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Localisation of phosphorylated MAP kinase during the transition from meiosis I to meiosis II in pig oocytes

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Summary

Mitogen-activated protein kinase (MAPK) has been reported to be involved in oocyte maturation in all animals so far examined. In the present study we investigate the expression and localisation of active phosphorylated MAPKs (p44^{ERK1}/p42^{ERK2}) during maturation of pig oocytes. In immunoblot analysis using anti-p44^{ERK1} antibody which recognised both active and inactive forms of p44^{ERK1} and p42^{ERK2}, we confirmed that MAPKs were phosphorylated around the time of germinal vesicle breakdown (GVBD) and the active phosphorylated MAPKs (pMAKs) were maintained until metaphase II, as has been reported. On immunofluorescent confocal microscopy using anti-pMAPK antibody which recognised only phosphorylated forms of MAPKs, pMAPK was localised at the spindle poles in pig mitotic cells. On the other hand, in pig oocytes, no signal was detected during GV stage. After GVBD, the area around condensed chromosomes was preferentially stained at metaphase I although whole cytoplasm was faintly stained. At early anaphase I, the polar regions of the meiotic spindle were prominently stained. However, during the progression of anaphase I and telophase I pMAPK was detected at the mid-zone of the elongated spindle, gradually becoming concentrated at the centre. Finally, at the time of emission of the first polar body, pMAPK was detected as a ring-like structure between the condensed chromosomes and the first polar body, and the staining was maintained even after the metaphase II spindle was formed. The inhibition of MAPK activity with the MAPK kinase inhibitor U0126 during the meiosis I/meiosis II transition suppressed chromosome separation, first polar body emission and formation of the metaphase II spindle. From these results, we propose that the spindle-associated pMAPKs play an important role in the events occurring during the meiosis I/meiosis II transition, such as chromosome separation, spindle elongation and cleavage furrow formation in pig oocytes.

Keywords: MAP kinase, Meiosis, Pig oocytes, Spindle, U0126

Introduction

In most vertebrates, fully grown oocytes which are arrested at meiotic prophase I resume meiosis by hormonal stimuli and progress to metaphase II where they

are arrested again until they finally complete meiosis by fertilisation. This progress from prophase I to metaphase II is called maturation. During maturation a series of conspicuous events such as chromosome condensation, nuclear disassembly and spindle formation occur. Most of these events are induced, at least in part, by phosphorylation of many intracellular substrates by mitotic kinases (Nigg, 1993). Maturation-promoting factor (MPF), which is a master kinase in M phase, plays important roles in the events during maturation, and is activated at both meiotic metaphases (Masui & Markert, 1971). In addition to MPF, mitogen-activated protein kinase (MAPK) has been reported to be involved in oocyte maturation in *Xenopus* (Gotoh *et al.*, 1991a), mouse (Sobajima *et al.*, 1993), rat (Zernicka-Goetz *et al.*, 1997), pig (Inoue *et al.*, 1995), goat (Dedieu

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et al., 1996), cattle (Fissore *et al.*, 1996) and human (Sun *et al.*, 1999). MAPKs are involved in various signal transduction pathways and are also called extracellular signal-regulated kinases (ERKs) (Waskiewicz & Cooper, 1995). The activity of MAPK is regulated by dual phosphorylation on threonine and tyrosine residues by an upstream kinase, MAPK kinase (MEK), and the phosphorylated MAPK is active as a kinase (Kosako *et al.*, 1992; Matsuda *et al.*, 1992). It has been shown that p44^{ERK1} and p42^{ERK2} are activated during oocyte maturation in mammals. In contrast to MPF, whose kinase activity drops transiently during the transition from meiosis I to meiosis II, MAPK activity is maintained during this period. Although MAPK activity has been measured in various species, only a few studies have reported the intracellular localisation of MAPK during oocyte maturation (Verlhac *et al.*, 1993; Inoue *et al.*, 1998).

Here we examined the expression and localisation of active MAPK during pig oocyte maturation, and found that the MAPK during pig oocyte maturation showed a different pattern of localisation from that which has been reported in mouse oocytes and which was examined in pig somatic cells. Since the localisation study implied that MAPK might be involved in the events occurring during the meiosis I/meiosis II transition by associating with the meiotic spindle, we inhibited MAPK activity with U0126, a specific inhibitor of MEK, to investigate the role of MAPK during this period.

Materials and methods

Culture of pig oocytes

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. Following three washes in Dulbecco's phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PBS-PVA), intact healthy antral follicles of 4–6 mm in diameter were dissected in PBS-PVA from ovaries following the technique described by Moor & Trounson (1977). After the follicles had been opened in 25 mM Hepes-buffered medium 199 (Earle's salt, GIBCO, Paisley, UK) containing glutamine and 0.08 mg/ml kanamycin sulphate (Sigma, St Louis, MO), cumulus–oocyte complexes with a piece of parietal granulosa tissue (cumulus–oocyte–granulosa cell complexes) were isolated from the follicles (Mattioli *et al.*, 1988). Following two washes in Hepes-buffered medium 199, cumulus–oocyte granulosa cell complexes were cultured in 2 ml of bicarbonate-buffered medium 199 supplemented with 10% fetal calf serum (FCS, Biocell, Carson, CA), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulphate, 0.1 IU/ml human menopausal gonado-

trophin (hMG, Pergonal, Serono, London, UK) and two everted theca shells with gentle agitation.

In some experiments, to inhibit the activity of MAPKs during the meiosis I/meiosis II transition, metaphase I oocytes which had been cultured for 27 h were denuded, transferred into medium containing various concentrations (0, 0.05, 0.1 or 0.2 mM) of MEK inhibitor U0126 (Promega, Madison, WI) (Favata *et al.*, 1998), and cultured for a further 24 h. All culture was carried out in an atmosphere of 5% CO₂ in humidified air at 38.5 °C.

After culture, some oocytes were denuded of cumulus cells by pipetting, mounted on slides, fixed in acetic acid-ethanol (1:3, v/v) solution, stained with 1% aceto-orcein, and observed under a Normarsky interference microscope. Others were used for immunocytochemistry or Western blot analysis.

Culture of pig somatic cells

Pig aortic endothelial cells were grown in Nutrient mixture F-12 (HAM) with L-glutamine (GIBCO) containing 10% FCS to 30% confluency on glass chamber slides (Lab-Tek 2 well, Nalge Nunc International, Naperville, IL). To obtain relatively high populations in mitosis, the 30% confluent cells were then cultured for 21–24 h in medium devoid of serum followed by a 23 h period in medium containing 10% FCS before fixation. The somatic cells were cultured under the same temperature and gas phase as the oocytes.

Electrophoresis and Western blotting

After denudation, oocytes were rinsed twice in PBS-PVA, dissolved in 15 µl of Laemmli sample buffer (Laemmli, 1970), boiled for 3 min, and frozen at –20 °C before use. Samples were run on 10% SDS-polyacrylamide gels, and proteins were electroblotted onto hydrophobic polyvinylidene difluoride membranes (Immobilin, Millipore, Bedford, MA) for 1.5 h. The membranes were blocked with 10% FCS in PBS containing 0.1% Tween20 (PBS-Tween) for 2 h, then incubated with either rabbit polyclonal rat ERK1 antibody (#SC-94, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution or rabbit polyclonal anti-phosphop44/42 MAPK antibody directed against human phosphorylated ERK1 (#9101S, New England Biolabs, Beverly, MA) at a dilution of 1:1000 for 4 h in 10% FCS in PBS-Tween. Both antibodies recognise not only (phosphorylated) ERK1 but also (phosphorylated) ERK2. After three washes in PBS-Tween, the membranes were treated with horseradish-peroxidase-labelled anti-rabbit immunoglobulins (1:1000, Dako, Glostrup, Denmark) in blocking buffer for 1 h at room temperature. After three washes of 10 min each with PBS-Tween, peroxidase activity was visualised using the

ECL Western blotting detection system (Amersham Life Science).

Immunocytochemistry

After being washed twice in PBS-PVA, denuded oocytes were fixed in 4% paraformaldehyde either in PBS-PVA or PHEM (60 mM PIPES, pH 6.9, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$) containing 0.2% Triton X-100 for 40 min. Both buffer solutions gave essentially the same results. The fixed oocytes were washed twice in PBS-PVA for 15 min each, then stored in 1% bovine serum albumin (BSA)-supplemented PBS-PVA (BSA-PBS-PVA) at least overnight. Thereafter oocytes were blocked with 10% goat serum in BSA-PBS-PVA for 45 min, and incubated with anti-phospho-p44/42 MAPK antibody (1:250 dilution) or rat anti- α -tubulin antibody (1:100 dilution, MAS 078, Harlan Sera, Sussex, UK) at 4 °C overnight. After washing three times in BSA-PBS-PVA for 15 min each, the oocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or anti-rat IgG (1:40, Dako) for 40 min at room temperature. After washing three times in BSA-PBS-PVA for 15 min each, DNA was counterstained with propidium iodide (Sigma).

Somatic cells were simply washed once with PBS-PVA, fixed in 4% paraformaldehyde in PHEM for 10 min, washed twice in PBS-PVA for 5 min each, then permeabilised with 0.2% Triton X-100 supplemented PHEM for 5 min. After a further two washes as above, cells were blocked with 10% goat serum in BSA-PBS-PVA, and incubated with the anti-phospho-MAPK antibody in BSA-PBS-PVA at appropriate dilutions for 1 h at room temperature. After washing twice, cells were incubated with the secondary antibody and subjected to DNA counterstaining with propidium iodide.

Confocal analysis was carried out using the Bio-Rad MRC 500 or 1024 system. At least 20 oocytes were stained and observed at each meiotic stage.

Results

Localisation of phosphorylated MAPK during pig oocyte maturation

The majority of the pig oocytes cultured in our culture system underwent germinal vesicle breakdown (GVBD) at 20–22 h and proceeded to metaphase I at 24–27 h, to anaphase I and telophase I at 30–33 h, and finally were arrested at metaphase II after 36 h. During maturation, each of the MAPKs (p44^{ERK1} and p42^{ERK2}) was detected as two bands, i.e. upper (u) and lower (l) bands in the immunoblot (Fig. 1). The u- and l-bands represent the active phosphorylated form and inactive

non-phosphorylated form of MAPKs respectively (Inoue *et al.*, 1995). During GV stage only 1-bands of p44^{ERK1} and p42^{ERK2} were detected (Fig. 1a). After GVBD, u-bands were seen. The active pMAPKs were detected throughout the progression from metaphase I to metaphase II. These results are consistent with previous reports in pig oocytes (Inoue *et al.*, 1995, 1998).

To examine the localisation of the active MAPK during pig oocyte maturation, we used the antibody against phosphorylated MAPK (pMAPK) in immunofluorescent confocal microscopy. The anti-pMAPK antibody reacted with only the u-bands of both p44^{ERK1} and p42^{ERK2}, indicating that the antibody specifically recognised only pMAPKs but not inactive MAPKs (Fig. 1b). Using this antibody, we first examined the localisation of pMAPK in pig somatic cells, and later compared these results with the localisation of pMAPK during meiosis in pig oocytes. In interphase (Fig. 2a), no signal or an extremely weak signal was detected diffusely in both nucleus and cytoplasm. During mitotic division, pMAPK was localised specifically at the spindle poles, then dissociated from the poles at the end of M phase (Fig. 2b–h). On the other hand, in pig oocytes, no signal was detected at GV stage (Fig. 2i). After GVBD, the entire cytoplasm of maturing oocytes was faintly stained until metaphase II (Fig. 2j–p). During metaphase I, the preferential staining in the vicinity of the condensed chromosomes was observed at a relatively early stage (Fig. 2j), but hardly detected at later stages (Fig. 1k). At early anaphase I, the polar regions of meiotic spindle were stained (Fig. 2l). Then, at late anaphase, the interzonal region of the spindle was stained (Fig. 2m). As the spindle elongated, the pMAPK signal became concentrated at the centre of the spindle mid-zone (Fig. 2n, o). The staining was finally detected as a ring-like structure between condensed chromosomes and the first polar body, and this staining was maintained during metaphase II (Fig. 2p).

Effects of MAPK inhibition by an MEK inhibitor U0126 on the meiosis I/meiosis II transition

To investigate the roles of MAPKs during the meiosis I/meiosis II transition, metaphase I oocytes which had been cultured for 27 h were cultured in medium containing various concentrations (0.05, 0.1 and 0.2 mM) of the MEK inhibitor U0126 for 24 h. First, in immunoblot analysis, we verified that these concentrations of U0126 completely inhibited the expression of active pMAPK in the treated oocytes while MAPKs remained phosphorylated in the control oocytes which were cultured in U0126-free medium for 24 h (Fig. 1c). Almost all the oocytes which were cultured in the presence of the inhibitor did not form a metaphase II spindle, while most of the oocytes which had been cultured in U0126-free medium did (Table 1). Further, in the

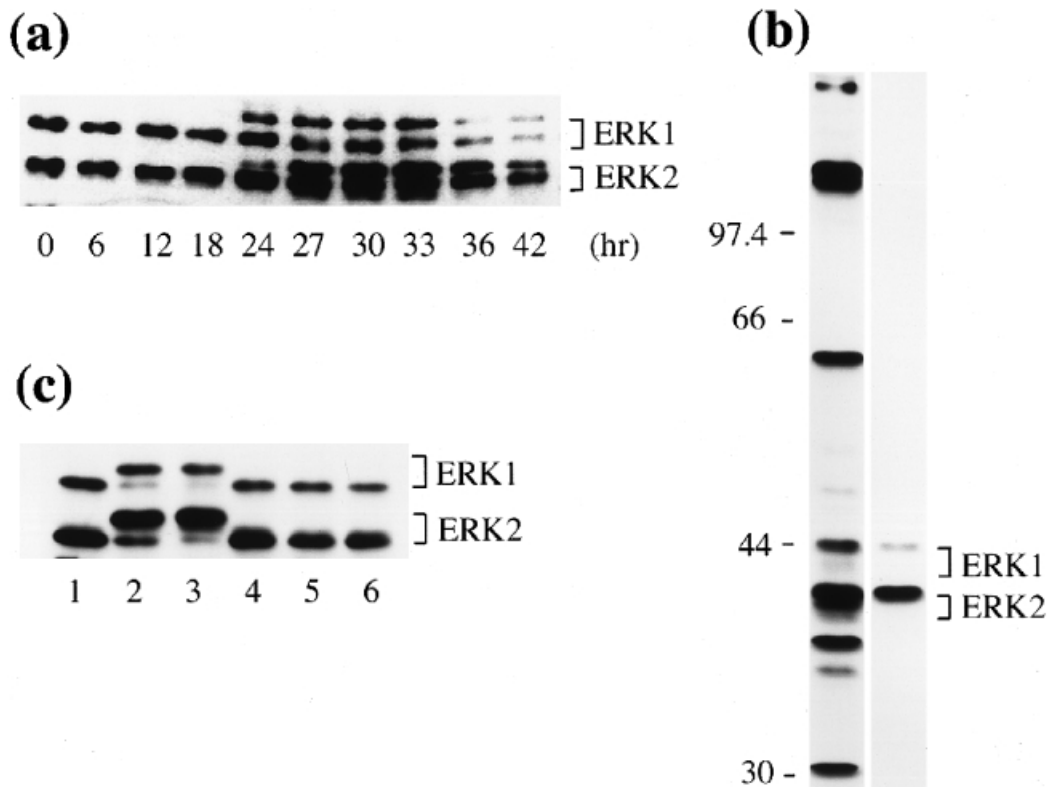


Figure 1 (a) Changes in the electrophoretic mobility of anti-MAPK antibody-reactive bands (p44^{ERK1} and p42^{ERK2}) during maturation of pig oocytes. Pig oocytes were cultured and harvested at various times indicated below the blot, then the whole lysates were subjected to immunoblot analysis with anti-MAPK antibody. Fifty oocytes were used per lane. (b) The specificity of anti-phosphorylated MAPK antibody. The whole lysates of 50 metaphase II oocytes were subjected to immunoblot analysis with anti-MAPK (left) and anti-phosphorylated MAPK antibodies. Positions of molecular weight markers are shown on the left. (c) The inhibition of MAPK phosphorylation by an MEK inhibitor, U0126, in metaphase I oocytes. Metaphase I oocytes which had been cultured for 27 h were cultured for a further 24 h in various concentrations of U0126-containing medium or U0126-free medium. The whole lysates of germinal vesicle stage oocytes (lane 1), metaphase I oocytes (lane 2), metaphase I oocytes which were cultured in U0126-free medium (lane 3), or in 0.05 mM (lane 4), 0.1 mM (lane 5) and 0.2 mM (lane 6) U0126-containing medium were subjected to immunoblot analysis with anti-MAPK antibody. Fifty oocytes were used per lane.

presence of relatively high concentrations (0.1 or 0.2 mM) of U0126, the majority of oocytes did not undergo either chromosome separation or first polar body emission, but had a chromatin cluster in the ooplasm after 24 h (Table 1). Irregular microtubule arrays, which apparently differed from the arrays of normal spindle microtubules, were observed around the chromatin cluster (Fig. 3). On the other hand, in the presence of a low concentration (0.05 mM) of U0126, the oocytes emitted the first polar body, and two-thirds of the oocytes eventually formed a pronucleus (Table 1).

Discussion

During pig oocyte maturation, MAPKs were activated around the time of GVBD and the active forms of MAPKs were maintained during the following maturation period, as has been reported in various animal oocytes. A new finding in the present study is pro-

vided by the immunofluorescent confocal microscopy using anti-pMAPK antibody. In mouse oocytes, MAPK has been found at microtubule-organising centres (MTOCs) at spindle poles as well as in the cytoplasm (Verlhac *et al.*, 1993). In the present study, active pMAPK was localised at the spindle poles in somatic cells during the process of spindle formation and chromosome segregation. It has been demonstrated in *Xenopus* egg extracts that the addition of active MAPK induces a change in the pattern of microtubule nucleation from the MTOCs from interphase to M phase states (Gotoh *et al.*, 1991b). Therefore, in mitotic cells and mouse oocytes, MAPK probably contributes to microtubule nucleation from the MTOCs. On the other hand, pMAPK in pig oocytes showed a different localisation pattern. After GVBD, the active pMAPK was localised in the vicinity of the condensed chromosomes at early metaphase I, around the polar regions of the spindle at early anaphase I, at the spindle mid-zone during anaphase I and telophase I, and finally at a ring-

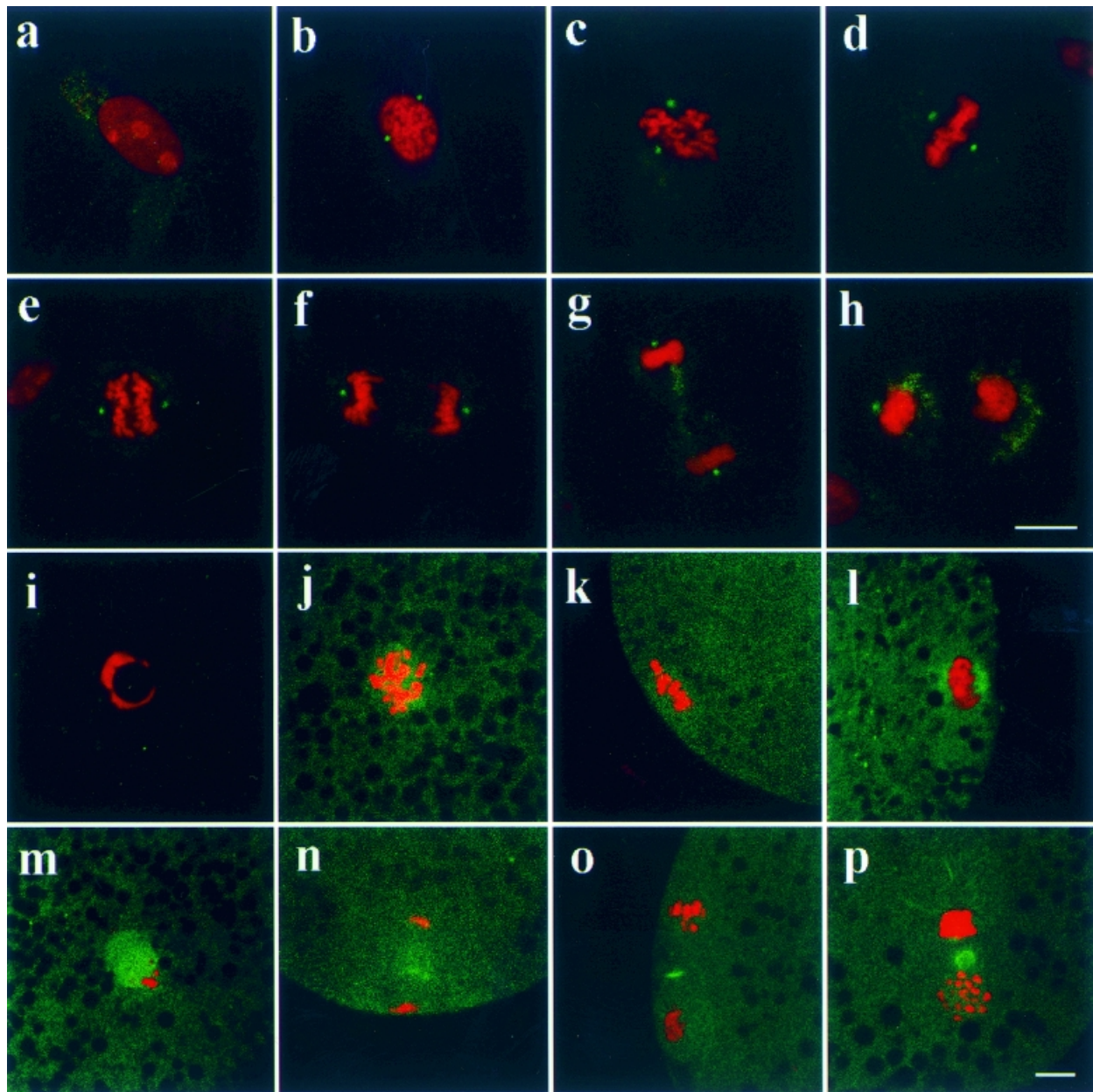


Figure 2 Immunofluorescent localisation of phosphorylated MAPK in pig aortic endothelial cells during mitosis (*a-h*) and in pig oocytes during meiosis (*i-p*). Phosphorylated MAPK appears in green and DNA appears in red. (*a*) Interphase, (*b*) prophase, (*c*) prometaphase, (*d*) metaphase, (*e*) early anaphase, (*f*) late anaphase, (*g*) telophase, (*h*) cytokinesis, (*i*) germinal vesicle stage, (*j*) early metaphase I, (*k*) late metaphase I, (*l*) early anaphase I, (*m*) late anaphase I, (*n*) telophase I, (*o*) cytokinesis, (*p*) metaphase II. Scale bar represents 10 µm.

like structure which, we presumed from its shape and the location, may be a contractile ring, during first polar body emission and metaphase II. The faint staining of the entire cytoplasm after GVBD probably reflects not non-specific signal but diffuse localisation of pMAPK, since the staining was not detected during GV stage. What causes the difference in pMAPK localisation at metaphase? We presume that the reason lies in the difference in the meiotic spindle between these two species. In contrast to mouse oocytes, maturing

pig oocytes do not have a MTOC either at the spindle poles or in the cytoplasm (Kim *et al.*, 1996). Further, discrete spots of γ -tubulin are not observed at pig meiotic spindle poles (our unpublished data) while mouse meiotic spindles have several γ -tubulin foci at their poles (Gueth-Hallonet *et al.*, 1993). However, interestingly, at early anaphase I we found pMAPK at the polar regions of pig meiotic spindle, although the signal was ambiguous and did not show clear spots as seen in somatic cells or mouse oocytes. The localisation

Table 1 Effects of U0126 on the progression from MI to MII during pig oocyte maturation

Time of U0126 treatment (h)	Concentration of U0126 (mM)	No. of oocytes examined	No. (%) of oocytes displaying normal maturation stages			No. (%) of oocytes existing from meiosis during MI–MII transition			No. (%) of oocytes degenerating
			MI	AI–TI	MI	1CC	1PB+1CC	1PB±1FP	
0	–	44	43 (98)	1 (2)	0	0	0	0	0
24	0	43	6 (14)	0	36 (84)	0	0	0	1 (2)
24	0.05	43	0	0	0	0	14 (33)	28 (65)	1 (2)
24	0.1	42	0	1 (2)	1 (2)	37 (88)	3 (7)	0	0
24	0.2	42	2 (5)	3 (7)	0	36 (86)	1 (2)	0	0

MI oocytes which had been cultured for 27 h were cultured in medium containing various concentrations of U0126 for 24 h. After culture, oocytes were fixed and stained, and the nuclear morphology determined under a Nomarsky interference microscope.

MI, first metaphase; AI, first anaphase; TI, first telophase; MII, second metaphase; CC, chromatin cluster; PB, first polar body; FP, female pronucleus.

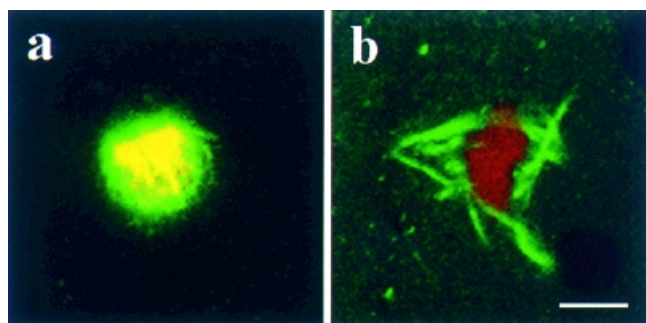


Figure 3. Disruption of spindle microtubule organisation by the MEK inhibitor, U0126. Metaphase I oocytes before (a) and after (b) treatment with 0.1 mM U0126 were stained with anti- α -tubulin antibody and propidium iodide. Microtubules appear in green and DNA appears in red. Scale bar represents 5 μ m.

of pMAPK in the spindle mid-zone during anaphase I and telophase I resembles the localisation of MAPK in tobacco cells. Recently, it has been reported that the pathway of spindle assembly in higher plant cells and animal oocytes, both of which are centrosome-free cells, differs from that in animal somatic cells (Vernos & Karsenti, 1995; Merdes & Cleveland, 1997). In tobacco cells, MAPK is transiently localised to the cell plate in anaphase cells, in the middle of the two microtubule arrays characteristic of the phragmoplast, a plant-specific structure involved in laying down the new cell wall, suggesting that MAPK may have a role in cytokinesis (Calderini *et al.*, 1998). The present localisation study also suggests that MAPK may be involved in spindle elongation and cytokinesis in animal oocytes.

To investigate the role of MAPKs during the meiosis I/meiosis II transition, MAPK activation was inhibited by an inhibitor of MEK, U0126, during the period. The effect of the inhibitor on meiotic progression varied according to the concentration, although every concen-

tration of U0126 inhibited normal progression to metaphase II. At higher concentrations, U0126 inhibited chromosome separation and the treated oocytes formed a chromatin cluster with irregular arrays of microtubules. Thus, MAPK might be involved in chromosome separation at the metaphase I/anaphase I transition either by directly associating with chromosomes (see Fig. 2j; some of the pMAPK staining appeared on chromosomes) or spindle microtubules (see Fig. 2l, m). At a lower concentration, the inhibitor did not prevent chromosome separation and the following first polar body emission, but prevented the formation of metaphase II spindle and induced pronucleus formation. Although it is not clear why the results differ according to the concentration of inhibitor, our interpretation is that at a lower concentration the suppression of MAPK activity might occur after the oocytes have accomplished first polar body emission while the higher concentrations of U0126 might take effect immediately. The formation of a pronucleus probably results from the failure of reactivation of MPF, which requires a Mos-MAPK pathway during meiosis I/meiosis II (Faruno *et al.*, 1994). On the other hand, the formation of a chromatin cluster at higher concentrations may arise from the maintenance of high MPF activity due to the failure of cyclin B degradation through the spindle assembly checkpoint (Hunt *et al.*, 1992; Minshull *et al.*, 1994). Alternatively, it may result from the failure of dissociation of synapses in bivalent chromosomes, which may be a prerequisite for the induction of chromosome decondensation following MPF inactivation (Hashimoto & Kishimoto, 1988).

In conclusion, pMAPK shows a unique localisation in a meiotic progression-dependent manner, and may have potential roles in chromosome separation, spindle elongation and first polar body emission during the meiosis I/meiosis II transition in pig oocytes.

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