

Localization of Cyclin B and MAP Kinase around Chromosomes of Cumulus Nuclei Transferred into Porcine Oocytes

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Abstract: Using immunohistochemical techniques, the localization of maturation promoting factor (MPF) and MAP kinase around chromosomes of cumulus nuclei transferred into the porcine mature oocytes was investigated. Nuclear condensation was observed in most of the transferred nuclei within 2 h and was maintained until 6 h after nuclear injection. Cyclin B, a subunit of MPF, and MAP kinase accumulated around the exogenous chromosomes in 47% of the injected oocytes at 6 h after injection. Tubulin assembly around the exogenous chromosomes was detected only in those accumulated by cyclin B and MAP kinase. The pronuclear formation rate after oocyte activation agreed well with the tubulin assembly rate. The present results indicate for the first time that MPF and MAP kinase accumulated around exogenous chromosomes in porcine mature oocytes and suggest that their accumulation correlates with tubulin assembly and pronuclear formation.

Key words: Cyclin B, MAP kinase, Localization, Porcine oocyte, Somatic nucleus

Maturation or M-phase promoting factor (MPF) is a protein phosphorylating enzyme composed of catalytic subunit, p34^{cdc2}, and the regulatory subunit cyclin B, and has been well accepted as a crucial regulator of both mitosis and meiosis. An increase in this kinase activity induces M-phase specific events, such as chromosome condensation and nuclear membrane breakdown [1]. The high MPF activity has also been suggested to be required for normal spindle formation, and the localization of cyclin B on the spindle during the M-phase has been reported in oocytes [2–4], embryos [5, 6] and cultured cells [7, 8].

Mitogen-activated protein (MAP) kinase belongs to a

group of serine/threonine protein kinase and is rapidly activated in response to various growth factors in mammalian cultured cells [9]. In oocytes, MAP kinase is activated around germinal vesicle breakdown (GVBD) and high MAP kinase activity is maintained until the second meiotic metaphase (M2) [9]. The necessity of MAP kinase activity has been suggested for GVBD in *Xenopus* oocytes, but not in mouse oocytes, transition from first meiosis to second meiosis and M2-arrest in both *Xenopus* and mouse oocytes [9, 10]. The relationship of MAP kinase activity to normal spindle formation has also been suggested and the localization of MAP kinase on the spindle has been reported in oocytes [11, 12] and cultured cells [13, 14].

Recently, somatic nuclear transfer into an enucleated M2 oocyte has been examined in several mammalian species including the pig [15–20]. Although normal birth has been reported from such re-constructed embryos, the successful rate was not satisfactory [16–18]. The distribution of MPF and MAP kinase in the re-constructed oocytes has never been investigated and, therefore, their localization on the exogenous nucleus and their relation to spindle formation are unclear.

In the present study we examined the distribution of MPF and MAP kinase in porcine M2 oocytes after cumulus nuclear injection, and the relationship of their localization on the exogenous chromosomes to subsequent processes was discussed.

Materials and Methods

Collection and maturation of porcine oocytes in vitro

Oocyte collection and in vitro maturation culture were performed as described previously [21] with some modifications. Briefly, the ovaries of prepubertal gilts were collected at a local slaughterhouse, and cumulus-oocyte complexes (COCs) were aspirated from follicles

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(2–5 mm in diameter). About 20 COCs were cultured in 0.1 ml maturation medium, consisting of modified Krebs-Ringer bicarbonate solution [22] supplemented with 1.0 IU/ml pregnant mare's serum gonadotropin (Peamex; Sankyo Co., Tokyo, Japan) and 20% porcine follicular fluid collected as described previously [21], for 48 h at 38°C, with 5% CO₂ in air. After culturing, the COCs were treated with 150 U/ml hyaluronidase (Type IV-S; Sigma, St. Louis, MO) for a few minutes, and then the surrounding cumulus cells were removed by pipetting. The isolated cumulus cells and the denuded oocytes were used for the cumulus nucleus transfer as described below.

Cumulus nucleus transfer into porcine oocytes

Injection pipettes (inner diameter 8–10 μm, tip angle 25°) were produced with a pipette puller (PN-3, Narishige) and a grinder (EG-4, Narishige). The denuded oocytes were put into 20 μl droplets of BMOC-II [23] on 60 mm dish lids (1010-060; IWAKI) and covered with silicon oil (14,615-3; Aldrich Chemical Company, Inc.). Micromanipulation of the oocytes was performed in the droplets using an inverted microscope (TMD200; Nikon Corp., Tokyo) fitted with micromanipulators (WR-50, Narishige). The isolated cumulus cells were put into 5 μl droplets of BMOC-II supplemented with 15% polyvinylpyrrolidone (PVP, Av. Mol. Wt. 360,000; SIGMA) on identical dish lids. The cumulus cells were aspirated in and out gently with an injection pipette while applying several piezo-pulses until their nuclei were largely devoid of visible cytoplasmic materials. The nuclei were washed once in BMOC-II to remove PVP and a single nucleus was injected into a denuded oocyte cytoplasm.

Examination of injected cumulus nuclei

The cumulus nuclear transferred oocytes were incubated for 0, 1, 2, 4 and 6 h in BMOC-II at 38°C, with 5% CO₂ in air. Some oocytes incubated for 6 h were activated with 200 μM Thimerosal (SIGMA) for 10 min followed by 8mM Dithiothreitol (SIGMA) for 30 min, then incubated for another 24 h in BMOC-II under the same conditions. The changes in the injected nuclei were examined under a phase-contrast microscope (Nikon) after being mounted on glass slides, fixed in ethanol-acetic acid fixative overnight, and stained with 0.75% acetic orcein solution.

Immunofluorescent microscopy

The cumulus nucleus-transferred oocytes incubated for 6 h in BMOC-II as described above were mounted on glass slides. The whole-mount preparations were

fixed in acetic alcohol-ethanol (1:1) for 20 min, then permeabilized in phosphate buffered saline (PBS) containing 0.1% polyoxyethylene (10) octylphenyl ether (Wako) for 30 min at 38°C and washed in PBS. Subsequent steps were all performed at 38°C in a moisture chamber. Blocking was performed for 1 h with 5% BSA in PBS (PBS-BSA). For the detection of cyclin B and tubulin, the oocytes were incubated for 2 h with both anti-porcine cyclin B polyclonal antibody (E-13, a gift from Dr. R.M. Moor of the Babraham Institute, Cambridge UK) and anti-tubulin alpha monoclonal antibody (DM1A, NeoMarkers) both diluted 1:30 in PBS-BSA. For the detection of MAP kinase and tubulin, anti-MAP kinase polyclonal antibody (K-23, Santa Cruz Biotechnology) diluted 1:30 in PBS-BSA was used instead of anti-porcine cyclin B antibody. The oocytes were washed in PBS-BSA, and incubated for 2 h with both FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) diluted 1:25 with PBS-BSA and rhodamine-conjugated goat anti-mouse IgG (Chemicon) diluted 1:50 with PBS-BSA. Chromosomes were stained in 10 μg/ml Hoechst 33342 (SIGMA) in PBS-BSA for 30 min. The oocytes were examined with a confocal laser microscope (LSM510-V2.01, Carl Zeiss) for the detection of MAP kinase, cyclin B and tubulin, and with a fluorescent microscope (IMT-2, OLYMPUS) for the detection of chromosomes.

Statistical analysis

Student's t-test was used for evaluation of the results. Probability of P<0.05 was considered to be statistically significant.

Results

Changes in cumulus nuclei injected into porcine oocytes

As the localization of cyclin B and MAP kinase around nuclei was generally shown after chromosome condensation but not during the interphase [7–9], the kinetics of chromosome condensation after cumulus nuclear transfer into porcine oocytes was first examined. The cumulus nuclei were unchanged both in size and shape just after injection into porcine oocytes (Fig. 1C) compared with before injection (Fig.1B). Condensation of injected nuclei was observed in more than half of the injected oocytes (58%) within 1 h and in most of the injected oocytes (92%) within 2 h of injection (Table 1). The condensed chromosomes were maintained until 6 h after injection (Table 1), but the condensed chromosomes formed a cluster-like structure (Fig. 1D), which

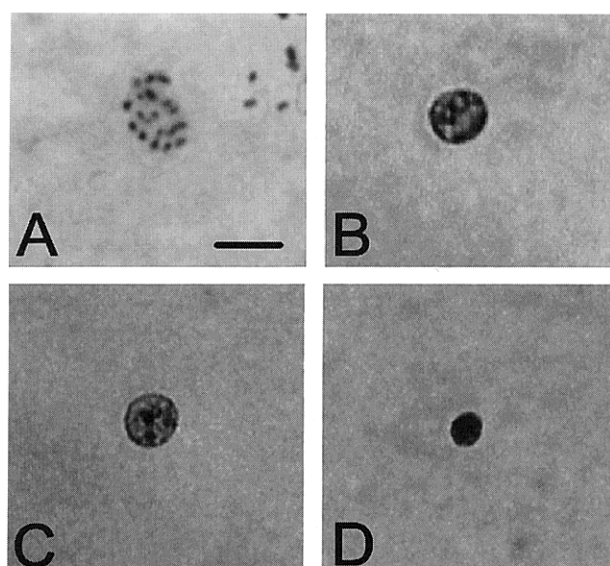


Fig. 1. Porcine chromosomes and cumulus nucleus before and after transfer into porcine oocytes. Porcine oocytes matured *in vitro* and injected each with a single cumulus nucleus were fixed at 0 h (A, C) or 6 h (D) after injection, and porcine chromosomes (A) or injected cumulus nuclei (C, D) were examined after staining with acetic orcein. A cumulus nucleus in an intact cumulus cell is also shown (B). Scale bar indicates 10 μ m.

was different from the metaphase plate of the oocytes own chromosomes (Fig. 1A).

We next examined whether the chromosomes in a cluster-like structure could form pronuclei after oocyte activation. Oocyte activation was induced in 74% of injected oocytes by Thimerosal and dithiothreitol, and 47% of such chromosomes in activated oocytes could form pronuclei (Table 2).

Distribution of cyclin B and MAP kinase in oocytes injected with cumulus nucleus

Examples of oocytes in which cyclin B and MAP kinase were localized around chromosomes of cumulus nucleus, are shown in Fig. 2 and Fig. 3, respectively. With regard to the oocyte's own spindle, cyclin B and tubulin were clearly localized on the poles of the spindle and whole spindle, respectively, although MAP kinase was dimly localized around the spindle. The described localization was observed in all of the oocytes examined. In contrast, the localization of these molecules around the exogenous chromosomes was not constant, and tubulin localization around the injected chromosomes was observed in only 48% of the injected oocytes (Table 3). The rate of tubulin localization agreed well with the

Table 1. Time required for condensation of injected cumulus nuclei

Incubation period (h)	Number of oocytes		
	injected	succeeded	condensed (%)
0	4	4	0 (0)
1	16	12	7 (58)
2	15	12	11 (92)
4	11	11	10 (91)
6	14	12	12 (100)

A single cumulus nucleus was injected into each porcine oocyte and the condensation of injected nuclei was examined at indicated incubation periods.

Table 2. Rate of pro-nucleus formation for injected nuclei in oocytes

Number of injected oocytes	Number (%) of oocytes		
	succeeded	activated	pro-nuclei formed
33	23	17 (74)	8 (47)

In vitro matured porcine oocytes each injected with a single cumulus nucleus were activated with 200 μ M Thimerosal for 10 min followed by 8 mM dithiothreitol for 30 min, and pro-nucleus formation was examined 24 h later.

rate of pronuclear formation (Table 2). Both cyclin B and MAP kinase also localized around the injected chromosomes in most of such oocytes (Table 3), although the degree of accumulation was less sharp than that on the oocyte's own spindle (Figs. 2 and 3). On the other hand, no localization of cyclin B or MAP kinase was detected around the injected chromosomes in the oocytes in which tubulin did not assemble around the injected chromosomes (Table 3).

Discussion

The purpose of the present study is to examine the localization of MPF and MAP kinase in porcine oocytes after somatic nuclear transfer. It has been reported that cyclin B and MAP kinase localized around condensed chromosomes but not in the decondensed interphase nucleus [7–9]. We therefore first examined the kinetics of chromosome condensation after cumulus nuclear transfer into porcine oocytes and found that most of the injected nuclei were condensed within 2 h after injection. This agreed well with a previous report on mouse oocytes, in which well condensed chromosomes were seen at 3 h after nuclear transfer [24]. The condensed chromosomes were unchanged until 6 h after injection, indicating that the present injection procedure did not

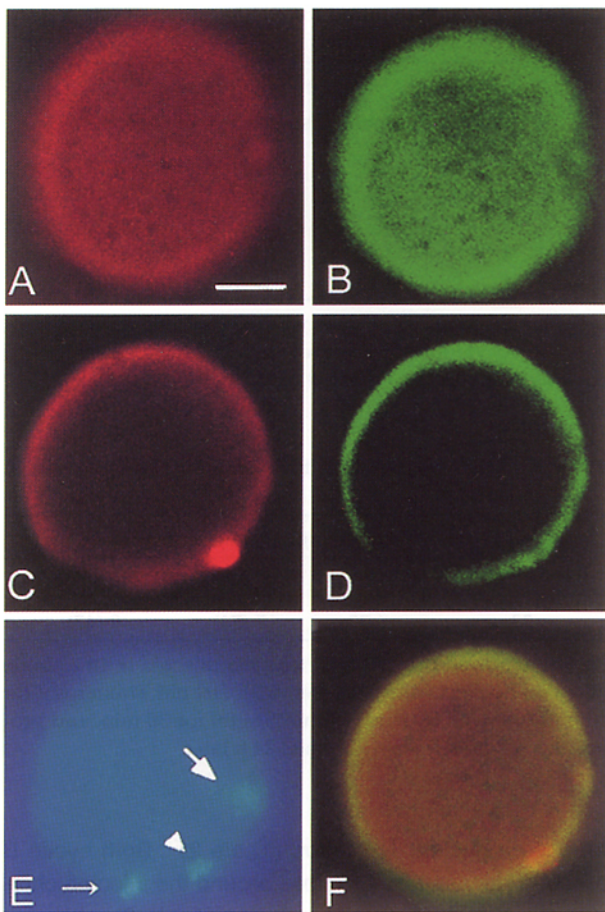


Fig. 2. Immunohistochemical analysis of cyclin B distribution in cumulus nucleus transferred porcine oocytes. A porcine oocyte was double stained with anti-tubulin and anti-cyclin B antibodies 6 h after cumulus nucleus injection. The anti-tubulin (A, C) and anti-cyclin B (B, D) antibodies were visualized with rhodamine (red) and FITC (green), respectively, and detected by confocal laser microscopy focused on the injected cumulus nucleus (A, B) or the oocyte's own chromosomes (C, D). A composite of all 4 photographs is shown in F. The oocyte was also examined after Hoechst 33342 staining (E), and the injected cumulus chromosomes (large arrow), the oocyte's own chromosomes (arrowheads) and first polar body (small arrow) are indicated. Scale bar indicates 30 μ m.

induce oocyte activation. In view of to these results, we decided to examine the distribution of cyclin B and MAP kinase at 6 h after nuclear transfer.

The immunohistochemical analysis revealed that cyclin B and MAP kinase accumulated around only half of the injected chromosomes in porcine oocytes and that the accumulation of these molecules correlated well with the tubulin assembly. This is the first report indi-

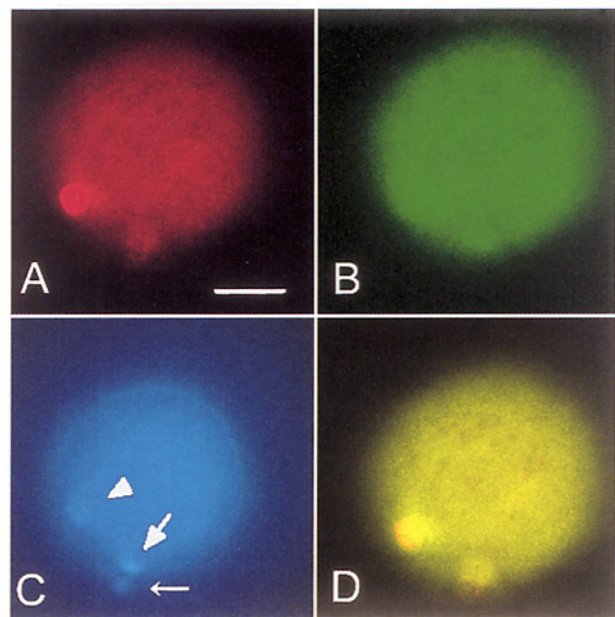


Fig. 3. Immunohistochemical analysis of MAP kinase distribution in cumulus nucleus transferred porcine oocytes. A porcine oocyte was double stained with anti-tubulin and anti-MAP kinase antibodies 6 h after cumulus nucleus injection. The anti-tubulin (A) and anti-MAP kinase (B) antibodies were visualized with rhodamine (red) and FITC (green), respectively, and detected by confocal laser microscopy. A composite of the photographs is shown in D. The oocyte was also examined after Hoechst 33342 staining (C), and the injected cumulus chromosomes (large arrow), oocyte's own chromosomes (arrowhead) and first polar body (small arrow) are indicated. Scale bar indicates 40 μ m.

cating the localization of these molecules around injected exogenous chromosomes in mammalian oocytes. The close correlation between the accumulation of these molecules and tubulin assembly might indicate the necessity for MPF and MAP kinase for spindle formation as suggested in previous reports [2–8, 11–14]. The close agreement between the rate of accumulation of cyclin B and MAP kinase around injected chromosomes and the rate of pronucleus formation after oocyte activation implies a relationship between these molecules, including tubulin, and normal pronuclear formation.

Analysis of the oocyte's own spindle revealed that cyclin B and MAP kinase were localized on spindle poles and around the whole spindle, respectively, in porcine mature oocytes. These results were different from previous reports on porcine oocytes, in which cyclin B was localized on condensed chromosomes [4] and active MAP kinase was localized between a spindle and a

Table 3. Localization of cyclin B or MAP kinase around the injected cumulus chromosomes

Number of oocytes		Tubulin accumulation	total	Number (%) of oocytes			
injected	succeeded			Cyclin B		MAP kinase	
				examined	localized	examined	localized
58	46	+	22 (48)	11	11 (100)	11	10 (91)
		-	24 (52)	10	0 (0)	14	0 (0)

A single cumulus nucleus was injected into each porcine oocyte and the localization of tubulin/cyclin B or tubulin/MAP kinase was examined 6 h later by immunofluorescent double staining.

second polar body [12]. The reasons for these discrepancies are unknown but probably may be attributed to differences in the specificity of the antibodies.

In the present study the shape of condensed chromosomes was different from that of normal metaphase plates and formed a cluster-like structure. These cluster-like chromosomes were similar to those in pro-metaphase porcine oocytes [25] and in cycloheximide treated-mouse oocytes [26], where MPF activity was higher than that in immature oocytes but lower than that in metaphase oocytes [26, 27]. Therefore, MPF activity of the nuclear injected-oocytes in the present study might be decreased a little, probably by the manipulation processes, although activity was not at the basal level that induced oocyte activation. About half of these cluster-like chromosomes were, however, able to form a pronucleus after oocyte activation as mentioned above. In a previous report in which granulosa cells were electrically fused with an enucleated porcine oocytes, pronuclear formation was observed in 57% of the fused oocytes 1 day after manipulation [17]. The present pronuclear formation rate was comparable with the reported rate and might not be abnormal despite the cluster-like shape of the condensed chromosomes.

In conclusion, we showed for the first time that cyclin B and MAP kinase accumulate around the injected chromosomes in porcine mature oocytes, and might contribute to tubulin assembly and pronuclear formation.

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