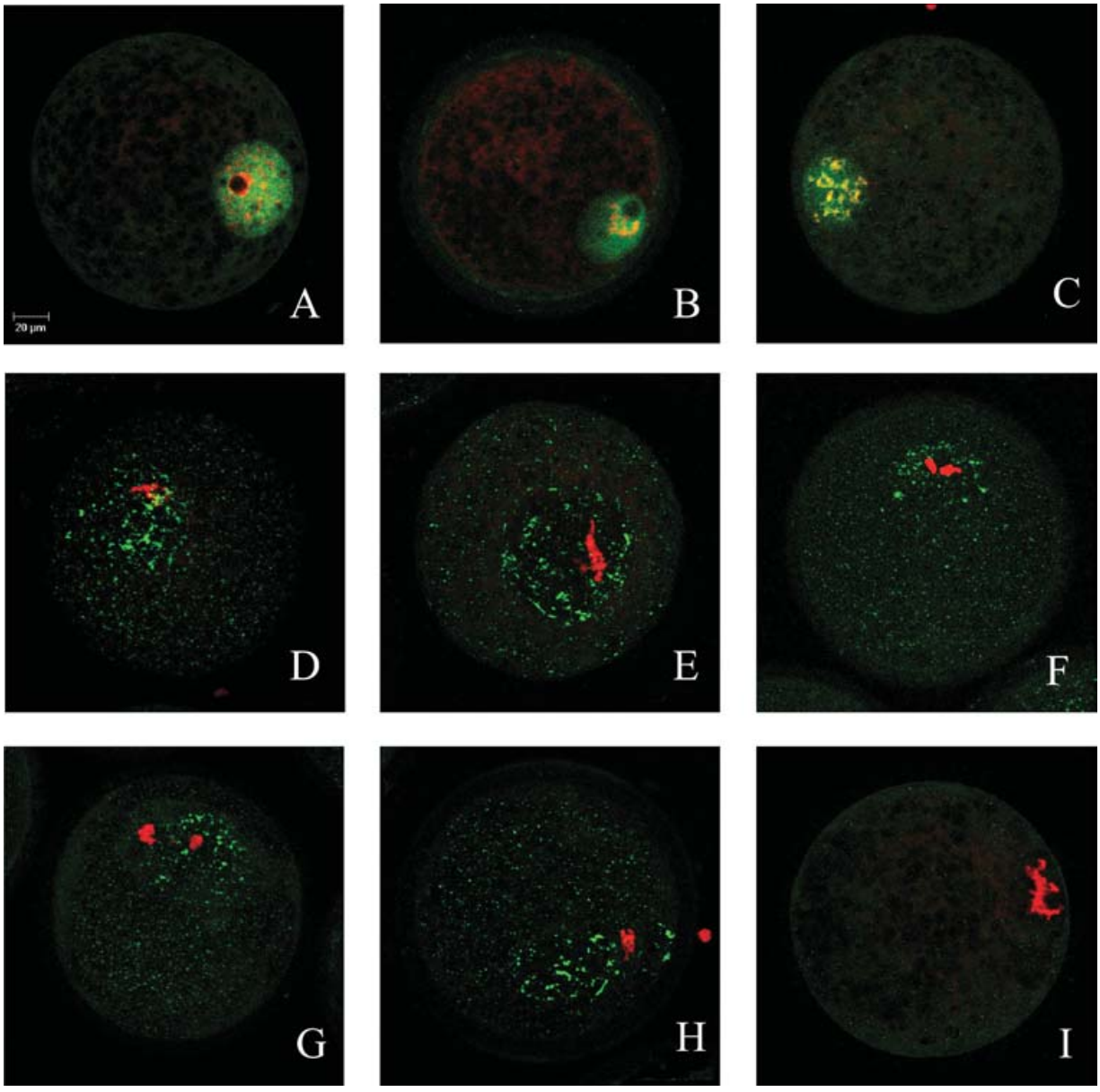

Figure 2 Immunofluorescent localization of Aurora-A during meiotic maturation. Green, Aurora-A; red, chromatin; yellow, overlapping of green and red. Aurora-A distributed in the germinal vesicle in GV stage oocytes (*A*, *B*). After GVBD, Aurora-A concentrated around the condensed chromatin (*C*). And when the chromosomes concentrated to the equatorial plate, Aurora-A migrated to around the spindle (*D*), and associated to spindle poles from MI (*E*). In anaphase I Aurora-A was localized around the chromosomes (*F*). After extrusion of the first polar body, Aurora-A distributed around the spindle (*G*), and then at the MII spindle poles (*H*). A GV 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 in the experimental group (*I*). Original magnification, ×400.

phenomenon was also found in mitotic cells (Kimura *et al*., 1997). Aurora-A is a centrosome kinase, and its structure is variable (Giet & Prigent, 2001). In order to fulfil its function, the Aurora-A centrosome kinase needs to interact with the centrosome structure. All these results suggest that Aurora-A may maintain the meiotic spindle organization and regulate the microtubule-organizing activity of spindle poles.

The spatial and temporal separation of several specific phase of the meiotic cycle makes maturing porcine oocytes and fertilized eggs an attractive model for studying the role of Aurora-A in meiotic progression of large mammals. In porcine oocytes at the GV stage and zygotes at the pronuclear stage, extensive Aurora-A staining was observed in the nucleus. The nuclear accumulation of active

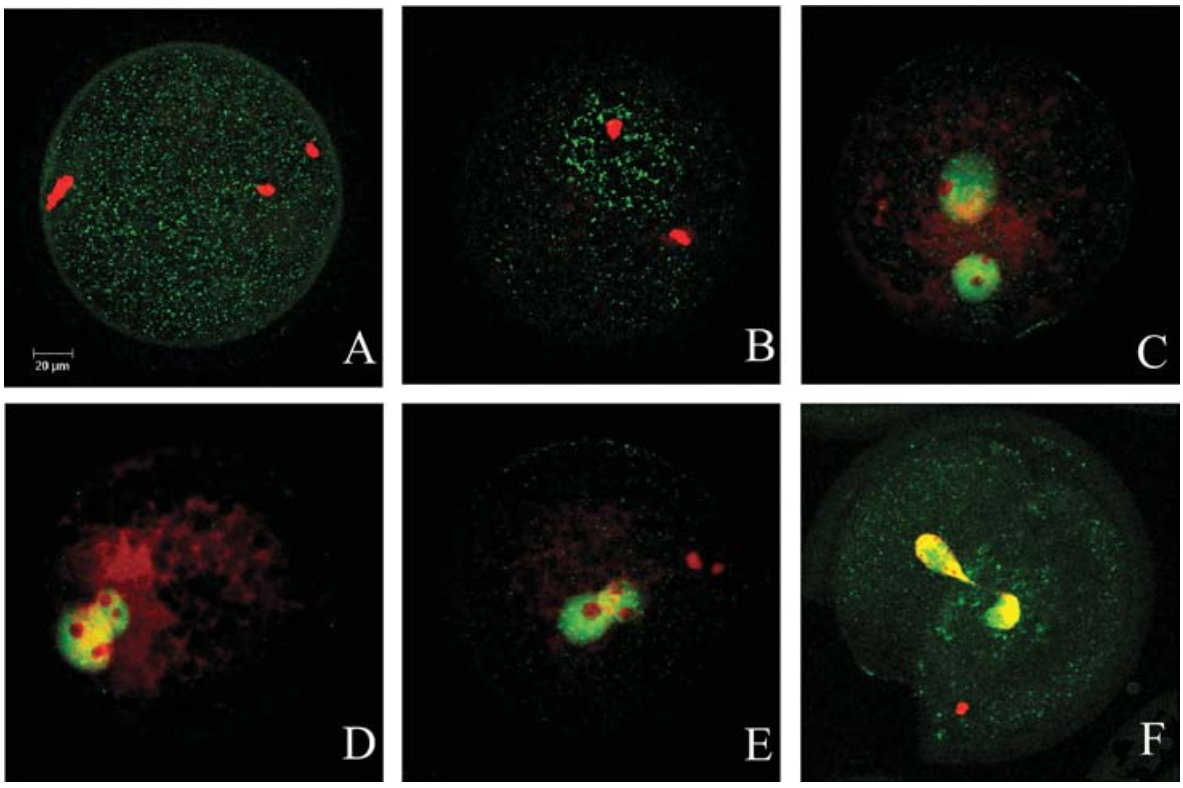

Figure 3 Localization of Aurora-A during fertilization and early embryonic mitosis. Aurora-A distributed evenly in the cytoplasm 6 h after insemination (*A*). Then the protein began to condense around the oocyte chromatin (*B*). After the pronucleus formation, Aurora-A was associated with the pronuclei (*C*). There were more Aurora-A dots between the two pronuclei when they began to go through nuclear membrane breakdown (*D, E*). After the first cleavage, Aurora-A was found in the nucleus (*F*). Original magnification, ×400.

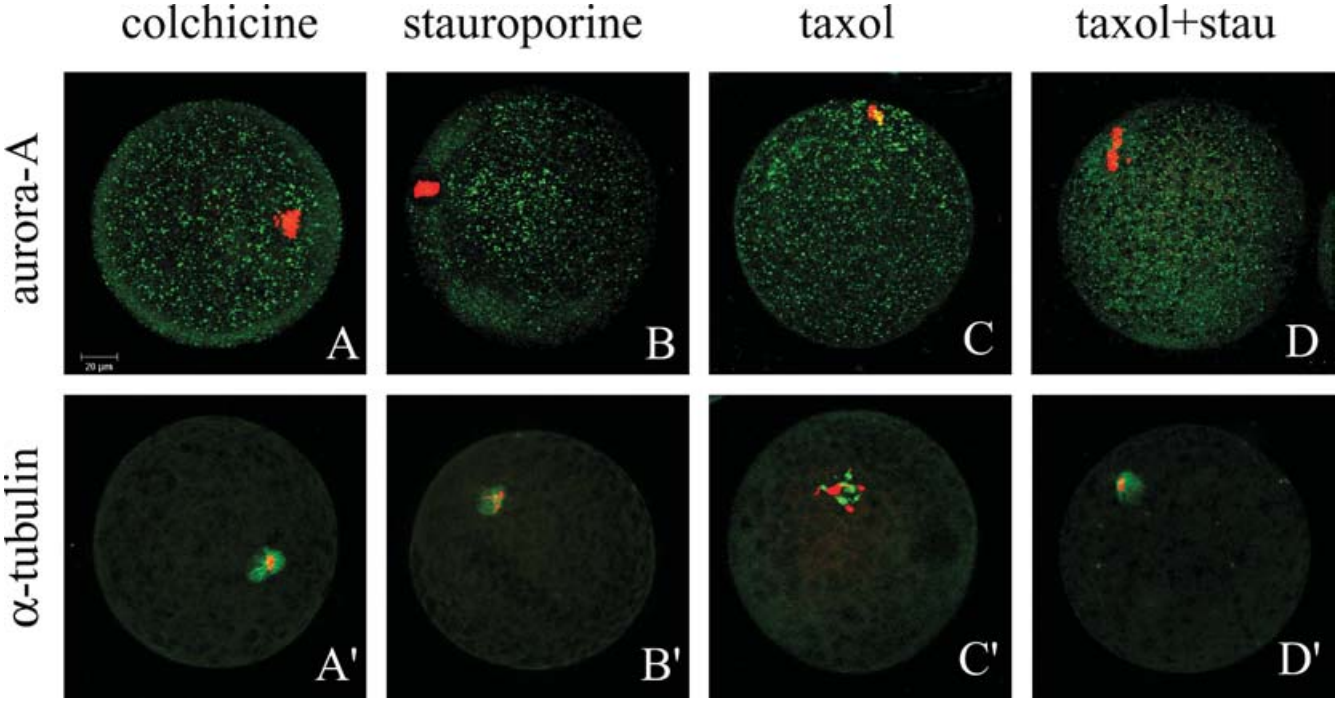

Figure 4 Effect of cytoskeleton modulators and staurosporine on Aurora-A (*A–D*) and α-tubulin (*A′–D′*) localization. When MII oocytes were cultured in medium containing colchicine, the spindle became disorganized (*A′*) and Aurora-A (*A*) distributed as several dots in the cytoplasm. When MII oocytes were cultured in staurosporine-containing medium, spindles were also disorganized (*B′*) and Aurora-A (*B*) distributed diffusely. When MII oocytes was treated with taxol, the spindle was abnormal with more poles (*C′*), Aurora-A was observed to exist as several dots in the cytoplasm (*C*). When MII oocytes were treated with stauroporine + taxol, the spindle was abnormal (*D′*), and Aurora-A distributed diffusely around the chromosomes (*D*). Original magnification, ×400.

MPF during prophase is essential for coordinating M-phase events in vertebrate cells (Kanatsu-Shinohara *et al*., 2000; Ledan *et al*., 2001). Aurora-A plays a role downstream of MPF activation in the oocyte, as described in human mitotic cell lines (Marumoto *et al*., 2002). And Aurora-A protein accumulates during meiotic maturation, in response to progesterone independently of MPF activation, but its kinase activity would be under the control of MPF (Frank-Vaillant *et al*., 2000). This suggests that Aurora-A functions downstream of MPF activation and controls the meiotic spindle. Moreover Aurora-A could be an early component of the progesterone pathway stimulating Mos synthesis upstream of Cdc2 activation. Maton *et al*. (2003) demonstrate that, *in vivo* during oocyte maturation as well as *in vitro* using prophase cytosolic extracts, active MPF is necessary and sufficient to induce Aurora-A phosphorylation and activation, independently of the Mos/MAPK pathway. 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 bring the kinase to the centrosomes, as has been shown for NuMA (Giet & Prigent, 2001).

In conclusion, Aurora-A protein is expressed in porcine oocytes, and the subcellular distribution of this kinase is highly relevant to microtubule formation. Aurora-A may be an important regulator of microtubule organization during porcine oocyte meiotic maturation, fertilization, and embryonic cleavage. In further research, the study of physiological upstream activators and target molecules of Aurora-A should be stressed, so as to deepen our understanding of its roles in cell cycle regulation.

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