Contribution of high p34^{cdc2} kinase activity to premature chromosome condensation of injected somatic cell nuclei in rat oocytes

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Abstract

The present study was undertaken to clarify the relationship between the p34^{cdc2} kinase activity of *in vitro*-aged or enucleated rat oocytes and the premature chromosome condensation (PCC) of microinjected cumulus cell nuclei. Wistar rat oocytes were placed *in vitro* up to 120 min after the animal was killed. The p34^{cdc2} kinase activity of the oocytes decreased in a time-dependent manner. The incidence of PCC was higher when nuclear injection into intact oocytes was completed in 15–45 min rather than 46–120 min. When rat oocytes were enucleated for subsequent nuclear injection, the p34^{cdc2} kinase activity transiently increased soon after enucleation but drastically decreased after 30 min. Removal of the cytoplasm instead of the metaphase-plate did not affect the p34^{cdc2} kinase activity even after 60 min. PCC occurred in intact and cytoplasm-removed oocytes but not in enucleated oocytes. In contrast, oocytes from BDF1 mice exhibited a p34^{cdc2} kinase level twice that of rat oocytes and supported PCC despite enucleation. The p34^{cdc2} kinase level of intact rat oocytes was reduced to the equivalent level of aged (120 min) or enucleated (+ 60 min) oocytes by a 45 min treatment with roscovitine, an inhibitor of p34^{cdc2} kinase. None of the roscovitine-treated oocytes supported PCC while half of the control oocytes did. When rat oocytes were treated with MG132, a proteasome inhibitor, delayed inactivation of the p34^{cdc2} kinase was observed in the MG132-treated oocytes. A significantly higher proportion of the MG132-treated oocytes supported PCC when compared with the control oocytes. Moreover, a higher proportion of MG132-treated and enucleated oocytes carried two pseudo-pronuclei after cumulus cell injection and developed to the two-cell stage when compared with the enucleated oocytes at the telophase-II stage. These results suggest that the decreased level of p34^{cdc2} kinase activity in aged or enucleated rat oocytes is responsible for their inability to support PCC of microinjected donor cell nuclei and that inhibition of p34^{cdc2} kinase inactivation by chemicals such as MG132 is in part effective for rat oocytes to promote PCC and further development.

Reproduction (2005) 129 171–180

Introduction

Successful animal cloning by somatic cell nuclear transfer (NT) has been reported in large domestic species (Wilmut *et al.* 1997, Kato *et al.* 1998, Baguisi *et al.* 1999, Onishi *et al.* 2000, Galli *et al.* 2003). As for small rodents, Wakayama *et al.* (1998) were the first to produce cloned mice using G0/G1-phase cumulus cells, and very recently Zhou *et al.* (2003) reported the production of fertile cloned rats using M-phase fetal fibroblast cells. However, the success rates of producing cloned rodents by NT are still low.

In the mouse, nuclei injected into enucleated oocyte cytoplasm are directly exposed to reprogramming factors,

resulting in premature chromosome condensation (PCC), with subsequent multiple pseudo-pronuclei formation following activation (Wakayama *et al.* 1998, 1999, Ogura *et al.* 2000). It is considered in rodents, at least, that PCC is a critical aspect contributing to successful cloning (Wakayama *et al.* 1999, Ogura *et al.* 2000). However, PCC is not essential for somatic cell cloning in all species, since telophase-II (TII) oocytes have promoted embryo development in goats (Baguisi *et al.* 1999) and cattle (Kurosaka *et al.* 2002).

There is a major difference between mouse and rat, in the behavior of somatic nuclei upon exposure to oocyte

cytoplasm. We have reported that the PCC of both mouse and rat cumulus cell nuclei occurred in enucleated mouse oocytes but not at all in enucleated rat oocytes (Hirabayashi *et al.* 2003a). Even if cumulus nuclei were injected into the rat oocytes without enucleation, the rate of PCC formation was less than half (Hirabayashi *et al.* 2003b).

We also reported that the ability of rat oocytes to promote PCC of the injected nuclei was dependent on the characteristics of the oocytes, such as age (4- to 5-week-old, superior to >10-week-old rats) or strain of the donor rats (Wistar and LEW, superior to the Donryu and F344 strains) and the timing of oocyte recovery (14 h post-human chorionic gonadotropin (hCG), superior to 17 h post hCG) (Hirabayashi *et al.* 2003b). In the same study, the time management of NT completion (less than 45 min after donor animal killing) and addition to the oocyte-handling media of *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), a neutral cysteine protease inhibitor, were also found important for intact rat oocytes to promote PCC. The successful production of cloned rats (Zhou *et al.* 2003) may be attributed to injecting the metaphase-arrested fetal fibroblasts into oocytes treated with MG132, a proteasome inhibitor, followed by quick removal of the recipient metaphase-plate. Therefore, it is a possibility that recipient oocytes arrested at the metaphase-II (MII) stage by the inhibition of protein degradation have essential roles in nuclear reprogramming including PCC formation.

Ovulated rat oocytes are known to activate spontaneously but abortively during *in vitro* culture (Zernicka-Goetz 1991). We also confirmed that more than half of the oocytes from Wistar rats progressed to anaphase-II (AII)/TII stages at 70 min after killing of the donor rats (Kato *et al.* 2001). In vertebrate oocytes, it has been considered that high activity of maturation promoting factor (MPF) affects the chromosome condensation and the maintenance of the meiotic spindle, both of which are involved in the meiotic arrest at the MII stage (Jones 2004). MPF is a heterodimer composed of p34^{cdc2} kinase and cyclin B1, controlling the cell cycle. When the activity was inactivated by the degradation of cyclin B (Nurse 1990), oocytes were released from the arrest at the MII stage. The p34^{cdc2} kinase in oocytes was maintained at a high level until ovulation in many species (Kubiak *et al.* 1993, Naito *et al.* 1995, Abrieu *et al.* 2001, Ito & Shimada 2005), suggesting that p34^{cdc2} kinase activity in rat ovulated oocytes was rapidly decreased *in vitro*. Moreover, destruction of the nuclear envelope in mitotic somatic cells occurred during the G2/M-phase transition by p34^{cdc2} kinase-related phosphorylation of lamin, which is one of the nuclear membrane proteins (Peter *et al.* 1990). It has been reported that ALLN, which was used in our previous study (Hirabayashi *et al.* 2003b), has an effect on the inhibition of cyclin B destruction (Sherwood *et al.* 1993). Therefore, it is possible that the low rate of PCC of injected somatic cell nuclei resulted from rapidly decreasing p34^{cdc2} kinase activity in rat ovulated oocytes,

while direct evidence that p34^{cdc2} kinase activity is involved in the PCC incidence has not yet been shown. Clarifying the relationship between p34^{cdc2} kinase activity and PCC incidence would contribute to understanding the nuclear reprogramming mechanism in mammalian oocytes.

In the present study, two experiments were conducted to examine the effects of *in vitro* aging and enucleation of rat oocytes on the kinetics of p34^{cdc2} kinase and the incidence of PCC of microinjected cumulus cell nuclei, with some comparisons with mouse oocytes. In addition, two more experiments were conducted to explain a possible role of p34^{cdc2} kinase for promoting PCC, by treatment of the rat oocytes with roscovitine, an inhibitor of p34^{cdc2} kinase (Lazar *et al.* 2002), and MG132, a proteasome inhibitor which has an effect on the inhibition of cyclin B1 degradation (Josefsberg *et al.* 2000).

Materials and Methods

Oocyte preparation

Specific-pathogen-free Crj:Wistar female rats (3–5 weeks old) or Crj:BDF1 female mice (7 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were housed under controlled lighting (lights on at 0600–1800 h), temperature (23 ± 2 °C) and humidity (55 ± 5%), with free access to laboratory diet (MF; Oriental Yeast Co., Tokyo, Japan) and water. Rats were superovulated by i.p. injections of 30 IU equine chorionic gonadotropin (eCG) (Nippon Zenyaku, Co., Fukushima, Japan) and 30 IU hCG (Sankyo Yell Yakuhin, Co., Tokyo, Japan) at 48 h intervals (Hirabayashi *et al.* 2001). Mice were superovulated as well with 5 IU eCG and 5 IU hCG.

Fourteen hours after the hCG injection, cumulus–oocyte complexes (COCs) were collected from the oviductal ampullae of donor rats or mice with modified Krebs–Ringer bicarbonate solution (mKRB) in Experiments 1 and 3 (Toyoda & Chang 1974) and modified rat one-cell embryo culture medium (mR1ECM) in Experiments 2, 4 and 5 (Oh *et al.* 1998) supplemented with 0.1% hyaluronidase (Sigma-Aldrich Corp., St Louis, MO, USA) respectively. Two to five minutes later, the denuded oocytes were washed three times with fresh mKRB or R1ECM and kept in the same medium at 37 °C until being subjected to the treatments. The time of killing of the donor animals was defined as 0 min, and it usually took 10–15 min from the animal killing to the preparation of denuded oocytes.

Measurement of p34^{cdc2} kinase activity

The p34^{cdc2} kinase assay was performed according to the method as described by Ito *et al.* (2001) with some modifications. In brief, the oocytes were washed several times with PBS and placed into cell lysis buffer composed of 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%

Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 mg/ml leupeptin (all chemicals from Cell Signaling Technology, Beverly, MA, USA) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The oocyte lysates were stored at -80°C until the ELISA assay.

The lysate (ten oocytes/5 μl cell lysis buffer) was mixed with 45 μl of kinase assay buffer-A composed of 25 mM Hepes buffer (pH 7.5) (MBL, Nagoya, Japan), 10 mM MgCl_2 (MBL), 10% (v/v) mouse vimentin peptide solution (SLYSSPGGAYC) (MBL) and 0.1 mM ATP (Sigma-Aldrich), and the mixture was incubated for 30 min at 30°C . The reaction was terminated by the addition of 200 μl PBS containing 50 mM EGTA (MBL). The phosphorylation of mouse vimentin peptides was detected using an ELISA analysis (MESACUP cdc2 kinase assay kit (code no. 5234); MBL). Data were expressed in terms of the strength of p34^{cdc2} kinase activity in denuded rat oocytes 15 min (Experiments 1, 3 and 4) or 45 min (Experiment 2) after animal killing.

Assessment of nuclear configuration

Rat oocytes immediately after recovery and 30, 60, 90 and 120 min after culture in mKRB were stained with 5 $\mu\text{g}/\text{ml}$ Hoechst 33342 (Sigma-Aldrich). Their nuclear configuration was then assessed under UV light at 330–380 nm, according to the classifications reported previously (Kato *et al.* 2001): MII, characterized by a metaphase spindle with the first polar body; AI, characterized by elongated spindle without the second polar body; and TII, characterized by elongated spindle with extrusion of the second polar body.

Nuclear injection and PCC evaluation

Cumulus cells dispersed from COCs by the hyaluronidase treatment were placed in mKRB (Experiments 1 and 3) or R1ECM (Experiments 2, 4 and 5) containing 22 mM Hepes and 5 mM NaHCO_3 (abbreviated hereafter as Hepes-mKRB and Hepes-R1ECM respectively). An aliquot (2 μl) of the cumulus cell suspension was transferred to 10 μl of Hepes-mKRB or Hepes-R1ECM supplemented with 12% w/v 360 kDa polyvinylpyrrolidone (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). Nuclei were removed from cumulus cells and gently aspirated in and out of the injection pipette (7–10 μm) until their nuclei were largely devoid of visible cytoplasmic materials. Each nucleus was injected into a separate enucleated oocyte within 5 min of its isolation, as described by Kimura & Yanagimachi (1995). One hour after the nuclear injection, the oocytes were stained with 5 $\mu\text{g}/\text{ml}$ Hoechst 33342 and evaluated for incidence of PCC under UV light at 330–380 nm, as described previously (Hirabayashi *et al.* 2003a,b). The injected somatic cell nucleus was distinguished from the oocyte nucleus by the position of the opening in the zona pellucida at the nuclear microinjection site.

Experimental design

Experiment 1: effect of *in vitro* aging

The effect of *in vitro* aging on the p34^{cdc2} kinase activity and the PCC incidence was investigated using rat oocytes. Ten oocytes for each test were sampled for *in vitro* p34^{cdc2} kinase assay immediately after recovery (15 min group), and after culture for 30 min (45 min group), 60 min (75 min group) or 105 min (120 min group) in mKRB at 37°C in 5% CO_2 in air. Nuclear configuration of the oocytes was also examined immediately after recovery and every subsequent 30 min up to 120 min. The other oocytes were placed in Hepes-mKRB, and the cumulus cell nuclei were then injected into the oocytes up to 120 min. The injected oocytes were divided into two groups based on the time from the animal killing to NT completion (15–45 min group and 46–120 min group). The incidence of PCC was evaluated 1 h after the last injection among each group.

Experiment 2: effect of enucleation

The effect of oocyte enucleation on the p34^{cdc2} kinase activity and the PCC incidence was investigated using both rat and mouse oocytes. For the *in vitro* p34^{cdc2} kinase assay, oocytes derived from the same donor rats and mice were allocated to the non-enucleation group and the enucleation group, and the non-enucleated and enucleated oocytes were sampled 45 min after the animal killing. Enucleation was performed by aspiration of the metaphase-plate with a part of the volume (~ 10 –20%) of surrounding cytoplasm in Hepes-R1ECM containing 5 $\mu\text{g}/\text{ml}$ cytochalasin B (Sigma-Aldrich). In addition to the non-enucleation and enucleation groups, denuded rat oocytes were allocated to a cytoplasm-removal group (a part of the cytoplasm was mechanically aspirated as a sham control.). All the treatments were completed within 45 min. The oocyte samples were harvested at 45, 75, 90 and 105 min (0, 30, 45 and 60 min after enucleation respectively). For PCC evaluation, nuclear injection into non-enucleated and enucleated mouse and rat oocytes, and into cytoplasm-removed rat oocytes, was completed within 45 min after animal killing.

Experiment 3: effect of roscovitine treatment

We investigated whether the decreased level of p34^{cdc2} kinase activity in non-enucleated rat oocytes negatively affected the PCC incidence of injected cell nuclei. Roscovitine (Sigma-Aldrich) dissolved at 10 mM in DMSO and stored at -80°C , a specific inhibitor of p34^{cdc2} kinase, was added to all media throughout the process of oocyte recovery and the *in vitro* culture at the final concentration of 150 μM , as reported by Lazar *et al.* (2002). Oocytes were sampled for *in vitro* p34^{cdc2} kinase assay at 15 (immediately after recovery), 45, 75 and 120 min as in Experiment 1. Because the 45 min treatment of rat oocytes with 150 μM roscovitine decreased their p34^{cdc2} kinase

activity to an equivalent level of oocytes 120 min after *in vitro* aging or 60 min after enucleation, nuclear injection into the roscovitine-treated oocytes was performed within 45 min, and the incidence of PCC was then evaluated after a further 1 h.

Experiment 4: effect of MG132 treatment

We investigated whether the treatment of rat oocytes with a proteasome inhibitor, by which the p34^{cdc2} kinase activity would be maintained, could support the PCC incidence of the injected donor cell nuclei. MG132 (Sigma-Aldrich), an inhibitor which can block cyclin B1 degradation, was used at the final concentration of 5 μ M, as reported by Josefsberg *et al.* (2000). Non-enucleated oocytes were exposed to MG132, throughout the process of oocyte recovery to *in vitro* culture or nuclear injection. Oocyte sampling for *in vitro* p34^{cdc2} kinase assay was performed at 15 min (immediately after recovery), 45, 75 and 120 min. Nuclear injection into the MG132-treated oocytes and PCC evaluation were performed as in Experiment 3.

Experiment 5: effect of PCC on NT embryo development

In the first group, oocytes exposed to MG132 from oocyte recovery to just before enucleation were used as recipients. From the result of Experiment 4, MG132 treatment is expected to support PCC even in enucleated oocytes, because the p34^{cdc2} kinase activity was maintained at a high level during the treatment. The procedure of enucleation was as described above. The cytoplasts were then injected with cumulus cell nuclei. One hour later, a high incidence of PCC was expected. In the second group, oocytes with the extrusion of a second polar body were selected from 2 h cultures of non-treated oocytes, and then enucleated and nuclear injected as well. The reduced incidence of PCC under a lowered MPF was expected. For both groups, the oocytes 1 h after nuclear injection were activated by two direct current pulses (100 V/mm, 99 μ s) and 4 h treatment with 2 mM 6-dimethylaminopurine (6-DMAP) (Sigma-Aldrich) in mR1ECM medium. The reconstructed oocytes were cultured in 100 μ l microdrops of mR1ECM at 37°C in 5% CO₂ in air, and number of oocytes forming one or two pseudo-pronuclei and cleaving to the two-cell stage was recorded at 6 and 24 h of culture respectively.

Statistical analysis

Each experiment had at least three replicates. Data on p34^{cdc2} kinase activity were compared by ANOVA and Fisher's protected least significant difference test using the StatView program (Abacus Concepts, Inc., Berkeley, CA, USA). Data on PCC incidence and NT embryo development were compared by Fisher's exact probability test. A value of $P < 0.05$ was chosen as an indication of statistical significance.

Results

Experiment 1: effect of *in vitro* aging

The time-dependent change in p34^{cdc2} kinase activity in rat oocytes is shown in Fig. 1. The p34^{cdc2} kinase activity of rat oocytes decreased significantly at 45 min (after 30 min cultivation) and more significantly at 75 min (after 60 min cultivation). The decreased activity was maintained until 120 min (after 105 min cultivation). Probably due to the decrease of p34^{cdc2} kinase activity, the release from meiotic arrest of ovulated oocytes occurred in a time-dependent manner, as shown in Fig. 2. Within 75 min after animal killing, more than half of the oocytes progressed beyond the MII stage. At 135 min, most of the oocytes were at either the AII or the TII stage. The proportion of *in vitro*-aged rat oocytes promoting PCC of the injected cumulus cell nuclei is shown in Table 1. When nuclear injection was completed within 45 min from animal killing, a positive sign of PCC was observed in 32.5% of the NT oocytes. On the other hand, PCC occurred in only 2.6% of the oocytes into which cumulus cell nuclei were injected between 46 and 120 min.

Experiment 2: effect of oocyte enucleation

The activities of p34^{cdc2} kinase in non-enucleated and enucleated oocytes within 45 min were compared in rats and mice, as shown in Fig. 3. In both species, there was a slight decrease in the p34^{cdc2} kinase activity after enucleation in both rat and mouse oocytes. A considerable

Figure 1 Kinetics of p34^{cdc2} kinase activity in rat oocytes during *in vitro* culture. ¹Data are expressed as relative percentage of the level of p34^{cdc2} kinase activity in rat oocytes immediately after recovery (15 min after the animal killing; defined as 100%). ^{a-c}Different superscripts on S.E.M. bars denote significant differences among the culture periods ($P < 0.05$).

Figure 2 Time-dependent changes of nuclear configuration during *in vitro* culture of ovulated rat oocytes. MII, metaphase-II; All, anaphase-II; TII, telophase-II. ¹Data are expressed as relative percentage of the level of p34^{cdc2} kinase activity in non-enucleated rat oocytes harvested at 45 min from the animal killing (defined as 100%).

difference was found in the p34^{cdc2} kinase activity between rat and mouse oocytes, because the mouse oocytes carried a p34^{cdc2} kinase activity 2.5 times that of rat oocytes either immediately or up to 60 min after enucleation or cytoplasm removal and compared with non-treated, age-matched controls, as shown in Fig. 4. The activities of p34^{cdc2} kinase were measured in rat oocytes immediately after or 60 min after one of three treatments (enucleated, cytoplasm-removed and non-treated), as shown in Fig. 4. Surprisingly, a higher p34^{cdc2} kinase activity was detected in the oocytes immediately after enucleation, although the p34^{cdc2} kinase activity of oocytes immediately after cytoplasm removal was not different from that of non-treated oocytes. The p34^{cdc2} kinase activity in enucleated oocytes dramatically decreased within 30 min after enucleation. At 45 min after enucleation, the activity was further decreased and thereafter maintained until 60 min. However, the activity in non-treated or cytoplasm-removed oocytes did not significantly differ by 60 min. The proportion of rat and mouse oocytes promoting PCC in relation to mechanical

Table 1 PCC of rat cumulus cell nuclei injected into rat oocytes kept *in vitro* for different periods.

Elapsed time until NT completion ^a	Injected ^b	PCC-positive (%)
15–45 min	40	13 (32.5) ^c
46–120 min	39	1 (2.6) ^d

^aTime from donor animal killing to NT completion.

^bNumber of oocytes injected, survived and evaluated.

^{c–d}Different superscripts denote significant difference ($P < 0.05$).

Figure 3 Changes in p34^{cdc2} kinase activity by enucleation in rat and mouse oocytes. ¹Data are expressed as relative percentage of the level of p34^{cdc2} kinase activity in non-enucleated rat oocytes harvested at 45 min from the animal killing (defined as 100%). *denotes significant difference from the corresponding value in the rat ($P < 0.05$).

treatment (enucleation or cytoplasm removal) is shown in Table 2. In rats, despite little reduction of p34^{cdc2} kinase activity, few of the oocytes that either had a small portion of cytoplasm-removed (17.1%) or the non-treated controls (28.2%) could support PCC at 105 min after animal killing. However, PCC was not observed at all in the

Figure 4 Effect of enucleation and cytoplasm removal on the kinetics of p34^{cdc2} kinase activity in rat oocytes. ¹Data are expressed as relative percentage of the level of p34^{cdc2} kinase activity in non-enucleated rat oocytes harvested at 45 min from the animal killing (defined as 100%). a, b; c, d; e, f; g, h Different superscripts on S.E.M. bars denote significant differences between the experimental groups in each culture period ($P < 0.05$). *An asterisk shows a significant difference compared with the activity in enucleated oocytes at 0 min ($P < 0.05$). NE, not examined.

Table 2 PCC of rat and mouse cumulus cell nuclei injected into rat and mouse oocytes.

Treatments	Rat		Mouse	
	Injected ^a	PCC-positive (%)	Injected ^a	PCC-positive (%)
Non-treated	46	13 (28.2) ^b	30	29 (96.7)
Cytoplasm-removed	41	7 (17.1) ^b	NE ^d	—
Enucleated	49	0 (0) ^c	29	27 (93.1)

^a Number of oocytes injected, survived and evaluated.

^{b–c} Different superscripts denote significant difference ($P < 0.05$).

^d Not examined.

enucleated oocytes where p34^{cdc2} kinase activity was significantly decreased at 60 min after completion of enucleation. In contrast, PCC of mouse cumulus cell nuclei occurred in most of the mouse oocytes before enucleation (96.7%) and even after enucleation (93.1%).

Experiment 3: effect of roscovitine treatment

The time-dependent decrease of p34^{cdc2} kinase activity in roscovitine-treated rat oocytes is shown in Fig. 5. The activity in roscovitine-treated oocytes was linearly decreased until 75 min and maintained until 120 min.

Figure 5 Effect of roscovitine treatment on the kinetics of p34^{cdc2} kinase activity in rat oocytes. ¹Data are expressed as relative percentage of the level of p34^{cdc2} kinase activity in non-treated rat oocytes immediately after recovery (15 min after the animal killing; defined as 100%). ^{a–c}; ^{d–f} Different superscripts on S.E.M. bars denote significant differences among different culture periods within each treatment group ($P < 0.05$). *Asterisk denotes a significant difference between the treatment groups ($P < 0.05$).

At 45, 75 and 120 min, each activity in the roscovitine-treated oocytes was significantly lower than that of non-treated control oocytes. The result of PCC incidence in the oocytes treated with or without roscovitine for 45 min is shown in Table 3. None of the roscovitine-treated oocytes promoted PCC, although 41.7% of non-treated oocytes promoted it.

Experiment 4: effect of MG132 treatment

The activities of p34^{cdc2} kinase in MG132-treated rat oocytes are shown in Fig. 6. The level of p34^{cdc2} kinase activity still decreased in the MG132-treated oocytes after 30 min of culture, but were significantly greater than in control oocytes. The p34^{cdc2} kinase activity was maintained during the further incubation up to 105 min. The kinase activities of MG132-treated oocytes at 60 and 105 min cultivation were significantly greater than those of control oocytes. As shown in Table 4, the proportion of MG132-treated oocytes promoting PCC (66.0%) was higher than that of the non-treated oocytes (46.5%) when the nuclear injection was completed within 45 min.

Experiment 5: effect of PCC on NT embryo development

After oocyte activation with the electrical stimulus plus 6-DMAP, the reconstructed embryos from both groups (MG132-treated MII oocytes and non-treated TII oocytes at the enucleation) formed one or two pseudo-pronuclei (91.7 and 87.5% in total respectively). After activation, a greater proportion of oocytes enucleated at the MII stage tended to form two pseudo-pronuclei compared with those enucleated at the TII stage. A significantly higher proportion of the NT embryos in the MG132-treated group vs the non-treated group (59.1 vs 7.1%) developed to the two-cell stage.

Discussion

Rat oocytes have poor potential for promoting PCC of injected somatic cell nuclei, and the rate was markedly decreased in aged or enucleated oocytes (Hirabayashi *et al.* 2003a,b). In the present study, rat oocytes into which cumulus cell nuclei were injected within 45 min

Table 3 PCC of rat cumulus cell nuclei injected into roscovitine-treated rat oocytes.

Roscovitine treatment ^a	Injected ^b	PCC-positive (%)
–	36	15 (41.7) ^c
+	35	0 (0) ^d

^a Oocytes were treated with 150 μ M roscovitine for 45 min, and nuclear injection was completed during the treatment.

^b Number of oocytes injected, survived and evaluated.

^{c–d} Different superscripts denote significant difference ($P < 0.05$).

Figure 6 Effect of MG132 treatment on the kinetics of p34^{cdc2} kinase activity in rat oocytes. ¹Data are expressed as relative percentage of the level of p34^{cdc2} kinase activity in non-treated rat oocytes immediately after recovery (15 min after the animal killing; defined as 100%). a, b; c–e Different superscripts on S.E.M. bars denote significant differences among culture periods within each treatment group ($P < 0.05$). *Asterisk denotes a significant difference between the treatment groups ($P < 0.05$).

promoted PCC (frequency 28–47% in Tables 1–4), but the incidence of PCC decreased with oocyte aging with a significant decrease in p34^{cdc2} kinase activity (Table 1, Fig. 1). In mouse oocytes, PCC was initiated at least 30 min after nuclear injection and lasted until 2 h (Chen *et al.* 2004), therefore the timing of PCC observation in the present study (60 min after nuclear injection) might be optimal. When oocytes are treated with roscovitine, which decreases the level of p34^{cdc2} kinase activity, PCC formation was never observed even though nuclear injection is completed within 45 min. Roscovitine is widely used as cyclin-dependent kinase inhibitor to suppress p34^{cdc2} kinase in rodent oocytes (Phillips *et al.* 2002). Therefore, the rapid decrease of p34^{cdc2} kinase activity in the roscovitine-treated oocytes found between 45 and 75 min (Fig. 5), when compared with the slow decrease in

the non-treated oocytes during the same period, may influence the outcome of PCC. The high concentration of roscovitine affects the other cyclin-dependent protein kinases such as cdk2 (De Azevedo *et al.* 1997). The unknown pathway and/or chemical toxicity of roscovitine cannot be ruled out for complete suppression of PCC induction. Moreover, the level of activity in rat oocytes was less than half compared with that in mouse oocytes, which had a high potential for promoting PCC. Judging from these results, we estimated that the high activity of p34^{cdc2} kinase is associated with promoting PCC of injected somatic cell nuclei, and the low level of p34^{cdc2} kinase activity is one of the reasons for the poor potential to promote PCC in rat oocytes compared with that of mouse oocytes. The reason why the potential level of p34^{cdc2} kinase activity is different between mouse and rat oocytes remains unclear. Our previous study indicated, at least, that the PCC-inducing potential is determined by the origin of the recipient cytoplasm, not by the origin of the donor nucleus, in the interspecies NT experiment between mouse and rat (Hirabayashi *et al.* 2003a). In somatic cell division, dissolution of the nuclear envelope occurs during G2/M transition in mitosis (Nurse 1990) and is induced by phosphorylation of lamin, one of the major nuclear membrane proteins (Peter *et al.* 1990). In oocytes, p34^{cdc2} kinase had the potential to phosphorylate lamins, inducing meiotic breakdown of the nuclear envelope and the meiotic resumption (Dessev & Goldman 1988). Therefore, it is possible that p34^{cdc2} kinase plays an important role in the destruction of the nuclear membrane of the injected somatic cell through phosphorylation of lamin.

One possible pathway known for inactivation of p34^{cdc2} kinase is the calmodulin-dependent pathway via Ca²⁺. We have reported that the Ca²⁺-dependent pathway triggers the degradation of cyclin B and results in inactivation of p34^{cdc2} kinase in porcine oocytes (Ito *et al.* 2003, 2004a). In *Xenopus* oocytes (Lorca *et al.* 1994, Morin *et al.* 1994), activation of calmodulin-dependent protein kinase II induces the degradation of cyclin B via the ubiquitin proteasome pathway. Based on these literature sources and preliminary data, we have investigated the effect of a proteasome inhibitor MG132 on p34^{cdc2} kinase activity and PCC incidence. The PCC incidence in rat oocytes was significantly improved by MG132 treatment (Table 4). This result matched well with the report by Josefsberg *et al.* (2000), who demonstrated that MG132-treated rat oocytes contained a high amount of cyclin B1. In the present study, MG132 also provided a suppressive effect on inactivation of p34^{cdc2} kinase (Fig. 6). Use of ALLN, a neutral cysteine protease inhibitor, was also slightly effective for improving the PCC incidence in rat oocytes (Hirabayashi *et al.* 2003b). Thus, the Ca²⁺-dependent degradation pathway, which may induce a decrease in the cyclin B1 level, is activated in aged rat oocytes, which results in a low level of p34^{cdc2} kinase activity, and a low potential to promote PCC. Our unpublished data (M Hirabayashi and J Ito, unpublished data) showing that the decreased

Table 4 PCC of rat cumulus cell nuclei injected into MG 132-treated rat oocytes.

MG 132 treatment ^a	Injected ^b	PCC-positive (%)
–	43	20 (46.5) ^c
+	47	31 (66.0) ^d

^aOocytes were treated with 5 μ M MG 132 for 45 min, and nuclear injection was completed during the treatment.

^bNumber of oocytes injected, survived and evaluated.

^{c–d}Different superscripts denote significant difference ($P < 0.05$).

Table 5 The effect of PCC induction on the pseudo-pronuclei (PN) formation and the first cleavage of reconstructed rat embryos.

Recipient oocytes	Nuclear injected	PN		Total	Two-cell
		1PN ^c	2PN ^c		
MG-treated MII ^a	24	8 (36.4%)	14 (63.6%)	22 (91.7%)	13 (59.1%) ^d
Non-treated TII ^b	16	11 (78.6%)	3 (21.4%)	14 (87.5%)	1 (7.1%) ^e

^a Oocytes were treated with 5 µM MG132 until just before enucleation after oocyte recovery.

^b Oocytes were cultured for about 2 h and only oocytes with a completely extruded polar body (the TII stage) were used.

^c Percentage of reconstructed embryos with 1 or 2 pronuclei.

^{d–e} Different superscripts denote significant difference ($P < 0.05$).

concentration of extracellular free calcium in the handling medium partially inhibits spontaneous activation of rat oocytes, support this hypothesis.

The other pathway for inactivation by phosphorylation of ¹⁴Thr and ¹⁵Tyr of p34^{cdc2} kinase has been reported in aged porcine oocytes (Kikuchi *et al.* 2000). In the same study, it has been reported that caffeine suppressed the shift from MPF to pre-MPF (¹⁴Thr/¹⁵Tyr-phosphorylated p34^{cdc2} kinase + cyclin B1) in the aged porcine oocytes. Okadaic acid is also known as a protein phosphatase inhibitor which prevents phosphorylation of ¹⁴Thr and ¹⁵Tyr of p34^{cdc2} kinase (Rime *et al.* 1995). In our preliminary study, PCC incidence of injected cumulus cell nuclei was slightly improved in rat oocytes treated with okadaic acid (M Hirabayashi and J Ito, unpublished data). Therefore, the phosphorylation of p34^{cdc2} kinase as well as the cyclin degradation may be involved in spontaneous activation of rat oocytes during *in vitro* culture.

The level of p34^{cdc2} kinase activity in non-treated rat oocytes was similar to that of cytoplasm-removed or enucleated oocytes in the case where the oocytes were sampled immediately after the treatment (Fig. 4). The mechanical puncture of the oocyte membrane and the aspiration of the cytoplasm or karyoplast did not influence the p34^{cdc2} kinase activity immediately after the enucleation procedure beyond the level expected from the relative volume of aspirated cytoplasm (~10–20%). These results strongly suggest that active p34^{cdc2} kinase is not accumulated near the metaphase spindles in rat oocytes. There was significant difference in the p34^{cdc2} kinase activity between 45 and 75 min in both non-treated and enucleated groups (Fig. 4). A dramatic decrease in the activity of p34^{cdc2} kinase was observed in the rat oocytes enucleated and cultured for 30 min compared with the non-treated group (Fig. 4), suggesting that the oocyte metaphases per se are involved in the maintenance of p34^{cdc2} kinase activity. We have previously reported in porcine oocytes that activation of p34^{cdc2} kinase was dependent on the synthesis of cyclin B and that the synthesis was induced by the activation of mitogen-activated protein (MAP) kinase (Ito *et al.* 2004b). The MAP kinase suppressed Myt1 activity that phosphorylated p34^{cdc2} kinase at ¹⁴Thr and ¹⁵Tyr (Mueller *et al.* 1995, Palmer *et al.* 1998). Because activated MAP kinase is localized on the meiotic spindle in porcine oocytes (Lee *et al.* 2000), it is

possible that enucleation results in the loss of MAP kinase from the recipient oocytes.

Nuclear injection into the MG132-treated, MII-enucleated rat oocytes resulted in a higher development to the two-cell stage when compared with non-treated, TII-enucleated rat oocytes after 24 h cultivation (Table 5). The favorable circumstance of recipient cytoplasm with a high MPF activity must induce PCC and promote subsequent development of NT embryos. Wakayama & Yanagimachi (2001) reported in mice that the injected cumulus cell nuclei undergoing PCC transformation to the two pseudo-pronuclei and such NT embryos are able to develop to full-term. In contrast, a different situation has been reported in the cloning of large domestic animals. The somatic cell NT in the livestock species is successfully reproducible when enucleated and activated oocytes with a low MPF activity are used as recipient cytoplasm (Baguisi *et al.* 1999, Kurosaka *et al.* 2002, Shin *et al.* 2002). Such a difference between rodents and large domestic animals remains unclear, but our present data suggest that induction of PCC in reconstructed embryos is related to the high activity of p34^{cdc2} kinase, which seems to play a key role in the successful rat cloning.

In conclusion, the decreased level of p34^{cdc2} kinase activity in aged or enucleated rat oocytes is responsible for their inability to promote PCC of microinjected donor cell nuclei. Inhibition of p34^{cdc2} kinase inactivation by chemicals such as MG132 is in part effective for rat oocytes to promote PCC and cleavage. These data may expand our existing knowledge on characteristics of rat oocytes and contribute to the development of a rat NT protocol using G0/G1-stage donor cells.

Acknowledgements

The authors thank Mrs A Ishikawa and Miss A Takata for their technical assistance. This work was supported in part by Grant-in-Aids for the Scientific Research (No. 15082211, No. 16300139; to M H and S H), and the 21st Century COE Program (to S H) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Research Fellowship for Young Scientists (No. 8254; to J I.) from the Japan Society for the Promotion of Science.

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Received 20 July 2004

First decision 16 August 2004

Revised manuscript received 8 September 2004

Accepted 12 October 2004