Cdk2 Activity is Essential for the First to Second Meiosis Transition in Porcine Oocytes

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Abstract. The meiotic progression of Xenopus oocytes has been suggested to depend on the activity of cyclin-dependent kinase 2 (Cdk2). We examined whether Cdk2 is involved in the regulation of mammalian oocyte meiosis by injecting porcine oocytes with anti-Cdk2 antibody. At first, the cross-reactivity of the anti-Cdk2 antibody with Cdc2 kinase was evaluated by immunoprecipitation and immunoblotting experiments using porcine granulosa cell extract, and no cross-reactivity with Cdc2 kinase was observed in the antibody used. In the anti-Cdk2 antibody-injected group, 50.7% of the oocytes were arrested in the second metaphase after 50 h of culture and this rate was significantly lower than those in the non-injected intact oocytes or the oocytes injected with mouse IgG (84.5% and 86.7%, respectively). Most of the other oocytes in the antibody-injected group formed a pronucleus without polar bodies or with only one polar body. The cyclin B1 amount in the antibody-injected and activated oocytes was dramatically decreased compared with that in the intact or mouse IgG-injected oocytes after 50 h of culture. These results suggest that Cdk2 is involved in the meiotic maturation of mammalian oocytes, and that the block of Cdk2 activity results in the failure of cyclin B1 accumulation and second meiosis induction.

Key words: Oocyte, Maturation, Cyclin-dependent kinase 2 (Cdk2), Cyclin B, Pig

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species. Therefore, it is important to study the contribution of Cdk2 activity to oocyte maturation in other species in addition to using *Xenopus* oocytes. In mammalian oocytes, however, although the presence of cdk2 mRNA in immature mouse oocytes has been reported [11], it has not yet been determined whether Cdk2 is involved in the regulation of oocyte meiosis.

In the present study, in order to evaluate the involvement of Cdk2 in the maturation of porcine oocytes, maturing porcine oocytes were injected with a Cdk2 antibody to inhibit its activity and were examined for their progression of meiotic maturation and the cyclin B1 accumulation.

**Materials and Methods**

**Preparation of porcine granulosa cell extract**

Pig ovaries were obtained from gilts at a commercial slaughterhouse. Granulosa cells in antral follicles (2–5 mm diameter) were collected by aspiration using an 18-G needle and syringe, then centrifuged at 3000 rpm for 30 min. The granulosa cell pellet was added to an equal volume of 2 × RIPA buffer, mixed well, then lysed for 30 min on ice. The suspension was centrifuged at 15,000 rpm for 5 min, and the supernatant (100 µl) was added to 20 µl of a 50% (v/v) slurry of protein-A agarose beads (Sigma, St. Louis, MO) in RIPA buffer, then incubated for 1 h at 4 C in order to remove the proteins bound to the agarose beads nonspecifically. The mixture was centrifuged at 15,000 rpm for 5 min and the supernatant was collected as granulosa cell extract.

**Immunoprecipitation**

One microliter aliquots of antibodies or mouse IgG solution (I-8765; Sigma) was added to 40 µl granulosa cell extract and incubated for 1 h on ice. Seven microliters of the protein-A beads slurry was added to the sample and incubated for 2 h at 4 C. The protein-A beads were recovered by centrifugation at 12,000 g for 0.5 min and washed three times in 200 µl RIPA buffer before 12.5 µl of SDS sample buffer [12] was added. After heating at 100 C for 5 min, the samples were stored at –80 C until immunoblotting. The antibodies used were mouse anti-Cdc2 antibody (sc-54; SantaCruz Biotechnology, Santa Cruz, CA) and mouse anti-Cdk2 antibody (C18520; Transduction Laboratories).

**Collection and maturation of porcine oocytes in vitro**

Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–5 mm in diameter) of gilt ovaries and washed four times in a modified Krebs-Ringer bicarbonate solution [13] containing 20% porcine follicular fluid, 1.0 IU/ml eCG (Pramex; Sankyo, Tokyo, Japan), and 3.2 mg/ml BSA (fraction V; WAKO Pure Chemical Ind., Osaka, Japan). Groups of 20 COCs were cultured for up to 50 h in 0.1 ml of the medium described above, and covered with liquid paraffin (Nakarai Tesque Inc., Kyoto, Japan) at 37 C, under an atmosphere of 5% CO2 and saturated humidity in air as reported previously [14, 15]. After culturing or before microinjection, oocytes were treated with 150 IU/ml hyaluronidase (type IV; Sigma), and the surrounding cumulus cells were removed by pipetting. The denuded oocytes were subjected to immunoblotting, microinjection or examination for their nuclear states after being mounted on glass slides, fixed with acetic acid-ethanol (1:3), and stained with 0.75% aceto-orcein solution.

**Microinjection**

The microinjection was performed after 30 h of culture using microinjectors (IM-5A/B; Narisige, Tokyo, Japan) equipped with manipulators (WR-50; Narishige) mounted on an inverted microscope (TMD200; Nicon Co., Tokyo, Japan). Approximately 50 pl of antibody or mouse IgG solution was injected into each ooplasm. After being injected, all oocytes were cultured as described above for an additional 20 h (for 50 h in total). After culture, the oocytes were subjected to immunoblotting or examination of nuclear states. The solvent of mouse anti-Cdk2 antibody and mouse IgG solution (I-8765; Sigma) was exchanged for PBS by Nanosep® 10K Omega (Pall Co. New York), and the antibody was concentrated 10-fold (approximately 2.5 mg/ml) and the mouse IgG was adjusted to a concentration of 10.5 mg/ml.

**Immunoblotting**

The micro-Western blotting method [16] was used with several modifications. Two microliters of immunoprecipitated samples of porcine granulosa cell extracts or 20 oocytes in 2.5 µl of SDS sample buffer were used for each lane of SDS-polyacrylamide gel electrophoresis (PAGE). For
the cyclin B1 immunoblotting of antibody-injected oocytes, the oocytes having a pronucleus were selected after staining with Hoechst 33342 (10 mg/ml) as described previously [17]. Proteins were separated on a 10% polyacrylamide gel by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (AE-6660; Atto Co., Tokyo, Japan) according to the manufacturer’s instructions. After blocking the membrane with 3% skim milk for 20 min, the membrane was treated with primary antibodies overnight at 4°C, then with secondary antibody for 90 min at room temperature. The signals were detected by an ECL blotting detection kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The primary antibodies used were the anti-Cdc2 and anti-Cdk2 antibodies described above and anti-cyclin B1 monoclonal antibody (CB169, Upstate Biochemistry Inc., Waltham, MA). The secondary antibody used was a peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc). All primary antibodies were diluted appropriately with 3% skim milk containing PBS.

Statistical analysis
The results were evaluated through the chi-square test. Values of P<0.05 were considered to indicate statistical significance.

Results

Specificity of anti-Cdk2 antibody
As shown in Fig 1A, a single band of about 33 kDa was detected by immunoblotting analysis of porcine granulosa cell extract with the anti-Cdk2 antibody (Fig. 1A). In order to examine the cross-reactivity of the anti-Cdk2 antibody with Cdc2 kinase, a catalytic subunit of MPF, the porcine granulosa cell extract was immunoprecipitated with the anti-Cdk2 and anti-Cdc2 antibodies, and then the precipitated proteins were detected by immunoblotting with these antibodies (Fig. 1B). In the sample immunoprecipitated with anti-Cdk2 antibody, a protein detected by anti-Cdk2 antibody but not by anti-Cdc2 antibody was present (Fig. 1B, lane 1). A faint band in the anti-Cdc2 immunoblotting (Fig. 1B, upper panel, lane 1) was also visible in the sample immunoprecipitated with mouse IgG (Fig. 1B, upper panel, lane 3), and such a non-specific band was also reported previously in a similar experiment [18]. Conversely, this anti-Cdk2 antibody did not detect the protein immunoprecipitated by anti-Cdc2 antibody (Fig. 1B, lower panel, lane 2), whereas a specific protein was detected by anti-Cdc2 antibody (Fig. 1B, upper panel, lane 2). In addition, the injection of this anti-Cdk2 antibody into immature porcine oocytes did not affect their germinal vesicle breakdown in a preliminary experiment (data not shown). These results indicate that the anti-Cdk2 antibody used is not cross-reactive with Cdc2 kinase and does not directly inhibit MPF activity.

Effects of anti-Cdk2 antibody injection on the maturation of porcine oocytes
To examine the effects of anti-Cdk2 antibody injection, porcine immature oocytes, freshly isolated from antral follicles, were cultured in vitro for 30 h and injected with either anti-Cdk2 antibody or mouse IgG. Then the injected oocytes were cultured for an additional 20 h and their nuclear states were examined (Table 1). Nuclear states of non-injected oocytes at 30 and 50 h are also shown in Table 1. Most of the oocytes were at the first
meiotic metaphase (M1) at the injection period. Fifty-point-seven percent of the oocytes injected with anti-Cdk2 antibody had reached M2 after 50 h of culture. This percentage was significantly lower than those of the non-injected intact oocytes and the oocytes injected with mouse IgG (84.5% and 86.7%, respectively). Forty-one percent of the antibody-injected oocytes had a pronucleus, and this rate was significantly higher than the rate for mouse IgG-injected oocytes (8.2%). Among the oocytes injected with anti-Cdk2 antibody and having a pronucleus, 50.0% and 43.4% had no polar body (Fig. 2A) or one polar body (Fig. 2B, C), respectively, and only 6.6% had two polar bodies (Fig. 2D, E), indicating that most of the oocytes with inhibited Cdk2 activity formed a pronucleus after M1 stage directly without entering into the second meiosis.

Cyclin B1 accumulation in the oocytes injected with anti-Cdk2 antibody

In intact maturing oocytes, cyclin B1 was accumulated gradually after 20 h of culture (Fig. 3A) as reported previously [19]. The cyclin B1 amount after 50 h of culture in the oocytes injected with mouse IgG was not decreased compared with that in intact oocytes (Fig. 3B). In contrast, the amount in the anti-Cdk2-injected and activated oocytes was dramatically decreased and became lower than that in the 30-h-cultured intact oocytes (Fig. 3B).

Discussion

In the present study, the involvement of Cdk2 activity in the regulation of meiotic maturation in porcine oocytes was examined. When porcine maturating oocytes were injected with an anti-Cdk2 antibody and cultured until the end of the maturation period, a significant percentage of the injected oocytes did not arrest at M2 but formed a pronucleus. A majority of the activated oocytes injected with anti-Cdk2 antibody had no or only one polar body, suggesting that the pronucleus is formed directly after M1 without reaching M2. The same phenomena have been reported in c-mos antisense RNA-injected Xenopus and porcine oocytes [20, 21], and c-mos deficient mouse oocytes [22], suggesting the failure of MPF reactivation after the first meiosis. The present results also suggest that the injection of anti-Cdk2 antibody inhibits the MPF reactivation and the first to second meiosis transition in porcine oocytes.

As Cdk2 has some homologies with Cdc2 kinase, the evaluation of cross-reactivity with Cdc2 kinase is essential for the anti-Cdk2 antibody used for the injection experiments. With immunoblotting analysis of porcine granulosa cell extract using the anti-Cdk2 antibody, only a single band was detected. Furthermore, the antibody used in the present study did not detect the proteins immunoprecipitated by an anti-Cdc2 antibody and, conversely, the proteins immunoprecipitated by the present anti-Cdk2 antibody were not detected by the anti-Cdc2 antibody. Moreover, the injection

<table>
<thead>
<tr>
<th>Injection</th>
<th>Culture period (h)</th>
<th>Total no. of oocytes</th>
<th>Number (%) of oocytes at the stage of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>53</td>
<td>7</td>
</tr>
<tr>
<td>(13.2)</td>
<td></td>
<td></td>
<td>(13.2)</td>
</tr>
<tr>
<td>None</td>
<td>50</td>
<td>110</td>
<td>3</td>
</tr>
<tr>
<td>(2.7)</td>
<td></td>
<td></td>
<td>(1.8)</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>50</td>
<td>166</td>
<td>8</td>
</tr>
<tr>
<td>(4.8)</td>
<td></td>
<td></td>
<td>(86.7)</td>
</tr>
<tr>
<td>Anti-Cdk2</td>
<td>50</td>
<td>73</td>
<td>2</td>
</tr>
<tr>
<td>(2.7)</td>
<td></td>
<td></td>
<td>(4.1)</td>
</tr>
</tbody>
</table>

Porcine oocytes were injected with mouse IgG or anti-Cdk2 antibody after 30 h of culture, and their nuclear states were examined after 50 h of culture. The non-injected control oocytes were also examined at 30 and 50 h. Different letter superscripts in the same column indicate significant differences. GV: germinal vesicle stage; PM1: first prometaphase; M1: first metaphase; AT1: first anaphase and first telophase; M2: second metaphase; PN: pronuclear stage.
of the anti-Cdk2 antibody into immature porcine oocytes did not affect their meiotic resumption in a preliminary experiment. These results show that the present anti-Cdk2 antibody did not cross-react with Cdc2 kinase and that the injected antibody did not inhibit MPF activity directly.

Cdk2 has been reported as regulating the cyclin B level by phosphorylating Cdh1, an activator of anaphase-promoting complex (APC) [23,24]. Cdk2 inhibits cyclin B degradation and increases the cyclin B level from the S phase to M phase in mammalian somatic cells [23, 24], because cyclin B is degraded by proteasome after being ubiquitinated by Cdh1-activated APC [25], and because phosphorylation of Cdh1 dissociates it from APC and inactivates APC [26]. The presence of Cdh1 protein has been reported during Xenopus oocyte maturation [27]. In the present results, cyclin B1 level in the oocytes having a pronucleus after the injection of anti-Cdk2 antibody was dramatically lower than that in the control oocytes. It is possible that Cdk2 regulates cyclin B degradation by inhibiting APC activity in porcine oocytes as in somatic cells, although further studies are required to assess this point.

Previously, the involvement of Cdk2 activity in the progression of meiotic maturation has been studied only in Xenopus oocytes [6, 8–10], and has produced conflicting results. The results of studies injecting cdk2 antisense RNA into immature oocytes [8] and adding active Cdk2/cyclin E complex in oocyte extracts [9] indicate important roles for Cdk2 activity during the meiotic cell cycle. Whereas, a study, using Cdk2 inhibitor, p21CIP, showed dispensability of Cdk2 activity for the oocyte meiosis [10], the present results, and those of some other studies indicate that Cdk2 activity is required for the normal progression of porcine
oocyte meiosis, especially for entering the second meiosis. Currently, whether other methods to inhibit Cdk2 activity, such as \textit{cdk2} antisense RNA injection and p21 Cip injection into mammalian oocytes, lead to contradictory results as in the studies using \textit{Xenopus} oocytes, is not clear. As contribution of Cdk2 activity had not yet been studied in mammalian oocytes, the present study is the first to report the requirement of Cdk2 activity during mammalian oocyte maturation, and that the block of Cdk2 activity results in the failure of second meiosis induction.

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