

Progression of Nuclear Maturation and $p34^{cdc2}$ Kinase Activity in Porcine Oocytes during In Vitro Culture in Different Media

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Abstract: In the present study, to elucidate the cause of different developmental potential in porcine oocytes matured in two media, mTCM199 and mNCSU37, we examined the time course of changes in the nuclear states and $p34^{cdc2}$ kinase activity of oocytes during meiotic maturation. The $p34^{cdc2}$ kinase activity of oocytes cultured in mTCM199 gradually increased from earlier in the culture time periods than that of oocytes in mNCSU37, and that of oocytes in mNCSU37 rapidly increased from the middle of the culture period, but the oocytes in both media had similar levels of $p34^{cdc2}$ kinase activity at the end of the culture period. Oocytes cultured in mTCM199 underwent GVBD and reached the MII stage earlier in the culture period than those in mNCSU37. Moreover, MII oocytes cultured in either mTCM199 or mNCSU37 had a similar level of $p34^{cdc2}$ kinase activity at the end of the culture period. It was therefore concluded that the difference in developmental competence of oocytes matured in different media seems to be independent of $p34^{cdc2}$ kinase activity.

Key words: Nuclear maturation, $p34^{cdc2}$ kinase, Porcine oocytes, GVBD, Culture medium

In most mammalian species, the oocytes enter the early stages of meiosis during fetal life and become arrested at the prophase stage of the first meiotic division until they are committed to ovulation. After triggering the resumption of meiotic maturation in response to the preovulatory LH surge, the oocytes complete germinal vesicle breakdown (GVBD), and then progress to metaphase II (MII) stage before ovulation.

During the meiotic progression of oocytes, $p34^{cdc2}$ kinase activation has been shown to be essential in mice [1], cattle [2, 3], and pigs [4]. High activity of

$p34^{cdc2}$ kinase before activation has also been found to be required for disintegration of the sperm nuclear membrane after its penetration into the oocyte [5]. This disintegration of the nuclear membrane has also been shown to be a key step in male pronucleus formation [5]. Conversely, Borsuk *et al.* [6] showed that low MPF activity in oocytes at the MII stage was responsible for formation of an abnormal male pronucleus. In the pig, Naito *et al.* [7] compared with H1 kinase activity (regarded as $p34^{cdc2}$ kinase) in two types of oocytes matured in either porcine follicular fluid (pFF) or Krebs-Ringer bicarbonate solution (KRB). Their results revealed that low activity of MPF/H1 kinase in MII oocytes cultured in KRB was insufficient to disintegrate the nuclear membrane of boar spermatozoa.

On the other hand, TCM199 is commonly used as a culture medium to mature porcine oocytes, and it was found that in oocytes cultured in TCM199, there was a relatively high maturation rate (about 85–90%) [8–10]. But Funahashi *et al.* [11, 12] compared the developmental competency of porcine oocytes cultured in TCM199 and modified North Carolina State University 37 (mNCSU37) supplemented with hormones and found that although both lots of oocytes had a high maturation rate (about 88%), the oocytes matured in TCM199 had a significantly lower cleavage rate after insemination (36%) than that of oocytes matured in NCSU37 (about 65%). It is therefore postulated that there are differences between oocytes cultured in NCSU37 and TCM199 in cytoplasmic maturation. The cause of the different developmental potential of oocytes cultured in various media remains to be clarified.

In the present study, to elucidate the cause of different developmental potential in porcine oocytes matured in two media, TCM199 and NCSU37, we examined the time course of changes in the nuclear states and $p34^{cdc2}$ kinase activity of oocytes during meiotic maturation.

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Materials and Methods

Isolation and culture of porcine cumulus oocytes complexes (COCs)

Porcine ovaries were collected from 5- to 7-month-old prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.85% (w/v) NaCl containing 0.1 mg/ml kanamycin (Meiji Seika, Tokyo, Japan) at about 30°C within 1.5 h. The surfaces of intact healthy antral follicles measuring from 3 to 8 mm in diameter were cut with a razor blade and the oocytes were collected by scraping the inner surface of the follicle walls with a surgical blade. The collected oocytes were placed in prewarmed phosphate-buffered saline (pH 7.4) supplemented with 0.1% (v/v) polyvinyl-pyrrolidone (PVP) (Sigma Chemical Co., St. Louis, USA). Oocytes having evenly granulated cytoplasm with at least 4 layers of unexpanded cumulus oophorus cells were selected under a stereomicroscope and then washed 3 times with maturation medium. The oocytes were cultured several times in 100 μ l drops of maturation medium (about 20 oocytes/drop) covered with mineral oil (Sigma) at 39°C in a humidified atmosphere of 5% CO₂ in air. Both modified NCSU37 [13] containing 10% (v/v) FCS (Gibco BRL, Grand Island, NY, USA), 0.6 μ g/ml pFSH (Sigma), 1.3 μ g/ml eLH (Sigma), 7 mM Taurine (Sigma), 2% (v/v) essential and 1% (v/v) non-essential amino acids (Gibco), and modified TCM199 containing 10% (v/v) FCS, 0.6 μ g/ml pFSH (Sigma), 1.3 μ g/ml eLH (Sigma) were used.

Assessment of nuclear maturation

At the end of the culture, the oocytes were mounted on slides, compressed with coverslips and fixed with ethanol: acetic acid (3:1) for more than 48 hr at room temperature. The oocytes were stained with 1% lacmoid in 45% acetic acid and sealed with acetoglycerol. These samples were examined by phase-contrast microscopy ($\times 600$).

In vitro p34^{cdc2} kinase assay

p34^{cdc2} kinase assay was performed with a MESACUP cdc2 kinase assay kit (MBL, Nagoya, Japan, code#5234), according to the method described by Shoujo *et al.* [14]. They showed that the correlation coefficients for p34^{cdc2} kinase activity examined with the MESACUP cdc2 kinase assay kit and histone H1 kinase activity measured by a radioactive method were as high as 0.9961.

The oocytes were lysed according to the method of Shimada and Terada [15]. Briefly 10 oocytes were

washed several times in PBS and then put into plastic tubes containing 5 μ l of cell lysis buffer [20 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml Leupeptin, and 1 mM PMSF (Sigma)]. All drugs except PMSF were purchased from New England Biolabs (Tozer Road Beverly, MA, USA). After suspension of the oocytes, the samples were frozen in liquid nitrogen and then the frozen samples were sonicated in an ultrasonic disruptor (UD-200, TOMY, Tokyo, Japan) fitted with CUP HORN (CH-0633, TOMY) 3 times for 25 sec each at 1°C. Cell extracts were frozen and stored at -80°C just before use.

Five μ l of the oocyte extract (containing 10 oocytes) was mixed with 45 μ l kinase assay buffer composed of 25 mM Hepes buffer (pH7.5) (MBL), 10 mM MgCl₂ (MBL), 10% (v/v) MV peptide solution (SLYSSPGGAYC) (MBL) and 0.1 mM ATP (Sigma), and the mixture was incubated for 30 min at 30°C. The reaction was terminated with 200 μ l PBS containing 50 mM EGTA (MBL). The phosphorylation of MV peptides was detected by ELISA analysis [MESACUP cdc2 kinase assay kit (MBL, code#5234)]. Data were expressed as the fold strength of p34^{cdc2} kinase activity in oocytes immediately after collection from their follicles. Each independent experiment was repeated three times.

Experimental design

In experiment 1, we investigated the time course of the changes in p34^{cdc2} kinase activity and nuclear progression in porcine oocytes during meiotic maturation in mTCM199 or mNCSU37. After COCs were cultured for various periods in mTCM199 or mNCSU37, the oocytes were treated with 150 U/ml hyaluronidase (Sigma, St. Louis, MO; Type IV-S) in PBS for a few minutes. Then the cumulus cells were removed by vortexing. The denuded oocytes were used immediately for p34^{cdc2} kinase assay.

In experiment 2, we investigated whether there is a difference between the MII oocytes matured in mTCM199 and mNCSU37 in p34^{cdc2} kinase activity. After COCs were cultured in mTCM199 or mNCSU37 for various periods from 36 to 52 hr, the oocytes were denuded by the abovementioned methods. The denuded oocytes were centrifuged at 15,000 $\times g$ for 5 min, in order to localize the lipid granules, and then stained with 50 μ g/ml Hoechst-33342 (H-33342; Calbiochem Co., FRG) in PBS for 15 min. The stained oocytes were examined under a fluorescence microscope (Leitz FROVERTFU Leica) and only MII oocytes were collected. The oo-

cytes were used immediately for p34^{cdc2} kinase assay.

Statistical analysis

Statistical analyses of data from three or four trials for treatment comparisons were carried out by analysis of variance (ANOVA) after the percentage data were subjected to arc-sine transformation, and Fisher's protected least significant difference test was done with the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program. Data were expressed as the mean \pm SEM. A probability of $p < 0.05$ was considered to be statistically significant.

Results

In experiment 1: the time course of changes in p34^{cdc2} kinase activity and nuclear status in porcine oocytes during meiotic maturation in mTCM199 or mNCSU37

The time courses of changes in p34^{cdc2} kinase activity in oocytes cultured in either mTCM199 or mNCSU37 are shown in Fig. 1. Cultivation in both mTCM199 and mNCSU37 displayed a low level of p34^{cdc2} kinase activity at the beginning of culture. The low level of p34^{cdc2} kinase activity in oocytes cultured in mNCSU37 was maintained until 20 hr of cultivation. Further cultivation significantly increased p34^{cdc2} kinase activity and then the activity had decreased temporarily at 32 hr, followed by reaching the maximal level at 36-hr cultivation. Thereafter, this kinase activity tended to decrease until 48 hr of cultivation. On the other hand, the p34^{cdc2} kinase activity in oocytes cultured in mTCM199 increased gradually from 4 hr to 28 hr of cultivation. A slight decrease in p34^{cdc2} kinase was observed at 32 hr of cultivation and the maximum level of the activity was noted at 44 hr of cultivation. Furthermore, significantly higher activity of p34^{cdc2} kinase was recognized in the oocytes cultured in mTCM199 at 12, 20, and 24 hr of cultivation compared with oocytes in the other medium.

As shown in Fig. 2, a small proportion of oocytes cultured in mTCM199 underwent GVBD at 16 hr of cultivation but this did not occur in the oocytes in mNCSU37. In oocytes cultured in mNCSU37, GVBD was realized at 20 hr of cultivation, although the proportion of GVBD oocytes in mNCSU37 differed significantly from that of oocytes in mTCM199. Both proportions of GVBD oocytes cultured in TCM199 for 24 and 28 hr were significantly higher than in mNCSU37, whereas 32-hr cultivation of COCs produced a similar proportion of GVBD oocytes in each medium.

As in the proportion of GVBD oocytes, a considerable proportion (20%) of oocytes cultured in mTCM199

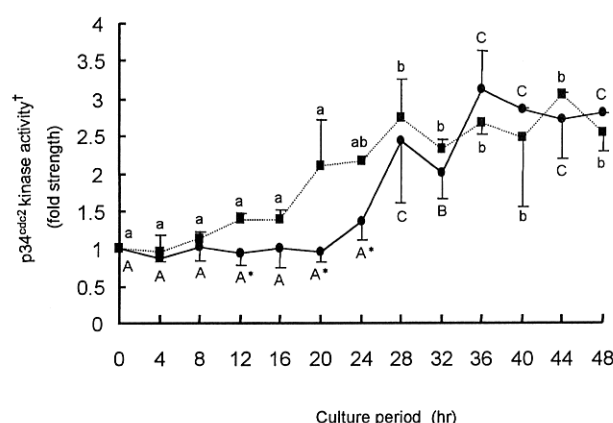


Fig. 1. Time course of p34^{cdc2} kinase activity in porcine COC matured in NCSU37 or TCM199. A-C, a-c Columns with different superscripts are significantly different ($p < 0.05$). *Significant difference from oocytes matured in TCM199. †Data are expressed as fold strength of control (oocytes just after collection from their follicles)■.... TCM199, —●— NCSU37.

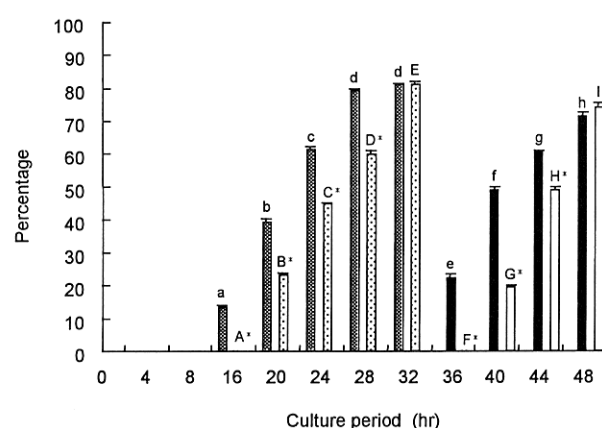


Fig. 2. Percentage of oocytes exhibited GVBD and oocytes reached MII stage when COCs were cultured in TCM199 or NCSU37. a-d, e-h, A-E, F-I Columns with different superscripts are significantly different ($p < 0.05$). *Significant difference from oocytes matured in TCM199. ▨GVBD oocytes in TCM199, ■MI oocytes in TCM199, ▤GVBD oocytes in NCSU37, □MI oocytes in NCSU37.

reached the MII stage at 36-hr cultivation, and a further 4-hr cultivation was required for a similar proportion of MII oocytes in mNCSU37. Even at 44-hr cultivation, the proportion of MII oocytes in mTCM199 was significantly higher than that in mNCSU37, but the proportion in mNCSU37 had increased rapidly at 48-hr cultivation and the difference between the proportions of oocytes in

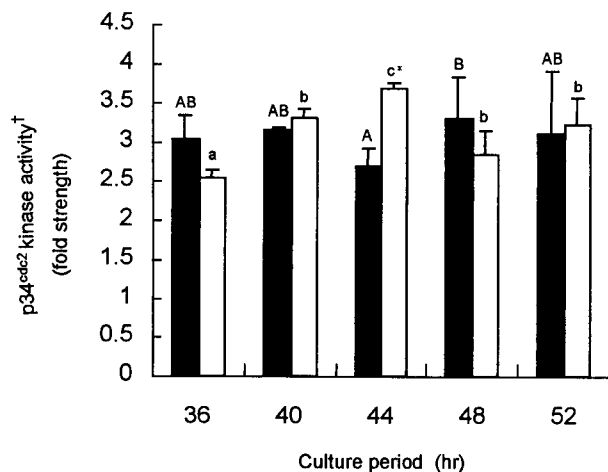


Fig. 3. Time course of p34^{cdc2} kinase activity in porcine MII oocytes cultured in mNCSU37 or mTCM199. *Data are expressed as fold strength of control (oocytes just after collection from their follicles). A-B, a-c Columns with no common superscripts are significantly within the same groups ($p < 0.05$). *Significant difference from oocytes matured in mTCM199. □ mTCM199, ■ mNCSU37.

both media was not significant.

In experiment 2: the difference between the MII oocytes matured in mTCM199 or mNCSU37 in the level of p34^{cdc2} kinase activity

In the experiment 2, to collect only oocytes arrested at the MII stage, we stained the oocytes cultured in mTCM199 and mNCSU37 for various culture periods, and the p34^{cdc2} kinase activities of these MII oocytes were determined. As shown in Fig. 3, the p34^{cdc2} kinase activity of MII oocytes cultured in mTCM199 for 48 hr was significantly lower than that for 44-hr cultivation, but was higher than the activity for 36-hr cultivation. The activity of oocytes cultured in mTCM199 for 48 hr was not significantly different from that at 40 or 52 hr. On the other hand, the activity of MII oocytes cultured in mNCSU37 for 48 hr was significantly higher than that at 44 hr, but not significantly different from these activities at any other times. Whereas at 44 hr, the activity of oocytes cultured in mTCM199 was significantly higher than that in mNCSU37, there were not significant differences between oocytes cultured in mTCM199 and mNCSU37 at any other times.

Discussion

The major findings of this study was that p34^{cdc2} kinase in oocytes cultured in mTCM199 were gradually

activated from 4 hr of cultivation, but in oocytes cultured in mNCSU37, the activity of p34^{cdc2} kinase dramatically increased from 20 hr of cultivation. This finding combined with the requirement of active p34^{cdc2} kinase for GVBD in porcine oocytes [4], may provide an explanation for the evidence that the proportion of GVBD oocytes cultured in mTCM199 increased earlier than that of oocytes in mNCSU37. Although from 28 to 48 hr of cultivation there were no differences between oocytes in mTCM199 and mNCSU37 in p34^{cdc2} kinase activities an earlier increase in the proportion of MII oocytes in mTCM199 was observed. The reason for this is not clear.

The fact that when p34^{cdc2} kinase activities were determined without the selection of MII oocytes, no significant difference between the oocytes cultured in mTCM199 and mNCSU37 was found at any time in this culture period (36–48 hr), in which many oocytes reached the metaphase II stage (Fig. 2), seems to be the result of including oocytes at various nuclear stages. We could therefore not exclude the possibility that the data would not reflect the exact dynamics of p34^{cdc2} kinase activity of the oocytes cultured in both media.

After only the MII stage oocytes were collected from those cultured in the two media for various times (36–52 hr) and assessed their p34^{cdc2} kinase activities. The results revealed that there was no statistically significant difference in p34^{cdc2} kinase activities between MII oocytes cultured in mTCM199 and mNCSU37 at any time except for 44 hr of cultivation. This indicates that cultivation in either mTCM199 or mNCSU37 produced oocytes with similar levels of p34^{cdc2} kinase activity. This further suggests that the difference in developmental competence of matured oocytes, which was reported by Funahashi *et al.* [12, 13], is not due to differences in p34^{cdc2} kinase activity of oocytes matured in different media.

In summary, the following conclusions can be drawn from the present study: 1) the p34^{cdc2} kinase activity was gradually increased during culture earlier in oocytes cultured in mTCM199 than in oocytes in mNCSU37, but the activity of oocytes in mNCSU37 rapidly increases from the intermediate culture period. The oocytes cultured in both media, however, exhibit similar levels of p34^{cdc2} kinase at the end of the culture period. 2) Oocytes cultured in mTCM199 undergo GVBD and reached the MII stage earlier in culture period than those in mNCSU37. 3) MII oocytes cultured in either mTCM199 or mNCSU37 had similar levels of p34^{cdc2} kinase activity at the end of the culture period. 4) The difference in developmental competence of oocytes ma-

tured in different media seems not to be independent of p34^{cdc2} kinase activity.

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