Acquisition of meiotic competence in growing pig oocytes correlates with their ability to activate Cdc2 kinase and MAP kinase

Kanayama, Nobuyuki
Miyano, Takashi
Lee, Jibak

(Citation)
Zygote,10(3):261-270

(Issue Date)
2002-08

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90000887

Zygote **10** (August), pp 261–270. © 2002 Cambridge University Press
DOI:10.1017/S0967199402002344 Printed in the United Kingdom

Acquisition of meiotic competence in growing pig oocytes correlates with their ability to activate Cdc2 kinase and MAP kinase

Nobuyuki Kanayama[1], *Takashi Miyano*[2] *and Jibak Lee*[1]
Kobe University, Kobe, Japan

Date submitted: 6.3.02. Date accepted: 3.4.02

Summary

Meiotic maturation of mammalian oocytes is under the control of cell cycle molecules Cdc2 kinase and MAP kinase (mitogen-activated protein kinase). In the present study, we investigated the relationship between the ability to activate Cdc2 kinase and MAP kinase and the acquisition of meiotic competence during pig oocyte growth. Growing and fully grown pig oocytes were collected from four groups of antral follicles of various diameters (A, 0.5–0.7 mm; B, 1.0–1.5 mm; C, 2.0–2.5 mm; D, 4.0–6.0 mm) and cultured *in vitro*. Fully grown oocytes from class D follicles, which have full competence to mature to metaphase II, had the ability to activate both Cdc2 kinase and MAP kinase. In contrast, growing oocytes from class A follicles, which have limited competence to resume meiosis, had no such ability. Cyclin B1 molecules did accumulate, however, with phosphorylated 35 and 36 kDa bands of $p34^{cdc2}$ appearing in the cultured oocytes. Of the growing oocytes from class B follicles, 60% resumed meiosis but arrested at metaphase I. Some of the oocytes in this class were capable of activating Cdc2 kinase, although they did not appear to have established a MAP kinase-activating pathway or the ability to activate MEK. These results suggest that limited meiotic competence in growing oocytes from class A follicles is due to their inability to activate Cdc2 kinase and their incomplete MEK-MAP-kinase pathway, although the oocytes are capable of accumulating cyclin B1 molecules. During the final growth phase, pig oocytes acquire the ability to activate Cdc2 kinase and then establish the MEK-MAP-kinase pathway for full meiotic competence.

Keywords: Cdc2 kinase, MAP kinase, Meiotic competence, MEK, Oocyte

Introduction

In pig ovaries, oocytes increase in volume and remain arrested at the dictyate stage of prophase I. After reaching full size, oocytes resume meiosis in response to pituitary gonadotropic hormones, mature to metaphase II, and are ovulated for fertilisation with spermatozoa. During the growth phase, oocytes acquire competence for meiotic maturation. Fully grown oocytes are able to mature to metaphase II. Although growing oocytes, which have acquired partial meiotic competence, undergo germinal vesicle breakdown (GVBD) and enter metaphase I, they cannot complete meiotic maturation (Sorensen & Wassarman, 1976; Motlik *et al.*, 1984). The process by which meiotically incompetent oocytes become meiotically competent is called acquisition of meiotic competence (Shultz, 1991).

It has been revealed in mammalian oocytes that meiotic maturation is controlled by cell cycle molecules such as Cdc2 kinase and MAP kinase (mitogen-activated protein kinase). Cdc2 kinase, a serine/threonine kinase composed of a regulatory subunit, cyclin B, and a catalytic subunit, $p34^{cdc2}$, has been found to be a fundamental regulator of the G2/M transition in both mitosis and meiosis in eukaryotic cells (Masui & Markert, 1971; Nurse, 1990). It is activated at

All correspondence to: T. Miyano, Laboratory of Reproductive Biology & Biotechnology, Faculty of Agriculture, Kobe University, Nada-ku, Kobe 657–8501, Japan. Tel: +81 78 803 5805. Fax: +81 78 803 5807. e-mail: miyano@ans.kobe-u.ac.jp
[1]The Graduate School of Science and Technology, Kobe University, Kobe 657-8501, Japan.
[2]Laboratory of Reproductive Biology & Biotechnology, Faculty of Agriculture, Kobe University, Kobe 657-8501, Japan.

the onset of oocyte maturation, and its activity, which essentially accounts for all histone H1 kinase activity in maturing oocytes, peaks at metaphase I and II (Hashimoto & Kishimoto, 1988; Choi *et al*., 1991; Naito & Toyoda, 1991). For the activation of Cdc2 kinase, dephosphorylation of threonine 14 (Thr 14) and tyrosine 15 (Tyr 15) residues of $p34^{cdc2}$ by Cdc25 phosphatase and phosphorylation of threonine 161 (Thr 161) of $p34^{cdc2}$ by cyclin-dependent kinase-activating kinase (CAK) are necessary.

MAP kinase is phosphorylated and activated during oocyte maturation in *Xenopus* (Gotoh *et al*., 1991), mouse (Sobajima *et al*., 1993), pig (Inoue *et al*., 1995) and cattle (Fissore *et al*., 1996). Its activity is demonstrated by its ability to phosphorylate myelin basic protein as a substrate. In mammalian somatic cells, MAP kinases are intermediates in signal-transduction pathways initiated by a variety of mitogens such as growth factors and tumour promoters, and located downstream from Ras (a signal-transducing, guanine nucleotide-binding protein), Raf (MAP kinase-kinase-kianse) and MEK (MAP kinase-kinase). In *Xenopus* oocytes there are at least three pathways for MAP kinase activation. One pathway involves Mos and MEK, and is activated primarily by progesterone stimulation (Nebreda & Hunt, 1993). The other two depend on receptor tyrosine kinases and include a Raf-dependent pathway (Muslin *et al*., 1993; Fabian *et al*., 1993) and a Raf-independent pathway (Itoh *et al*., 1993), respectively. MAP kinase has been implicated in the cascade leading to Cdc2 kinase activation (Kosako *et al*., 1994; Gotoh & Nishida, 1995). Activated MAP kinase may directly or indirectly regulate some proteins controlling Cdc2 kinase activity such as Cdc25 phosphatase, Wee1 kinase, Myt1 kinase and CAK. It has been established that MAP kinases are activated during oocyte maturation in mammals, but a MAP kinase cascade(s) has not been elucidated. In mouse oocytes, however, MAP kinase is thought to be activated through a Mos–MEK cascade (Verlhac *et al*., 1996).

It is not understood how oocytes acquire meiotic competence during their growth phase to become competent to resume and complete meiosis. Pig and cattle oocytes continue growing in their follicles, which have formed an antrum. Therefore, oocytes from antral follicles of different sizes have different meiotic competence (Hyttel *et al*., 1997). The configuration of chromatin and nucleolus of growing oocytes changes during the development of antral follicles (Motlik *et al*., 1984). Current evidence suggests that acquisition of meiotic competence in oocytes during the growth phase correlates with changes in the accumulation and activation of cell cycle molecules. It has been shown that growing oocytes accumulate $p34^{cdc2}$ molecules (Hirao *et al*., 1995; de Vantry *et al*., 1996) and develop the ability to activate MAP kinase (Harrouk & Clarke, 1995). In the present experiment, we collected growing pig oocytes with different meiotic competence from antral follicles of various sizes and compared their ability to activate Cdc2 kinase, MAP kinase and MEK.

Materials and methods

Oocyte culture

Pig ovaries were obtained form prepubertal gilts at a local slaughterhouse. Following three washes in Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA), intact healthy antral follicles 0.5–6.0 mm in diameter were dissected in PBS-PVA from ovaries following the technique described by Moor & Trounson (1977). The follicles were classified into four classes according to diameter: early antral (0.5–0.7 mm, class A), small antral (1.0–1.5 mm, class B), middle antral (2.0–2.5 mm, class C) and large antral follicles (4.0–6.0 mm, class D). Follicles in each class were opened in HEPES-buffered medium 199 (Nissui Pharmaceutical, Tokyo, Japan) and oocyte–cumulus granulosa cell complexes (OCCs) were isolated from the follicles. In some experiments, oocyte diameters (excluding the zona pellucida) were measured with an ocular micrometer (CSM-4, Olympus, Tokyo, Japan) attached to an inverted microscope.

After two washes, OCCs in the four classes were cultured for 27 and 42 h in 2 ml of bicarbonate-buffered medium 199 supplemented with 10% fetal calf serum (FCS; BIO. Whittaker, ML), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, 0.1 IU/ml human menopausal gonadotropin (hMG; Pergonal, Teikokuzoki, Toyko, Japan), and two everted theca shells with gentle agitation. All cultures were carried out in an atmosphere of 5% CO_2 in humidified air at 38.5 °C. After culture, OCCs were collected and treated with 0.05% hyaluronidase (Sigma Chemical Co., St Louis, MO) to remove cumulus cells. Oocytes were then denuded completely by pipetting with a small-bore pipette. Some oocytes were mounted on slides, fixed in an acetic acid–ethanol (1:3, v/v) solution, stained with 1% aceto-orcein, and observed under a Nomarsky interference microscope. Others were used for Western blotting analysis or a kinase activity assay.

Statistical differences in the mean oocyte diameters were analysed by Student's *t*-test. Statistical differences in other data were analysed by the chi-square test. A p value < 0.05 was considered statistically significant.

Western blotting

Groups of 77–86 growing oocytes from class A follicles,

65 growing oocytes from class B follicles and 50 fully grown oocytes from class D follicles were collected for each sample before and after 27 and 42 h of culture. Denuded oocytes were rinsed twice in PBS-PVA, dissolved in 15 µl of Laemmli sample buffer (Laemmli, 1970), boiled for 5 min, and frozen at –20 °C before use. Samples were run on 13% SDS-polyacrylamide gels, and proteins were electroblotted onto hydrophobic polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA) for 1.5 h at 2 mA/cm^2. The membranes were blocked with 10% FCS in PBS containing 0.1% Tween20 (PBS-Tween) for 2 h, then incubated with the following primary antibodies at room temperature: rabbit polyclonal anti-MAP kinase antibody (#sc-94, 1:1000, Santa Cruz, CA) for 2 h, mouse monoclonal anti-Cdc2 antibody (#sc-54, 1:500, Santa Cruz), mouse monoclonal anti-MEK antibody (#sc-6250, 1:500, Santa Cruz), or rabbit affinity purified anti-pig cyclin B1 antibody (1:1000, generously donated by Drs Y. Dai and R.M. Moor) for 4 h. After three washes in PBS-Tween, membranes were treated with horseradish-peroxidase-labelled anti-mouse (1:1000, Dako Japan, Tokyo, Japan) or anti-rabbit immunoglobulin antibody (1:1000, Amersham Life Science, UK) according to the primary antibody in the blocking buffer for 1 h at room temperature. After three washes of 10 min each with PBS-Tween, peroxidase activity was visualised with the ECL Western blotting detection system (Amersham Life Science).

Histone H1 and MAP kinases double assay

OCCs from different-sized follicles were cultured, and oocytes were collected as described above. After denudation of oocytes and three washes in PBS-PVA, each oocyte was transferred into an Eppendorf tube with 1 µl of PBS-PVA. Thereafter, 4 µl of ice-cold extraction buffer was added, and the mixture was frozen at –80 °C before the kinase assay. The extraction buffer was composed of 80 mM β-glycerophosphate, 25 mM HEPES (pH 7.2), 10 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM PMSF, 0.1 mM Na_3VO_4, 1 µg/ml aprotinin (Sigma) and 1 µg/ml leupeptin (Sigma) (Nebreda *et al.*, 1995). After oocytes had thawed, they were centrifuged at 13 000 *g* for 10 min, added to 5 µl of kinase buffer and 5 µl of substrate solution, and incubated for 10 min at 37 °C. The kinase buffer was composed of 75 mM HEPES (pH 7.2), 75 mM β-glycerophosphate, 75 mM $MgCl_2$, 6 mM DTT, 0.1 mM EGTA, 10 mM ATP, 15 µM cAMP-dependent protein kinase inhibitor peptide (sequence: TTYADFIASGRTGRRNAIHD, Sigma) and 0.3 µCi/µl [γ–^{32}P]ATP (250 µCi/25 µl, Amersham Pharmacia Biotech, UK). The substrate solution was composed of 4.25 µl of histone H1 (5 mg/ml, from calf thymus; Boehringer, Tokyo, Japan) and 0.75 µl of myelin basic protein (5 mg/ml, from bovine brain; Sigma). The reaction was terminated by the addition of 5 µl of 4-times-concentrated SDS sample buffer. The samples were boiled for 5 min and loaded onto a 15% gel for separation of labelled myelin basic protein and histone H1. After running, the gels were dried and autoradiographed.

EGF treatment of cumulus granulosa cells

For the detection of MEK in pig oocytes, EGF-treated granulosa cells were Western blotted as a control. We collected OCCs from 4–6 mm follicles in the manner described above. Intact OCCs were used as the source of granulosa cells, as each OCC contained one oocyte and approximately 1.5×10^4 cumulus granulosa cells (Nagai *et al.*, 1993). After three washes in HEPES-199, OCCs were cultured in 0.5 ml of medium for 5 and 60 min in an atmosphere of 5% CO_2 in humidified air at 38.5 °C. The medium was bicarbonate-buffered medium 199 supplemented with 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate and 10 ng/ml EGF (human recombinant epidermal growth factor; Genzyme Diagnostics, MA). After culture, OCCs were rinsed twice in PBS-PVA, dissolved in 15 µl of Laemmli sample buffer by boiling for 5 min, and frozen at –20 °C before use.

Results

Meiotic competence in growing oocytes

The nuclear morphology and meiotic competence of oocytes from different-sized antral follicles were compared. The mean diameters of oocytes isolated from the A, B, C and D class follicles were 103.9 ± 2.2, 109.1 ± 2.4, 114.4 ± 2.9 and 121.5 ± 2.5 µm, respectively (Table 1). All the oocytes examined contained a germinal vesicle (GV) including a nucleolus. The oocytes from class A follicles had a nucleolus with large nucleolar vacuoles and filamentous chromatin spreading throughout the GV (Fig. 1*a*). In class B, chromatin was clearly thicker than in class A oocytes (Fig. 1*d*). In classes C and D, oocytes had a condensed chromatin ring around a compact lucent nucleolus (Fig. 1*g*).

Oocytes were cultured for 27 and 42 h to examine their meiotic competence (Table 1). In all classes, meiotic resumption was observed after 27 h of culture. In class A, however, 61% of oocytes still remained at the GV stage (Fig. 1*b*). In class B, 26% and 29% of oocytes reached diakinesis and metaphase I, respectively (Fig. 1*e*). In class D, 71% of oocytes reached metaphase I (Fig. 1*h*). Progression to metaphase II was not observed in oocytes from any class of follicles after 27 h. After 42 h of culture, 55% of class A oocytes remained at the GV stage (Fig. 1*c*). In class B, 60% of oocytes reached

Table 1 Meiotic resumption and arrest of pig oocytes isolated from ovarian follicles at various sizes

Follicular diameter (mm)	Oocyte diameter (μm)	Time of culture (h)	No. of oocytes examined	No. (%) of oocytes at stage of						No. (%) of oocytes degenerating
				GV	D	MI	AI–TI	MII	FP	
A: 0.5–0.7	103.9 ± 2.2[a]	0	28	28 (100)	0	0	0	0	0	0
		27	34	21 (61)[a]	8 (24)[ab]	0	1 (3)	0	0	4 (12)
		42	33	18 (55)[a]	6 (18)[ac]	2 (6)[a]	2 (6)	1 (3)[a]	2 (6)	2 (6)
B: 1.0–1.5	109.1 ± 2.4[b]	0	24	24 (100)	0	0	0	0	0	0
		27	34	8 (24)[b]	9 (26)[ab]	10 (29)[b]	3 (9)	0	2 (6)	2 (6)
		42	32	3 (9)[bc]	2 (6)[bc]	19 (60)[bc]	2 (6)	0	1 (3)	5 (16)
C: 2.0–2.5	114.4 ± 2.9[c]	0	28	28 (100)	0	0	0	0	0	0
		27	33	1 (3)[c]	11 (33)[a]	13 (40)[b]	0	0	0	8 (24)
		42	35	1 (3)[c]	1 (3)[c]	11 (31)[b]	2 (6)	16 (45)[b]	1 (3)	3 (9)
D: 4.0–6.0	121.5 ± 2.5[d]	0	29	29 (100)	0	0	0	0	0	0
		27	31	1 (3)[c]	3 (10)[bc]	22 (71)[c]	0	0	0	5 (16)
		42	36	0	1 (3)[c]	1 (3)[a]	1 (3)	33 (91)[c]	0	0

Pig oocytes isolated from ovarian follicles at various sizes were cultured for 27 and 42 h. GV, germinal vesicle; D, diakinesis; MI, metaphase I; AI–TI, anaphase I–telophase I; MII, metaphase II; FP, female pronucleus.
[a–d]Values with different superscripts in the same column differ significantly ($p < 0.05$).

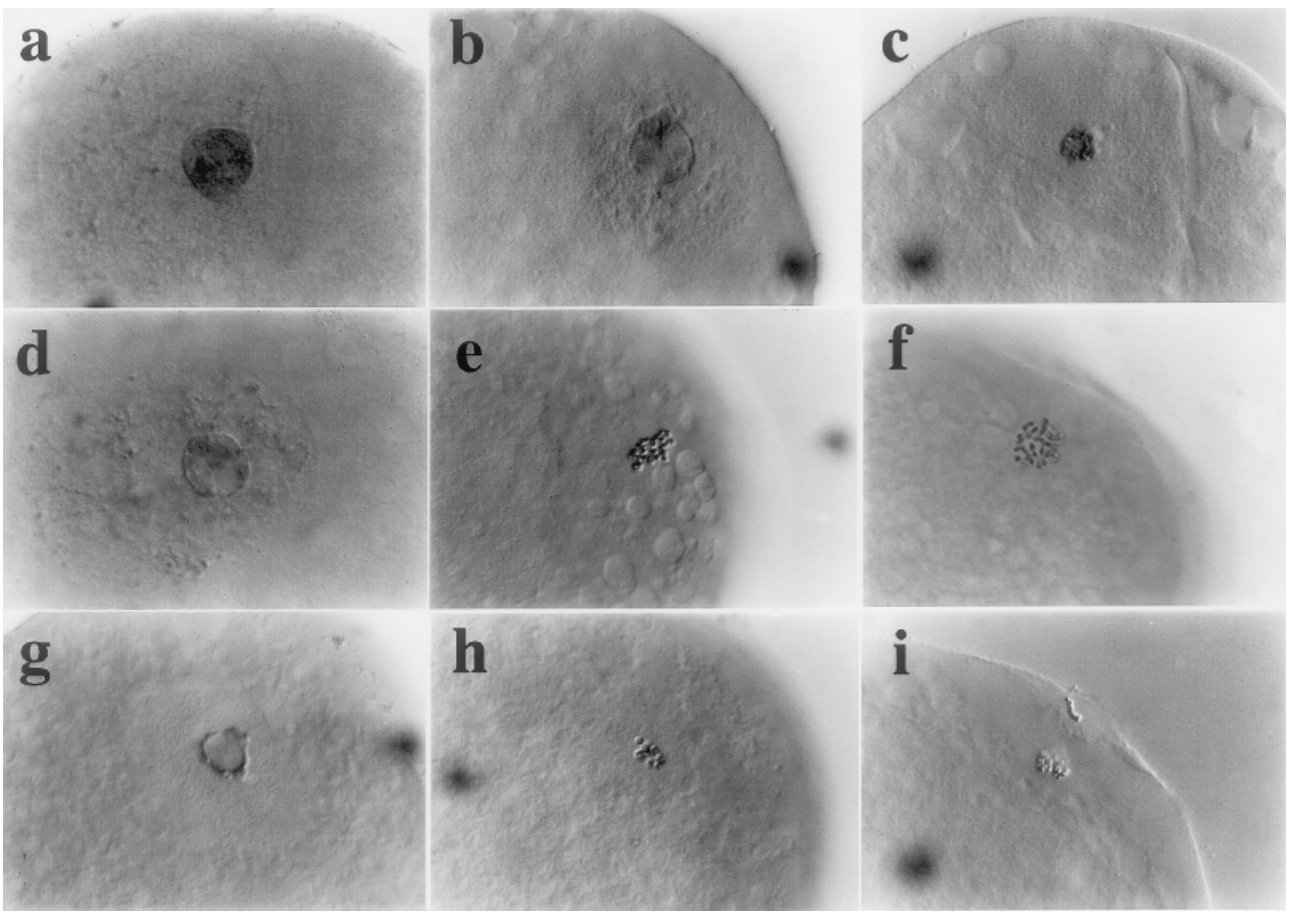

Figure 1 Nuclear morphology of cultured pig oocytes. The oocytes at various growth stages were cultured, fixed in acetic alcohol, and stained with 1% aceto-orcein. Growing oocytes isolated from 0.5–0.7 mm follicles were cultured for 0 (*a*), 27 (*b*) and 42 h (*c*). Growing oocytes isolated from 1.0–1.5 mm follicles (*d–f*) and fully grown oocytes isolated from 4.0–6.0 mm follicles (*g–i*) were also cultured for 0 (*d* and *g*), 27 (*e* and *h*) and 42 h (*f* and *i*).

metaphase I, although no oocytes progressed to metaphase II (Fig. 1*f*). In class C, 31% of oocytes reached metaphase I and 45% progressed to metaphase II. In class D, 91% of oocytes matured to metaphase II (Fig. 1*i*). These results show that (1) growing oocytes from class A follicles have limited meiotic competence; (2) growing oocytes from class B follicles resume meiosis but do not have the meiotic competence to go through the first meiotic division; and (3) fully grown oocytes from class D follicles have full meiotic competence to mature to metaphase II.

Activation of Cdc2 kinase in growing oocytes

To examine the ability to activate Cdc2 kinase in growing oocytes during their acquisition of meiotic competence, oocytes from three classes of follicles which had different meiotic competence (A, B and D) were subjected to kinase assay using histone H1 as a substrate. Changes in components of Cdc2 kinase, $p34^{cdc2}$ and cyclin B1 in the oocytes were also examined by Western blotting.

Fully grown oocytes from class D follicles did not contain cyclin B1 molecules before culture (Fig. 2*a*). Cyclin B1 molecules accumulated after 27 and 42 h, and a 35 kDa upper phosphorylated band of $p34^{cdc2}$ (Lee *et al.*, 1999) was detected after 42 h of culture. In the immunoblots with anti-cyclin B1 antibody, another two bands were detected at the upper position of the cyclin B1 band (62 kDa). Since the upper two bands are detected in pig oocytes at any stage, including fertilised eggs, in which the 62 kDa cyclin B1 band has completely disappeared, we recognise them as non-specific bands (Lee *et al.*, 1999; Miyano *et al.*, 2000).

Fig. 2*b* shows the typical pattern of activation of the Cdc2 kinase of oocytes. Fully grown oocytes from class D follicles accumulated cyclin B1 molecules after 27 h, and a high histone H1 kinase activity was observed in all the oocytes examined (12/12). Growing oocytes from class B follicles had the ability to resume meiosis and to proceed to metaphase I as described above. After 27 and 42 h of culture, a 35 kDa phosphorylated band of $p34^{cdc2}$ and a cyclin B1 band were detected. Some of the oocytes (4/12) in this class had a high Cdc2

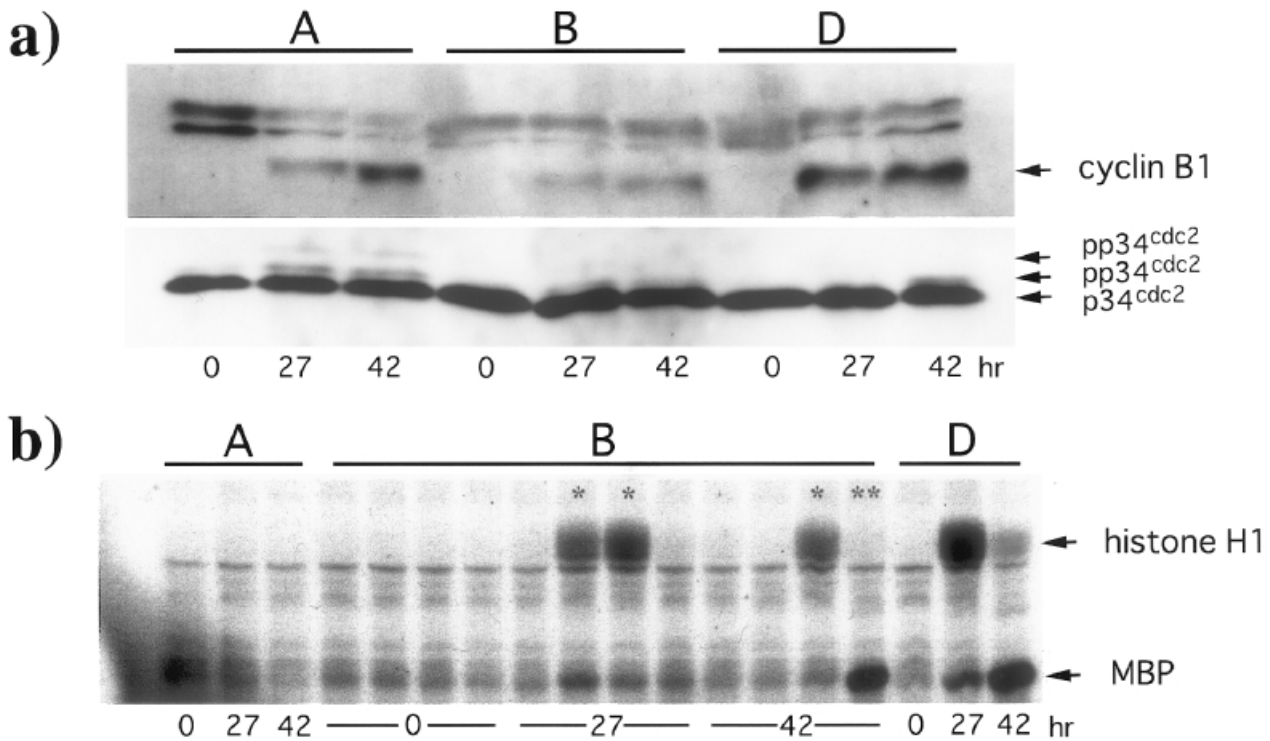

Figure 2 (*a*) Changes in cyclin B1 and $p34^{cdc2}$ during maturation culture of pig oocytes at various stages of growth. Groups of 77–86 growing oocytes from class A follicles, 65 growing oocytes from class B follicles and 50 fully grown oocytes from class D follicles were collected before and after 27 and 42 h of culture. Whole oocyte lysates were subjected to SDS-PAGE, transferred to membranes, and reacted with anti-cyclin B1 and anti-Cdc2 antibodies. Upper two arrows in the lower panel indicate phosphorylated $p34^{cdc2}$ ($pp34^{cdc2}$). (*b*) Changes in the activities in Cdc2 kinase and MAP kinase during maturation of pig oocytes at various stages of growth. Oocytes were collected before and after 27 and 42 h of culture, and changes in the activities of Cdc2 kinase and MAP kinase were detected with phosphorylation of histone H1 and myelin basic protein (MBP) in every oocyte. Lanes with an asterisk (*) show strong phosphorylation of histone H1 and weak phosphorylation of MBP; a lane with a double asterisk (**) shows weak phosphorylation of histone H1 and strong phosphorylation of MBP by growing pig oocytes from class B follicles.

kinase activity, and others showed no activity. In contrast to oocytes having the ability to resume meiosis, growing oocytes from class A follicles did not activate Cdc2 kinase (0/12) (Fig. 2*b*). However, cyclin B1 molecules accumulated in the oocytes cultured for 27 and 42 h, and 35 kDa and 36 kDa phosphorylated bands of $p34^{cdc2}$ were detected in the oocytes (Fig. 2*a*).

Activation of MAP kinase in growing oocytes

In oocytes from all classes of follicles, two MAP kinases ($p44^{ERK1}$ and $p42^{ERK2}$) were detected before culture (Fig. 3). They were found to be inactive non-phosphorylated forms of the MAP kinases. In fully grown oocytes from class D follicles, phosphorylated forms of MAP kinases were detected after 27 and 42 h of culture. The changes in these bands coincided with an increase in kinase activity using myelin basic protein as a substrate (Fig. 2*b*). In contrast, in growing oocytes from the class B follicles, MAP kinases were partially activated after 27 and 42 h. Although H1 kinase activity in some oocytes in this class increased as described above, MAP kinase activity increased in only one oocyte of the examined 12 oocytes cultured for 27 and 42 h. In growing oocytes from class A follicles, MAP kinases were not activated after culture (0/12) (Figs. 2*b*, 3). These results show that growing

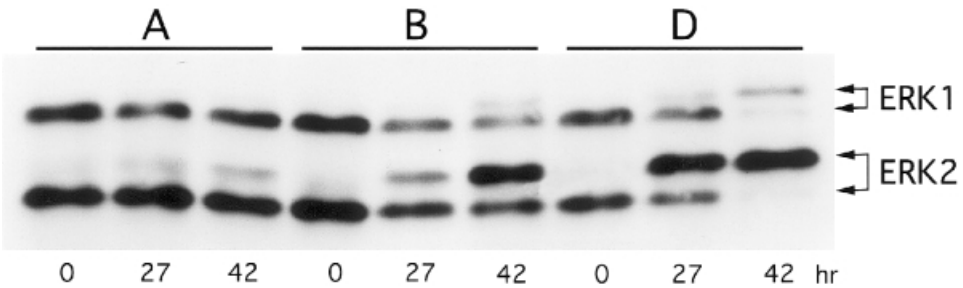

Figure 3 Changes in MAP kinase during maturation culture of pig oocytes at various stages of growth. Groups of 43 growing oocytes from class A, 33 growing oocytes from class B and 25 fully grown oocytes from class D follicles were collected before and after 27 and 42 h of culture. Whole oocyte lysates were subjected to SDS-PAGE, transferred to membranes and reacted with anti-MAP kinase antibodies.

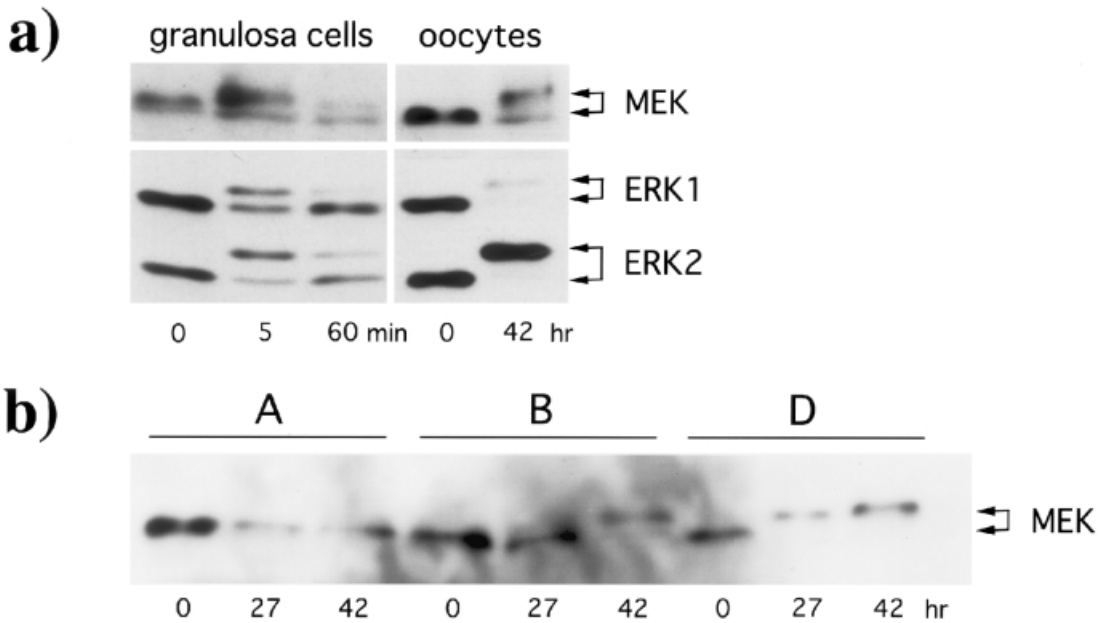

Figure 4 Changes in MEK in pig granulosa cells and oocytes. (*a*) Changes in MEK and MAP kinase in pig granulosa cells after EGF stimulation. Oocyte–cumulus granulosa cell complexes were cultured for 5 and 60 min under the stimulation of 10 ng/ml EGF. Groups of 50 fully grown oocytes were collected before and after 42 h of culture. Whole lysates were subjected to SDS-PAGE, transferred to membranes and reacted with anti-MEK and anti-MAP kinase antibodies. (*b*) Change in MEK during maturation culture of pig oocytes at various stages of growth. Groups of 86 growing oocytes from class A follicles, 65 growing oocytes from class B follicles and 50 fully grown oocytes from class D follicles were collected before and after 27 and 42 h of culture. Whole oocyte lysates were subjected to SDS-PAGE, transferred to membranes and reacted with anti-MEK antibodies.

oocytes that have limited ability to resume meiosis cannot activate MAP kinases, and growing oocytes that stop at metaphase I have an inferior ability to activate MAP kinases.

In somatic cells, MAP kinase participates in a signalling cascade downstream from the growth factors, Ras, Raf and MEK. We first confirmed the activation of MEK in EGF-stimulated granulosa cells and then in growing oocytes. When granulosa cells were stimulated by EGF, the phosphorylated upper bands of two MAP kinases appeared in the cells after 5 min, and the bands then shifted down after 60 min (Fig. 4*a*). These changes are in good agreement with the reported pattern of MAP kinase activation in PC-12 cells treated with nerve growth factor (NGF) or EGF (Miyasaka *et al*., 1991). MEK showed a similar change. An upper 46 kDa band of MEK was detected after 5 min, and the band then shifted down to the 45 kDa forms after 60 min (Fig. 4*a*). The 45 kDa band is thought to be an inactive non-phosphorylated form of MEK, and the 46 kDa band is the active phosphorylated form (Pages *et al*., 1994; Haraguchi *et al*., 1998). In fully grown oocytes from class D follicles, MEK was detected as a 45 kDa band that shifted up to 46 kDa in mature oocytes. In the fully grown oocytes, the phosphorylated form of MEK was detected after 27 and 42 h of culture (Fig. 4*b*). In contrast, in growing oocytes from class B follicles, MEK was activated after 42 h, but not after 27 h. MEK in growing oocytes from class A follicles was not activated during the culture period. These results indicate that growing oocytes from class A follicles have no potency to activate MEK-MAP kinase, and that oocytes in class B follicles have limited potency.

Discussion

Meiotic incompetence of growing pig oocytes has been attributed to an inability to activate Cdc2 kinase (Christmann *et al*., 1994). In the present study, we have shown that most of the growing pig oocytes from class A follicles that do not resume meiosis have no ability to activate Cdc2 kinase, and that some growing oocytes from class B follicles and fully grown oocytes from class D follicles that resume meiosis have the ability to activate the kinase. In our culture experiment, 24% and 27% of growing oocytes from class A follicles were at metaphase (diakinesis, metaphase I and metaphase II) after 27 and 42 h of culture, respectively. However, none of the growing oocytes in this class showed detectable activity of Cdc2 kinase. It is thought that none of the metaphase oocytes in the class were chosen for the kinase assay by chance, or Cdc2 kinase of the oocytes was too low to be detected, because we carried out the kinase assay for each oocyte to establish the individual status of the kinase. Nevertheless, the result is in good agreement with those reported previously. It has been suggested that the acquisition of meiotic competence during the growth phase of mammalian oocytes is related to an accumulation of $p34^{cdc2}$ molecules (Hirao *et al*., 1995; de Vantry *et al*., 1996; Dedieu *et al*., 1998). In pig oocytes, $p34^{cdc2}$ molecules are accumulated during the early growth phase, while follicles develop from the preantral to the antral stage (Hirao *et al*., 1995). In the present study using the later growth stages of oocytes harvested from various-sized antral follicles, we did not recognise any further accumulation of $p34^{cdc2}$ in the pig oocytes. Growing oocytes from class A follicles remained at the GV stage, and the Cdc2 kinase activity was kept low during the culture. Interestingly, the cyclin B1 band as well as the phosphorylated 35 and 36 kDa bands of $p34^{cdc2}$ appeared in the cultured oocytes. These bands have also been observed when pig oocytes at metaphase II are treated with vanadate (a tyrosine phosphatase inhibitor) or olomoucine (a Cdk kinase inhibitor) and released from metaphase II arrest to interphase (Lee *et al*., 1999). Based on this previous study, it was thought that Cdc2 kinase was inactivated by tyrosine phosphorylation of $p34^{cdc2}$ molecules rather than degradation of cyclin B1 in response to the treatment. In the present study, it is obvious that cultured growing oocytes accumulate the inactive form of Cdc2 kinase. We presume that the growing oocytes are already equipped with an ability to synthesise cyclin B1, but they cannot resume meiotic maturation because of the lack of mechanisms inverting the inactive form of Cdc2 kinase to the active form through post-translational modifications, that is, dephosphorylation of $p34^{cdc2}$ on Thr 14 and Tyr 15.

Cdc25 phosphatase dephosphorylates Thr 14 and Tyr 15 residues of $p34^{cdc2}$, thereby inducing activation of Cdc2 kinase. In *Xenopus* oocytes, there are two distinct signal transduction pathways for activation of Cdc2 kinase; one including Plk (polo-like kinase), which activates Cdc25, and the other including MAP kinase and $p90^{RSK}$, which suppresses Myt1 (Gross *et al*., 2000). Kosako *et al.* (1994) have suggested that activated MAP kinase directly or indirectly regulates some proteins controlling Cdc2 kinase activity such as Cdc25, Wee1 and CAK. Similar to previous reports on mouse (Harrouk & Clark, 1995; Chesnel & Eppig, 1995) and *Xenopus* oocytes (Chesnel *et al*., 1997), MAP kinase was not activated in incompetent pig oocytes. *Xenopus* oocytes accumulate MEK and MAP kinase during their growth phase, and only fully grown oocytes are capable of activating MAP kinase (Chesnel *et al*., 1997). In the present study, we observed no obvious change in the quantity of MEK and MAP kinase during oocyte growth in the pig. However, growing oocytes from class A follicles neither activated MAP kinase nor resumed meiosis.

The role of MAP kinase in mammalian oocytes is still equivocal. MAP kinase is not likely to be necessary for meiotic resumption of oocytes of species such as mouse, pig, rat and goat, as the activation occurs after GVBD (Inoue *et al*., 1995; Zernicka-Goetz *et al*., 1997; Dedieu *et al*., 1996). Mos−/− mouse oocytes, in which MAP kinase fails to be activated, undergo GVBD and extrusion of the first polar body (Verlhac *et al*., 1994, 1996). Inhibition of MAP kinase activation by U0126, a potent inhibitor of MEK, has no effect on GVBD or Cdc2 kinase activation in mouse oocytes (Kagii *et al*., 2000). Moreover, meiotic resumption and progression through meiosis occurred in bovine oocytes which lacked MAP kinase activity by microinjection of MKP-1 mRNA, which encodes a dual specific MAP kinase phosphatase (Gordo *et al*., 2001). These results clearly demonstrate that at least mouse and bovine oocytes do not require MAP kinase activation in order to resume meiosis. On the other hand, microinjection of active MAP kinase into GV accelerates GVBD in immature pig oocytes (Inoue *et al*., 1998). In addition, Kagii *et al*. (2000) reported in their study using pig oocytes that suppression of MAP kinase activity by U0126 induced inhibitory effects on the activation of Cdc2 kinase and subsequently half the oocytes did not undergo GVBD. They suggested that MAP kinase acts as an indispensable mediator of Cdc2 kinase activation and GVBD in pig oocytes, as in *Xenopus* oocytes, which require MAP kinase activation to inactivate Cdc2-inhibitory kinases (Myt1 or Wee1) in meiotic resumption (Abrieu *et al*., 2001). In the present study, it is unlikely that in growing pig oocytes MAP kinase functions in meiotic resumption, because some of the growing oocytes from class B follicles resumed meiosis by only Cdc2 kinase activation. However, we can not deny the possibility that pig oocytes are equipped with a supportive MAP kinase activating pathway in meiotic resumption during their final growth phase.

Although more than 70% of growing pig oocytes isolated from class B follicles resumed meiosis, it was found that most of them were arrested at metaphase I. These oocytes synthesise cyclin B1 similar to fully grown oocytes from class D follicles. Cdc2 kinase was found to be activated in some of the growing oocytes, although MAP kinase was not completely activated. This result coincided with the Western blotting analysis. There were some oocytes that did not activate either kinase. These oocytes probably remained at the GV stage after culture. Only one growing oocyte activated MAP kinase without activation of Cdc2 kinase. When Cdc2 kinase is blocked by butyrolactone I (a Cdk kinase inhibitor) in fully grown pig oocytes and oocytes treated with okadaic acid (an inhibitor of type 1 and 2A protein phosphatase), they resumed meiosis by means of only MAP kinase activity without activating Cdc2 kinase (Kulbelka *et al*., 1996). Similarly, it has been reported that meiotic resumption occurs by only MAP kinase activation in incompetent mouse oocytes (Chensel & Eppig, 1995).

Progression to metaphase I in growing oocytes from class B follicles was shown to relate to activation of Cdc2 kinase in the present study. After 27 and 42 h of culture, a phosphorylated band of $p34^{cdc2}$ was observed. This band was observed in fully grown oocytes after 42 h. It is possible that $p34^{cdc2}$ in growing oocytes is more susceptible to phosphorylation after meiotic resumption than that in fully grown oocytes. From the present study, we cannot fully explain why the growing oocytes stop at metaphase I. Several recent results suggest that MAP kinase and Cdc2 kinase regulate the metaphase I/anaphase I transition and chromosome separation. MAP kinase has been suggested to be involved in chromosome separation at the metaphase I/anaphase I transition of fully grown pig oocytes, since active MAP kinase localises at the midzone of the elongated spindle (Lee *et al*., 2000). The failure of the metaphase I/anaphase I transition of growing oocytes in the present study may have been due to the incomplete activation of MAP kinase.

Stimulation of growth factor is thought to be transmitted by intracellular signals, activating MAP kinase through the Ras-Raf-MEK pathway (Nishida & Gotoh, 1993; Marshall, 1994). Our results in pig granulosa cells and oocytes coincided well with the hypothesis that their patterns of MEK activation are similar to those of MAP kinase activation. The activation cascade of MAP kinase is well understood in *Xenopus* oocytes. Treatments of *Xenopus* oocytes with progesterone and insulin/IGF-1 lead to activation of MEK-MAP kinase (Fabian *et al*., 1993; Gotoh *et al*., 1995; Gebauer & Richter, 1997). In pig oocytes, when the fully grown oocytes at metaphase I are treated with MEK inhibitor, the activity of the MAP kinase decreases (Lee *et al*., 2000). Meiotically incompetent pig oocytes have shown no activation of MAP kinase and MEK, and growing pig oocytes from class B follicles do not have a sufficient MAP kinase activating pathway to include the activation of MEK, although they are capable of activating Cdc2 kinase.

In summary, pig oocytes acquire meiotic competence in a stepwise manner during their growth; they first become competent to resume meiosis and progress to metaphase I, and then to pass through the metaphase I/anaphase I transition and mature to metaphase II. Keeping step with the acquisition of meiotic competence, the important kinase-activation pathways are established; at first Cdc2 kinase and then MEK-MAP kinase become susceptible to full activation. Therefore, we presume from the present study that the acquisition of competence to resume meiotic maturation is associated with the establishment of the Cdc2 kinase-activation cascade in oocytes which

already have the ability to accumulate cyclin B1 molecules, and the acquisition of competence to complete meiotic maturation is associated with full establishment of the MEK-MAP kinase cascade.

Acknowledgements

The authors would like to thank Drs Yanfeng Dai and R.M. Moor (The Babraham Institute, Cambridge, UK) for their generous gift of anti-pig cyclin B1 antibody. We would like to thank the staff of the Kobe Meat Inspection Office for supplying pig ovaries. This work was supported in part by a grant for Creative Scientific Research (13GS0008) from The Japan Society for the Promotion of Science and a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Abrieu, A., Doree, M. & Fischer, D. (2001). The interplay between cyclin-B-Cdc2 kinase (MPF) and MAP kinase during maturation of oocytes. *J. Cell Sci.* **114**, 257–67.

Chesnel, F. & Eppig, J.J. (1995). Induction of precocious germinal vesicle breakdown (GVB) by GVB-incompetent mouse oocytes: possible role of mitogen-activated protein kinases rather than $p34^{cdc2}$ kinase. *Biol. Reprod.* **52**, 895–902.

Chesnel, F., Bonnec, G., Tardivel, A. & Boujard, D. (1997). Comparative effects of insulin on the activation of the Raf/Mos-dependent MAP kinase cascade in vitellogenic versus postvitellogenic *Xenopus* oocytes. *Dev. Biol.* **188**, 122–33.

Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y. & Kohmoto, K. (1991). Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. *Development* **113**, 789–95.

Christmann, L., Jung, T. & Moor, R.M. (1994). MPF components and meiotic competence in growing pig oocytes. *Mol. Reprod. Dev.* **38**, 85–90.

Dedieu, T., Gall, L., Crozet, N., Sevellec, C. & Ruffini, S. (1996). Mitogen-activated protein kinase activity during goat oocyte maturation and the acquisition of meiotic competence. *Mol. Reprod. Dev.* **45**, 351–8.

Dedieu, T., Gall, L., Hue, I., Ledan, E., Crozet, N., Ruffini, S. & Sevellec, C. (1998). $p34^{cdc2}$ expression and meiotic competence in growing goat oocytes. *Mol. Reprod. Dev.* **50**, 251–62.

de Vantry, C., Gavin, A.C., Vassalli, J.D. & Schorderet-Slatkine, S. (1996). An accumulation of $p34^{cdc2}$ at the end of mouse oocyte growth correlates with the acquisition of meiotic competence. *Dev. Biol.* **174**, 335–44.

Fabian, J.R., Morrison, D.K. & Daar, I.O. (1993). Requirement for Raf and MAP kinase function during the meiotic maturation of *Xenopus* oocytes. *J. Cell Biol.* **122**, 645–52.

Fissore, R.A., He, C.L. & Vande Woude, G.F. (1969). Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. *Biol. Reprod.* **55**, 1261–70.

Gebauer, F. & Richter, J.D. (1997). Synthesis and function of Mos: the control switch of vertebrate oocyte meiosis. *Bioessays* **19**, 23–8.

Gordo, A.C., He, C.L., Smith, S. & Fissore, R.A. (2001). Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. *Mol. Reprod. Dev.* **59**, 106–14.

Gotoh, Y. & Nishida, E. (1995). Activation mechanism and function of the MAP kinase cascade. *Mol. Reprod. Dev.* **42**, 486–92.

Gotoh, Y., Moriyama, K., Matsuda, S., Okamura, E., Kishimoto, T., Kawasaki, H., Suzuki, K., Yahara, I., Sakai, H. & Nishida, E. (1991). *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. *EMBO J.* **10**, 2661–8.

Gotoh, Y., Masuyama, N., Dell, K., Shirakabe, K. & Nishida, E. (1995). Intiation of *Xenopus* oocyte maturation by activation of the mitogen-activated protein kinase cascade. *J. Biol. Chem.* **270**, 25898–904.

Gross, S.D., Schwab, M.S., Taieb, F.E., Lewellyn, A.L., Qian, Y.W. & Maller, J.L. (2000). The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by $p90^{Rsk}$. *Curr. Biol.* **10**, 430–8.

Haraguchi, S., Naito, K. & Sato, E. (1998). MAP kinase cascade, but not ERKs, activated during early cleavage of mouse embryos. *Mol. Reprod. Dev.* **51**, 148–55.

Harrouk, W. & Clarke, H.J. (1995). Mitogen-activated protein (MAP) kinase during the acquisition of meiotic competence by growing oocytes of the mouse. *Mol. Reprod. Dev.* **41**, 29–36.

Hashimoto, N. & Kishimoto, T. (1988). Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. *Dev. Biol.* **126**, 242–52.

Hirao, Y., Tsuji, Y., Miyano, T., Okano, A., Miyake, M., Kato, S. & Moor, R.M. (1995). Association between $p34^{cdc2}$ levels and meiotic arrest in pig oocytes during early growth. *Zygote* **3**, 325–32.

Hyttel, P., Fair, T., Callesen, H. & Greve, T. (1997). Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* **47**, 23–32.

Inoue, M., Naito, K., Aoki, F., Toyoda, Y. & Sato, E. (1995). Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes. *Zygote* **3**, 265–71.

Inoue, M., Naito, K., Nakayama, T. & Sato, E. (1998). Mitogen-activated protein kinase translocates into the germinal vesicle and induces germinal vesicle breakdown in porcine oocytes. *Biol. Reprod.* **58**, 130–6.

Itoh, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsuura, Y., Maeda, A., Shimizu, K. & Takai, Y. (1993). A protein factor for ras p21-dependent activation of mitogen-activated protein (MAP) kinase through MAP kinase kinase. *Proc. Natl. Acad. Sci. USA* **90**, 975–9.

Kagii, H., Naito, K., Sugiura, K., Iwamori, N., Ohashi, S., Goto, S., Yamanouchi, K. & Tojo, H. (2000). Requirement of mitogen-activated kinase activation for the meiotic resumption of porcine oocytes. *J. Reprod. Dev.* **46**, 249–56.

Kosako, H., Gotoh, Y. & Nishida, E. (1994). Requirement for the MAP kinase kinase/MAP kinase cascade in *Xenopus* oocyte maturation. *EMBO J.* **13**, 2131–8.

Kulbelka, M., Kalab, P., Kalous, J., Nagyova, E. & Motlik, J. (1996). The effect of butyrolactone I, a specific inhibitor of cdk-kinase, on GVBD and chromosome condensation during the first meiotic division of pig and cattle oocyte. *Arch. Tierz.* **39**, 53.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–5.

Lee, J., Hata, K., Miyano, T., Yamashita, M., Dai, Y. & Moor, R.M. (1999). Tyrosine phosphorylation of $p34^{cdc2}$ in metaphase II-arrested pig oocytes results in pronucleus formation without chromosome segregation. *Mol. Reprod. Dev.* **52**, 107–16.

Lee, J., Miyano, T. & Moor, R.M. (2000). Localisation of phosphorylated MAP kinase during the transition from meiosis I to meiosis II in pig oocytes. *Zygote* **8**, 119–25.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.* **4**, 82–9.

Masui, Y. & Markert, C.L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129–46.

Miyano, T., Dai, Y., Lee, J., Kano, K. & Moor, R.M. (2000). Degradation of pig cyclin B1 molecules precedes MAP kinase dephosphorylation during fertilisation of the oocytes. *Zygote* **8**, 153–8.

Miyasaka, T., Sternberg, D.W., Miyasaka, J., Sherline, P. & Saltiel, A.R. (1991). Nerve growth factor stimulates protein tyrosine phosphorylation in PC-12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* **88**, 2653–7.

Moor, R.M. & Trounson, A.O. (1977). Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro*. *J. Exp. Zool.* **198**, 155–62.

Motlik, J., Crozet, N. & Fulka, J. (1984). Meiotic competence *in vitro* of pig oocytes isolated from early antral follicles. *J. Reprod. Fertil.* **72**, 323–8.

Muslin, A.J., MacNicol, A.M. & Williams, L.T. (1993). Raf-1 protein kinase is important for progesterone-induced *Xenopus* oocyte maturation and acts downstream of mos. *Mol. Cell Biol.* **13**, 4197–202.

Nagai, T., Ding, J. & Moor, R.M. (1993). Effect of follicle cells and steroidogenesis on maturation and fertilization *in vitro* of pig oocytes. *J. Exp. Zool.* **266**, 146–51.

Naito, K. & Toyoda, Y. (1991). Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocytes. *J. Reprod. Fertil.* **93**, 467–73.

Nebreda, A.R. & Hunt, T. (1993). The c-*mos* proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs. *EMBO J.* **12**, 1979–86.

Nebreda, A.R. Gannon, J.V. & Hunt, T. (1995). Newly synthesized protein(s) must associate with $p34^{cdc2}$ to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes. *EMBO J.* **14**, 5597–607.

Nishida, E. & Gotoh, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends, Biochem. Sci.* **18**, 128–31.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503–8.

Pages, G., Brunet, A., L'Allemain, G. & Pouyssegur, J. (1994). Constitutive mutant and putative regulatory serine phosphorylation site of mammalian MAP kinase kinase (MEK1). *EMBO J.* **13**, 3003–10.

Schultz, R.M. (1991). Meiotic maturation on mammalian oocytes. In *Elements of Mammalian Fertilization*, vol. I, *Basic Concepts* (ed. P.M. Wassarman), pp. 77–104. Boston: CRC Press.

Sobajima, T., Aoki, F. & Kohmoto, K. (1993). Activation of mitogen-activated protein kinase during meiotic maturation in mouse oocytes. *J. Reprod. Fertil.* **97**, 389–94.

Sorensen, R.A. & Wassarman, P.M. (1976). Relationship between growth and meiotic maturation of the mouse oocyte. *Dev. Biol.* **50**, 531–6.

Verlhac, M.H., Kubiak, J.Z., Clarke, H.J. & Maro, B. (1994). Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development* **120**, 1017–25.

Verlhac, M.H., Kubiak, J.Z., Weber, M., Geraud, G., Colledge, W.H., Evans, M.J. & Maro, B. (1996). Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. *Development* **122**, 815–22.

Zernicka-Goetz, M., Verlhac, M.H., Geraud, G. & Kubiak, J.Z. (1997). Protein phasphatases control MAP kinase activation and microtubule organization during rat oocyte maturation. *Eur. J. Cell Biol.* **72**, 30–8.