The Drop in the cAMP Level Due to the Closure of Gap Junctional Communication Between Cumulus Cells and Oocytes is Essential for Meiotic Progression Beyond the MI Stage in Porcine Oocytes

Masayuki Shimada and Takato Terada*

Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8528, Japan

Abstract: *Mammalian oocytes are surrounded by numerous layers of cumulus cells and the loss of gap junctional communication between cumulus cells and oocytes induces meiotic progression to the MII stage in oocytes. The closure of gap junctional communication was associated with the phosphorylation of connexin-43, gap junctional protein, in cumulus cells. In this study we investigated the effects of the phosphorylation of connexin-43 in cumulus cells on the cAMP level, MAP kinase activity and meiotic progression beyond the MI stage in the oocytes. The connexin-43 in cumulus cells was phosphorylated after 32-hr cultivation of COCs and up to 48 hr, whereas most of the connexin-43 was unphosphorylated in cumulus cells from COCs cultured for 24 hr. When the phosphorylation of connexin-43 in cumulus cells was suppressed by the addition of either PI 3-kinase inhibitor or PKC inhibitor, a significantly higher level of cAMP in the oocyte and a significantly lower proportion of oocytes at the MII stage were produced, as compared to those of oocytes cultured for 48 hr without these drugs. The activity of MAP kinase was also significantly inhibited by the addition of both drugs. It was therefore concluded that the closing of the gap junctional communication via the phosphorylation of connexin-43 might induce a decrease in the cAMP level, resulting in activation of MAP kinase and meiotic progression beyond the MI stage to the MII stage in porcine oocytes.*

Key words: *Connexin-43, cAMP, MAP kinase, Gap junction, Porcine oocytes*

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*To whom correspondence should be addressed.
e-mail: mashimad@hiroshima-u.ac.jp

Oocyte maturation depends on the activation of maturation promoting factor (MPF), which is composed of $p34^{cdc2}$ kinase and cyclin B [1, 2]. Activated MPF induces germinal vesicle breakdown (GVBD) in amphibian oocytes [3, 4], mouse oocytes [5], porcine oocytes [6], and bovine oocytes [7]. After GVBD in porcine oocytes, a further increase in MPF activity is required for meiotic progression beyond the MI stage; MPF is then transiently inactivated and reactivated again to induce the MII stage [8]. Consistent with the increase in MPF activity after GVBD, mitogen-activated protein kinase (MAP kinase) in porcine oocytes is also activated [9]. In our previous study [10], when porcine oocytes were cultured in the presence of U0126, MAP kinase kinase inhibitor, MAP kinase activity in oocytes was maintained at a similar low level to that of oocytes arrested at the GV stage, and consequently neither a further increase in MPF activity nor meiotic progression beyond the MI stage could not be observed in this condition. Therefore, in porcine oocytes MAP kinase activation seems to be required for meiotic progression beyond the MI stage.

The activation of MAP kinase is reduced by cyclic AMP (cAMP) in Xenopus oocytes [11] and rat oocytes [12]. cAMP was synthesized in cumulus cells stimulated by LH or FSH and was transported into oocytes via numerous gap junctions in mammalian oocytes [13, 14]. The gap junctions, the specialized regions in opposite membranes between neighboring cells, are channels that allow the passage of low-molecular-weight substances such as cAMP and ions in order to enhance cellular interactions [15]. These channels are formed by hexametric structures consisting of connexin molecules (connexon) in many tissues [15]. The

connexin-43 protein has multiple phosphorylated sites, and these phosphorylations play a key role in regulatory mechanisms governing the assembly of connexons into gap junctions in the plasma membrane, and gating the formed gap junction [16, 17]. In porcine cumulus cells surrounding oocytes, the connexin-43 was phosphorylated by the addition of FSH and LH to the maturation medium during meiotic progression to the MII stage [18, 19]. When the phosphorylation of connexin-43 in cumulus cells was suppressed by the addition of either PKC or PI 3-kinase inhibitor, the majority of oocytes were arrested at the MI stage [10, 19, 20]. These observations suggest that the closure of gap junctional communication between cumulus cells and oocytes induces a decrease in the cAMP level in oocytes, leading to meiotic progression beyond the MI stage in porcine oocytes, but the mechanisms by which the cAMP level in oocytes is decreased during meiotic progression beyond the MI stage remain unclear.

In the present study we investigated the time dependent changes in phosphorylated connexin-43 in cumulus cells. Furthermore, when the phosphorylation of connexin-43 was suppressed by PKC or PI 3-kinase inhibitor, the level of cAMP, nuclear status and MAP kinase activity in oocytes were also examined.

Materials and Methods

Isolation and culture of porcine cumulus oocyte complexes (COCs)

Porcine ovaries were collected from 5- to 7-month-old prepubertal gilts at a local slaughterhouse and transported within 1.5 hr to the laboratory in 0.85% (w/v) NaCl containing 0.1 mg/ml kanamycin (Meiji Seika, Tokyo, Japan) at about 30°C. The surfaces of intact healthy antral follicles measuring from 3 to 8 mm in diameter were cut with a razor blade and oocytes were collected with a surgical blade to scrape the inner surface of follicle walls. The collected oocytes were placed in prewarmed phosphate-buffered saline (pH 7.4) supplemented with 0.1% (w/v) polyvinyl-pyrrolidone (PVP) (Sigma Chemical Co., St. Louis, MO, USA). Oocytes having evenly granulated cytoplasm with at least 4 layers of unexpanded cumulus oophorus cells were selected under a stereomicroscope and were washed 3 times with maturation medium. COCs were cultured for various time periods in 100 μl drops of the maturation medium covered with mineral oil (Sigma) at 39°C in a humidified atmosphere of 5% CO_2 in air (about 20 COCs/drop). The maturation medium was modified NCSU37 [21] containing 10% (v/v) FCS (Gibco BRL, Grand Island, NY, USA), 7 mM Taurine (Sigma), 2% (v/v) essential amino acids (Gibco), 1% (v/v) non-essential amino acids (Gibco), 0.6 μg/ml porcine FSH (Sigma) and 1.3 μg/ml equine LH (Sigma).

Assessment of nuclear maturation

After incubation, the oocytes were freed from cumulus cells, then mounted on slides, fixed with acetic acid/ethanol (1:3) for 48 hr, and stained with aceto-lacmoid before examination under a phase-contrast microscope (400 ×) for evaluation of their chromatin configuration.

Detection of connexin-43 by immunoblotting analysis

Cumulus cells were lysed according to our previous report [19]. The cumulus cell extract was diluted two-fold with 2 × Laemmli sample buffer [22]. After denaturing by boiling for 5 min, the protein samples were separated by SDS-PAGE on 12.5% polyacrylamide gel (Pharmacia Biotech, Uppsala, Sweden), then transferred onto PVDF membrane (Amersham, Arlington Heights, IL, USA) by means of the PhastTransfer system (Pharmacia Biotech). The membrane was blocked with SuperBlock blocking buffer (PIERCE, Rockford, IL, USA), then incubated with mouse monoclonal anti-connexin 43 antibody (Chemicon International, Temecula, CA, USA) at 1:2000 overnight at 4°C in 10% (v/v) SuperBlock blocking buffer in 0.1% (v/v) Tween 20-PBS (T-PBS). After three washes in T-PBS, the membranes were treated with horseradish-peroxidase-labeled anti-mouse IgG (1:7000, Amersham) in 10% (v/v) SuperBlock blocking buffer in T-PBS for 1 hr at room temperature. After three 10 min washes with T-PBS, peroxidase activity was visualized by ECL Plus Western blotting detection (Amersham), according to the manufacturer's instructions. The intensity of the bands was analyzed with a Gel-Pro Analyzer (Media Cybernetics, MD, USA). Each experiment was repeated three times.

Quantification of cAMP by HPLC-UV analysis

Oocytes were stripped of cumulus cells by repeatedly aspirating COCs through a glass pipette in the presence of 50 μM IBMX (Sigma) in the basic medium. Forty denuded oocytes were transferred to a 100 μl assay buffer, 0.01 M ammonium acetate, pH 6.7, containing 50 μM IBMX. The samples were stored at −80°C until assayed.

Oocyte extracts were separated on a reverse-phase Eicompak CA-5DS column (2.1 × 150 mm) (Eicom, Kyoto, Japan). Column temperature was kept at 25°C by a temperature controller (TSK CO-8000, TOSOH,

Tokyo, Japan). The solvent delivery system (TSK CCPD, TOSOH) contained 97.2% (v/v) 0.01M ammonium acetate (Nakarai, Oosaka, Japan) and 2.8% (v/v) acetonitrile (Nakarai), pH 6.7. The buffer was filtered through a Millicup-HV filter, 0.45 μm (Milipore, Mississauga, Canada) and degassed, then the flow rate was adjusted to 200 μl/min. Samples (100 μl) were injected onto the column with an auto sampler (AS-8020, TOSOH, Tokyo, Japan). The detection was performed at 254 nm with a UV detector (UV 8020, TOSOH) and peak heights were measured with a computer integrator (Sic chromatocorder 11, TOSOH).

A standard solution of 100 μM cAMP (Sigma) was prepared in the assay buffer and kept frozen at −80°C. Prior to analysis, the 100 μM cAMP solution was diluted in the assay buffer to various concentrations.

In vitro MAP kinase assay

A p44/42 MAP kinase assay kit (New England BioLabs, Tozer Road, Beverly, MA, USA) was used for measuring MAP kinase activity. Oocytes were lysed according to our previous report [19]. The 5 μl oocyte extract (containing 20 oocytes) was mixed with 25 μl kinase assay buffer A [25 mM Tris (pH 7.5), 5 mM β-Glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$], 0.1 mM ATP (Sigma) and 2 μg Elk 1 fusion protein. The mixture was incubated for 30 min at 30°C. All drugs except ATP were purchased from New England BioLabs. The reactions were terminated by the addition of 10 μl 4 × Laemmli sample buffer, and after boiling for 5 min the samples were subjected to 12.5% SDS-PAGE. Phosphorylation of Elk 1 fusion protein was detected by immunoblotting and chemiluminescence with anti-phospho-specific Elk 1 antibody.

Treatment of COCs with LY294002 or Calphostin C

After COCs had been cultured for 24 hr in the maturation medium, COCs were cultured further in medium supplemented with 5.0×10^{-5} M LY294002 (Sigma) and 1×10^{-6} M Calphostin C (Sigma), respectively. LY294002 and Calphostin C were dissolved in DMSO (Sigma) at 5×10^{-2} M and 1×10^{-3} M, respectively. The final concentrations of LY294002 (5×10^{-5} M) and Calphostin C (1×10^{-6} M) were obtained by dilution with the medium. Inhibitor-free medium supplemented only with 0.098% (v/v) DMSO served as a control. This concentration of DMSO did not affect porcine oocyte maturation [23].

Experimental design

In order to investigate the time dependent changes in the phosphorylation of connexin-43 in cumulus cells surrounding oocytes, the somatic cells which were removed form COCs cultured in the maturation medium for 24, 32, 40 and 48 hr were used for immunoblotting analysis of connexin-43. We next examined whether the phosphorylation of connexin-43 in cumulus cells after the first 24-hr cultivation was controlled by PKC or PI 3-kinase. COCs cultured for 24 hr in the basic maturation medium were further cultured for 24 hr with either 5.0×10^{-5} M LY294002 (PI 3-kinase inhibitor) or 1×10^{-6} M Calphostin C (PKC inhibitor). The cumulus cells were subjected to analysis of the phosphorylation state of connexin-43 by immunoblotting. The cumulus cell-free oocytes were employed for estimation of the level of cAMP, MAP kinase activity and the proportion of oocytes which reached the MII stage.

Statistical analysis

Statistical analyses of all data from three or four replicates for comparison were carried out by analysis of one-way ANOVA followed by Duncan's multiple ranges test with STATVIEW (Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to arc-sine transformation before statistical analysis.

Results

Time dependent changes in phosphorylation of connexin-43 in cumulus cells surrounding oocytes during meiotic progression beyond the MI stage

Fig. 1 shows the results of the time dependent changes in the phosphorylated connexin-43 in cumulus cells. Three bands situated at 43, 45, and 47 kDa on SDS-PAGE were detected in cumulus cells isolated from COCs cultured for 24 hr. After 32-hr cultivation of COCs, the staining intensities of faster (43 kDa) and moderate (45 kDa) migrating bands were significantly decreased as compared to those of cumulus cells from COCs cultured for 24 hr. Significant increases in the staining intensity of the slower migration form (47 kDa), the phosphorylated form of connexin-43, were found in cumulus cells from COCs cultured for 32 hr. Further cultivation (40 hr) of COCs resulted in a significant increase in the intensity of the phosphorylated band compared to that of COCs at 32 hr. The limited increase in the intensity was detectable at 48-hr cultivation, but this increase was not significantly different from that at 40-hr cultivation.

Effects of PI 3-kinase inhibitor or PKC inhibitor on the phosphorylation of connexin-43 in cumulus cells

In order to define the role of PI 3-kinase or PKC in

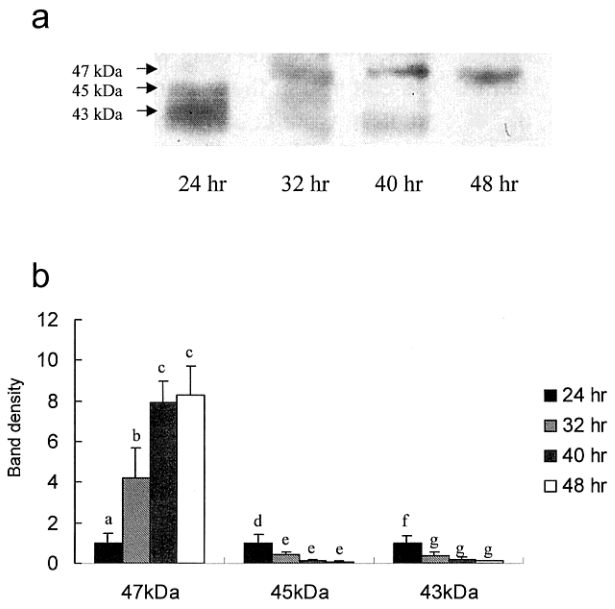

Fig. 1. Time dependent changes in the phosphorylation of connexin-43 in cumulus cells surrounding oocytes during meiotic progression beyond the MI stage (a): Immunoblot probed with anti-connexin-43 monoclonal antibody; (b): The intensity of connexin-43 bands as determined by scanning densitometry, (the data in each band were expressed as the fold strength of the intensity in cumulus cells from COCs cultured for 24 hr); a–c, d, e, and f, g: Different superscripts indicate significant differences within the same migrated bands at the $p<0.01$ level.

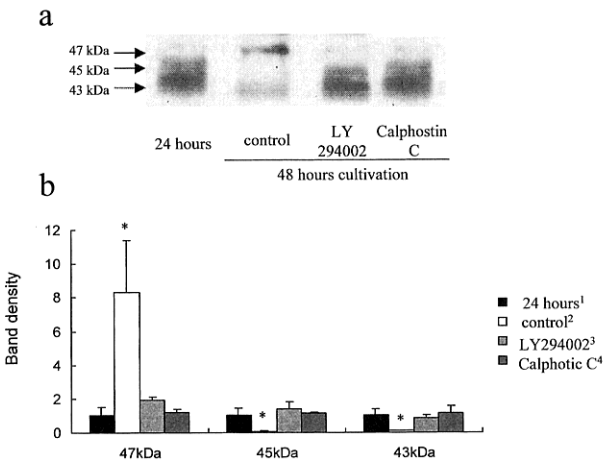

Fig. 2. Effects of LY294002 on the phosphorylation of connexin-43 in cumulus cells from porcine COCs. (a): Immunoblot probed with anti-connexin-43 monoclonal antibody; (b): The intensity of connexin-43 bands as determined by scanning densitometry, (the data in each band express the fold strength of the intensity in cumulus cells from COCs cultured for 24 hr); *: Significant difference was observed within the same migrated bands at the $p<0.01$ level; 1: COCs were cultured for 24 hr; 2: COCs were cultured without LY294002 for 48 hr; 3: After COCs had been cultured for 24 hr, COCs were cultured further with LY294002 for 24 hr; 4: After COCs had been cultured for 24 hr, COCs were cultured further with Calphostin C for 24 hr.

the phosphorylation of connexin-43 in cumulus cells, COCs were precultured for 24 hr in the maturation medium (to allow GVBD and progression to the MI stage) and then transferred to medium supplemented with either 5.0×10^{-5} M LY294002 (PI 3-kinase inhibitor) or 1×10^{-6} M Calphostin C (PKC inhibitor), followed by the second 24-hr cultivation. In lysates of cumulus cells from COCs cultured for 24 hr, three bands of connexin-43 were detected on SDS-PAGE. At 48 hr, the staining intensity of the slower migration form (phosphorylated form of connexin-43) was increased significantly. In sharp contrast, treating COCs with LY294002 for 24 hr (a total of 48-hr cultivation) produced a significant decrease in the intensity of the 47 kDa band (phosphorylated form of connexin-43) in cumulus cells compared to that in COCs cultured without these drugs (Fig. 2). Concomitantly, significant increases in the intensities of the 43-kDa and 45-kDa bands were observed after 48-hr cultivation of COCs with LY294002, and the intensities of three bands of connexin-43 at 48-hr cultivation of COCs treated with LY294002 were comparable to those from COCs cultured for 24 hr.

In cumulus cells from COCs which were further cultured with Calphostin C for 24 hr after the first 24-hr cultivation, the phosphorylation of connexin-43 was significantly suppressed compared to that in cumulus cells form COCs cultured for 48 hr in the basic maturation medium. No significant difference in the intensities of three connexin-43 bands was noted between cumulus cells from COCs cultured for 48 hr with either LY294002 or Calphostin C and COCs cultured for 24 hr.

Effects of the phosphorylation of connexin-43 in cumulus cells on the level of cAMP, activity of MAP kinase and meiotic progression to the MII stage in oocytes

In order to determine whether the phosphorylation of connexin-43 in cumulus cells is required for a decrease in cAMP, activation of MAP kinase and meiotic progression from the MI stage to the MII stage in oocytes, COCs were cultured for 24 hr with LY294002 or Calphostin C after the 24-hr first cultivation. The level of cAMP in oocytes cultured for the first 24 hr, was 4.12 ± 0.48 fmol/oocyte (Table 1). The further 24-hr cultiva-

Table 1. Levels of cAMP, maturation rates and MAP kinase activity in oocytes after COCs were cultured in LY294002 or Calphostin C for 24 hr after the first 24-hr cultivation in the basic maturation medium

Treatment	Level of cAMP (f mol/oocyte)	MI stage (%)	MII stage (%)	MAP kinase activity[1]
24 hr	4.12 ± 0.48^{a}	48^{d}	0^{f}	1.15 ± 0.13^{i}
control	1.85 ± 0.31^{b}	10^{e}	81^{g}	31.33 ± 3.49^{j}
LY294002	6.37 ± 1.04^{c}	51^{d}	35^{h}	8.21 ± 1.05^{k}
Calphostin C	6.18 ± 0.82^{c}	57^{d}	28^{h}	10.07 ± 2.13^{k}

[a–c; d, e; i–j]; Columns with no common superscript are significantly different ($p<0.01$).
[1]: Activity is expressed as fold MAP kinase activity in which positive control, 5 ng active MAP kinase activity is defined as 100. Each independent experiment was repeated three times (level of cMAP and MII stage) or four times (MAP kinase activity).

tion of control COCs significantly decreased the level of cAMP in oocytes (1.85 ± 0.31 fmol/oocyte) (Table 1), but treatments with each drug produced significantly higher levels of cAMP as compared to those of oocytes cultured for 48 hr without any drug (Table 1). There is no significant deference between the levels of cAMP in oocytes cultured with LY294002 and Calphostin C (Table 1). In oocytes containing the high level of cAMP which were cultured with either LY294002 or Calphostin C, the MAP kinase activity and proportion of oocytes which reached the MII stage were significantly lower than those in control oocytes (Table 1). Nearly 50 % of oocytes cultured for 24 hr with LY294002 or Calphostin C after the first 24-hr cultivation were arrested at the MI stage (Table 1). The low activity of MAP kinase and the proportion of oocytes which reached the MII stage when oocytes were cultured with LY294002 were remarkably similar to those of oocytes cultured with Calphostin C (Table 1).

Discussion

During meiotic progression beyond the MI stage, gap junctional communications between cumulus cells and oocytes are disrupted, resulting in meiotic progression to the MII stage in pig [24,25] and rat [26]. Removal of cumulus cells from COCs after 24-hr cultivation has been reported to lead to a significant increase in the proportion of oocytes which reached the MII stage [27]. Moreover, it was shown that in denuded porcine oocytes the activation of MAP kinase was accelerated more than that in oocytes attached to cumulus cells [19]. These observations, and our own, imply that closing the channels between cumulus cells and oocytes blocks the transport of unknown factor(s) from cumulus cells into oocytes, which prevents meiotic progression and MAP kinase activation, leading to meiotic progression beyond the MI stage.

The results of the present study demonstrated that connexin-43, the gap junctional protein in cumulus cells, was phosphorylated after 32-hr cultivation of COCs and up to 48 hr, whereas most of the connexin-43 was as an unphosphorylated form in cumulus cells from COCs cultured for 24 hr. When the phosphorylation of connexin-43 in cumulus cells was suppressed by either PI 3-kinase inhibitor or PKC inhibitor, the increase in the cAMP level in oocytes was recognized. In rat liver cells, the phosphorylation of connexin-43 has been shown to control the channel [28]. In addition, Motlik *et al.* [24] reported that during in vivo porcine oocyte maturation, the gap junctional communication was decreased by 32 hr after hCG stimulation. Judging from these findings, it was estimated that the phosphorylation of connexin-43 in cumulus cells induced closure of the gap junctional channel between cumulus cells and oocytes after 32-hr cultivation with gonadotropins.

In our previous study (unpublished data), the level of cAMP was continuously decreased in oocytes during meiotic progression beyond the MI stage, though a very high level of cAMP in cumulus cells surrounding oocytes was maintained in up to 48-hr cultivation. Moreover, the addition of forskolin or IBMX induced an increase in cAMP levels in oocytes and suppressed meiotic progression to the MII stage, MAP kinase activity, and $p34^{cdc2}$ kinase activity. In the present study, a significant increase in cAMP in oocytes which was induced by the maintenance of an unphosphorylated form of connexin-43 in cumulus cells, suppressed meiotic progression to the MII stage and MAP kinase activity. Very recently we demonstrated that the activation of MAP kinase which was reduced by cAMP was required for an increase in MPF activity after GVBD in porcine oocytes

[10]. These observations by us regarding cAMP in oocytes, strongly support our hypothesis that the closure of the channel via the phosphorylation of connexin-43 inhibits transport of cAMP from cumulus cells into oocytes and decreases the cAMP level in oocytes, leading to the activation of MAP kinase and meiotic progression to the MII stage in porcine oocytes.

In summary, connexin-43, the gap junctional protein in cumulus cells, was phosphorylated after 32-hr cultivation of COCs and up to 48 hr, whereas most connexin-43 was unphosphorylated in cumulus cells from COCs cultured for 24 hr. When the phosphorylation of connexin-43 in cumulus cells was suppressed by a further 24-hr treatment with either PI 3-kinase inhibitor or PKC inhibitor, a significantly higher level of cAMP in the oocytes and a significantly lower proportion of oocytes at the MII stage were recognized, as compared to those of oocytes cultured for 48 hr without this drug. The activity of MAP kinase activity in the oocytes was also significantly inhibited by the addition of both drugs. These results reveal that the closing of the gap junctional communication via the phosphorylation of connexin-43 in cumulus cells may induce a decrease in the cAMP level, resulting in activation of MAP kinase and meiotic progression beyond the MI stage to the MII stage in porcine oocytes.

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References

1) Gautier, J., Norbury, C., Lohka, M., Murse, P. and Maller, J. (1988): Purified maturation-promoting factor contains the product of *Xenopus* homolog of fission yeast cell cycle control gene cdc2+. Cell, 54, 433–439.
2) Pines, G. and Hunter, T. (1989): Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell, 58, 833–846.
3) Masui, Y. and Markert, G.E. (1971): Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool., 177, 129–146.
4) Taieb, F., Thibier, C. and Jessus, C. (1997): On cyclin, oocytes, and eggs. Mol. Reprod. Dev., 48, 397–411.
5) Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y. and Kohmoto, K. (1991): Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycle in mouse oocytes and embryos. Development, 113, 789–795.
6) Naito, K. and Toyoda, Y. (1991): Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocytes. J. Reprod. Fertil., 93, 467–473.
7) Tatemoto, H. and Terada, T. (1996): Activation of $p34^{cdc2}$ kinase around the meiotic resumption in bovine oocytes cultured in vitro. Theriogenology, 45, 427–437.
8) Naito, K., Daen, F.P. and Toyoda, Y. (1992): Comparison of histone H1 kinase activity during meiotic maturation between two types of porcine oocytes matured in different media in vitro. Biol. Reprod., 47, 43–47.
9) Inoue, M., Naito, K., Aoki, F., Toyoda, Y. and Sato, E. (1995): Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes. Zygote, 3, 265–271.
10) Shimada, M., Zeng, W.X. and Terada, T. (2001a): Inhibition of PI 3-kinase or MEK leads to suppression of $p34^{cdc2}$ kinase activity and meiotic progression beyond the MI stage in porcine oocyte surrounded with cumulus cells. Biol. Reprod., 65, 442–448.
11) Matten, W., Daar, I. and Vande-Woude, G.F. (1994): Protein kinase A acts at multiple points to inhibit *Xenopus* oocyte maturation. Mol. Cell. Biol., 14, 4419–4426.
12) Lu, Q., Smith, G.D., Chen, D.Y., Yang, Z., Han, Z.M., Schatten, H. and Sun, Q.Y. (2001): Phosphorylation of mitogen-activated protein kinase is regulated by protein kinase C, cyclic 3',5'-adenosine monophosphate, and protein phosphatase modulators during meiotic resumption in rat oocytes. Biol. Reprod., 64, 1444–1450.
13) Schultz, R.M., Montgomery, R.R. and Belanoff, J.R. (1983): Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. Dev. Biol., 97, 264–273.
14) Racowsky, C. (1985): Effect of forskolin on meintenance of meiotic arrest and stimulation of cumulus expansion, progesterone and cyclic AMP production by pig oocyte-cumulus complexes. J. Reprod. Fertil., 74, 9–21.
15) Grazul-Bilska, A.T., Reynolds, L.P. and Redmer, D.A. (1997): Gap junctions in the ovaries. Biol. Reprod., 57, 947–957.
16) Masil, L.S., Cunningham, B.A., Edelman, G.M. and Goodenough, D.A. (1990): Differential phosphorylation of the gap junction protein connexin 43 in junctional communication-competent and -deficient cell lines. J. Cell. Biol., 111, 2077–2088.
17) Lau, A.F., Kurata, W.E., Kanemitsu, M.Y., Loo, L.W.M., Warn-Cramer, B.J., Eckhart, W. and Lampe, P.D. (1996): Regulation of connexin 43 function by

activated tyrosine protein kinases. J. Bioenerg. Biomembr., 28, 359–367.

18) Shimada, M., Maeda, T. and Terada, T. (2001): Dynamic changes of connexin-43, gap junctional protein, in outer layers of cumulus cells are regulated by PKC and PI 3-kinase during meiotic resumption in porcine oocytes. Biol. Reprod., 64, 1255–1263.

19) Shimada, M. and Terada, T. (2001): PI 3-kinase in cumulus cells and oocytes is responsible for activation of oocyte MAP kinase during meiotic progression beyond the MI stage in pigs. Biol. Reprod., 64, 1106–1114.

20) Shimada, M., Anas, M.K.I. and Terada, T. (1998): Phosphatidylinositol 3-kinase in cumulus cells is responsible for meiotic progression from MI to MII stage in porcine follicular oocytes. J. Mamm. Ova Res., 15, 68–76.

21) Petters, R.M. and Reed, M.L. (1991): Addition of taurine or hypotaurine to culture medium improves development of one- and two-cell pig embryos in vitro. Theriogenology, 35, 253.

22) Laemmli, U.K. (1970): Cleavage of structual proteins during the assembly of the heat of bacteriophage T4. Nature, 227, 680–685.

23) Shimada, M., Anas, M.K.I. and Terada, T. (1998): Effects of phosphatidylinositol 3-kinase inhibitors, wortmannin and LY294002, on germinal vesicle breakdown (GVBD) in porcine oocytes. J. Reprod. Dev, 44, 281–288.

24) Motlik, J., Fulka, J. and Flechon. J.E. (1986): Changes in intercellular coupling between pig oocytes and cumulus cells during maturation in vivo and in vitro. J. Reprod. Fertil., 76, 31–37.

25) Isobe, N., Maeda, T. and Terada, T. (1998): Involvement of meiotic resumption in the disruption of gap junctions between cumulus cells attached to pig oocytes. J. Reprod. Fettil., 113, 167–172.

26) Larsen, W.J., Wert, S.E. and Brunner, G.D. (1987): Differential modulation of rat follicle cell gap junction populations at ovulation. Dev. Biol., 122, 61–71.

27) Isobe, N., Fujihara, H. and Terada, T. (1996): Cumulus cells suppress meiotic progression in pig oocytes cultured in vitro. Theriogenology, 45, 1479–1489.

28) Masil, L.S., Cunningham, B.A., Edelman, G.M, and Goodenough, D.A. (1990): Differential phosphorylation of the gap junction protein connexin 43 in junctional communication-competent and -deficient cell lines. J. Cell Biol., 111, 2077–2088.