MAPK regulates cell cycle progression in pig oocytes and fertilized eggs

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Abstract Oocytes collected from prepubertal gilt ovaries were matured *in vitro* (IVM), and fertilized *in vitro* (IVF) or electrically activated. Phosphorylation of mitogen-activated protein kinase (MAPK) was detected with SDS-PAGE and Western blotting, and translocation of ERK$_2$ was observed with immunofluorescent cytochemistry. We found that the quantity of MAPK kept unchanged during oocyte maturation. There was no phosphorylated MAPK in porcine oocytes at the germinal vesicle (GV) stage; a little MAPK was phosphorylated at 20 h of IVM; a high level phosphorylation was detected at 30 h, while MAPK phosphorylation decreased at 36 h; and then MAPK phosphorylation increased again to the peak level from 40 to 60 h. ERK2 translocated from the peripheral cytoplasm to inner cytoplasm and nuclear area during oocyte maturation. There was nearly no phosphorylated MAPK at 18 and 20 h of electrically activated oocytes, but phosphorylation increased at 22 h. There was no phosphorylated MAPK at 12 h of IVF, while phosphorylation resumed at 16 h. These results suggest that MAPK may play an important regulatory role in MI-MII transition, pronucleus formation and the initiation of the first mitosis in pig eggs.

Keywords: porcine oocyte, MAPK, IVM, IVF.

Mitogen-activated protein kinase (MAPK), also termed extracellular signal-regulated protein kinase (ERK), is the central transducer of extracellular signals, such as hormones, growth factors and cytokines. It is one of the key signal molecules in the regulation of cell cycle and is widely expressed in somatic cells.

Mammalian oocytes are generated in fetus and are arrested at diplotene stage of the first meiosis. During oocyte growth, preparing for fertilization and development of early embryos, much mRNA is transcribed and a large quantity of proteins is synthesized and stored. After puberty oocytes resume the first meiosis as indicated by germinal vesicle breakdown (GVBD) under the stimulation of gonadotropin, followed by condensation of chromatin, organization of spindle and translocation of organelle[1,2]. Meiosis of oocytes was mainly controlled by phosphorylation and dephosphorylation of protein kinase cascade[3,4], and MAPK cascade plays an important role in this process[5]. We have reported the regulatory function of MAPK on maturation and fertilization of oocytes in rodents and humans[6—10]. Recently, we also reported the regulatory roles of MAPK and its downstream molecule p90rsk in maturation, fertilization and microtubule organization of porcine oocytes[11—15]. But the function of MAPK during MI-MII transition and first mitosis initiation of porcine oocytes has not been investigated.

In this note, phosphorylation/dephosphorylation of MAPK during IVM, IVF and electrical activation of porcine oocytes were examined with an antibody reacted with phosphorylated form of MAPK, and the functions of MAPK during MI-MII transition and the first mitosis of porcine oocytes were discussed.

1 Materials and methods

（ⅰ）Reagents. All the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz,CA,USA): ERK2 (C-14, cat# sc-154): rabbit polyclonal IgG against rat ERK2; p-ERK(E4, cat# sc-7383): mouse monoclonal IgG$_{2a}$ against human p-ERKs; second antibodies include HRP-labeled rabbit antimouse IgG, HRP-labeled goat anti-rabbit IgG; all chemicals used in this study were purchased from Sigma Chemical Company (St.Louis, MO) unless otherwise stated.

（ⅱ）Collection of porcine oocytes. Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory within 1—1.5 h in a thermos filled with 0.9% (*w/v*) NaCl solution containing 75 mg/L potassium penicillin G and 50 mg/L streptomycin sulphate at 30—35℃. Follicular fluid from antral follicles of 2—5 mm in diameter was aspirated. After washing, only intact cumulus-oocyte complexes (COCs) with uniform cytoplasm and several layers of cumulus cells were selected for maturation culture.

（ⅲ）*In vitro* maturation of oocytes. *In vitro* maturation of oocytes was carried out basically according to the previous reports[16,17]. The medium used for the maturation culture was TCM-199 (with Earle's salts, Gibco, Grand Island, NY) supplemented with 2.92 mmol/L calcium lactate, 0.91 mmol/L sodium pyruvate, 0.57 mmol/L cysteine, 3.05 mmol/L D-glucose, 75 mg/mL potassium penicillin G, 50 mg/mL streptomycin sulphate, 10% heat inactivated fetal calf serum (FCS, Gibco), 10 IU/mL PMSG and 10 IU/mL hCG. Each 50—60 oocytes were cultured in a 500 mL droplet of the same medium which had previously been covered with warm paraffin oil (light mineral oil; Fisher) in a polystyrene culture dish and equilibrated in an atmosphere of 5% CO_2 in air for more than 3 h at 38.5℃. After 22 h culture the oocytes were washed 3 times and transferred into another 500 μL droplet of the same maturation medium without hormonal supplements and cultured for another 22 h or the designed hours. After culture, oocytes were free of cumulus and

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corona cells by treatment with maturation medium containing 0.02% (*w/v*) hyaluronidase, followed by repeated passage through a fine pipette. Only cumulus-free oocytes were used in the following experiments.

(ⅳ) *In vitro* fertilization of oocytes. Cumulus-free oocytes were washed three times and a group of 25—30 oocytes were transferred into a 50-μL droplet of insemination medium covered with paraffin oil. The insemination medium was modified Tris-buffered medium (mTBM), consisting of 110.0 mmol/L NaCl, 0.47 mmol/L KCl, 7.5 mmol/L $CaCl_2$, 0.5 mmol/L Na-pyruvate, 10 mmol/L glucose, 20 mmol/L Tris, 2 mmol/L caffeine, and 2 mg/mL BSA(A-7888, Sigma). The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. Central part semen with rich spermatozoa was collected. Before use, the semen was diluted in Dubecco's phosphate-buffered saline (DPBS), washed by centrifuge at $1900 \times g$ for three times, 5 min each time. Spermatozoa were resuspended with mTBM to get a concentration of 2×10^5 cells/mL. For frozen semen, one 0.2-mL frozen semen pellet was thawed at 39℃ in 10 mL of DPBS containing 1 mg/mL BSA and antibiotics. After being washed three times, spermatozoa were resuspended with mTBM to give a concentration of 1×10^6 cells/mL. And 50 μL of the sample was added to a 50 μL fertilization drop containing the oocytes. Six hours after insemination at 39℃ in an atmosphere of 5% CO_2 in air, oocytes were cultured in 500 μL of culture medium, NCSU23 containing 4 mg/mL BSA (A-8022, Sigma), in a four-wall culture plate until examination.

(ⅴ) Parthenogenetic activation. The oocytes were activated by electrical pulse. Briefly, after washing three times in the electroportion medium (0.28 mol/L mannitol, 0.05 mmol/L $CaCl_2$, 0.1 mmol/L $MgSO_4$, and 0.01% BSA), cumulus-free oocytes were put in a fusion chamber. Two 80 vs. pulses at 120 KV/cm DC were selected using an embryo manipulation system. The oocytes were then washed six times and cultured in NCSU23 containing 0.04% BSA.

(ⅵ) Electrophoresis and immunoblotting. Electrophoresis and immunoblotting were conducted basically by the method we previously reported[12]. A total of 30 eggs were collected in sodium dodecyl sulfate(SDS) sample buffer and heated to 100℃ for 4.5 min. After cooling on ice for 4 min and centrifuging at $16000 \times g$ for 5 min, samples were frozen at −70℃ until use. The proteins were separated by SDS-PAGE with a 4% stacking gel for 30 min at 90 V and a 10% running gel for 2 h at 120 V, then electrophoretically transferred onto PROTRAN nitrocellulose membrane for 2 h, at 200 mA and 4℃. After blocking for 2 h in TBS buffer (20 mmo/L Tris, 137 mmol/L NaCl, pH7.4) containing 0.1% Tween-20(TBS-T)

and 5% skimmed milk, the membrane was incubated for 2 h at 37℃ with TBS-T buffer containing 0.5% nonfat milk and 1∶1000 monoclonal p-MAPK antibody that selectively recognizes the dually phosphorylated form of $p42ERK_2$ and $p44ERK_1$.

After two washes of 15 min each in TBS-T, the membrane was incubated for 1 h at 37℃ with rabbit anti-mouse IgG diluted 1∶2000 in TBS-T containing 0.25% skimmed milk. The membrane was washed twice in TBS-T and then processed with the enhanced chemiluminesence (ECL) detection system.

For reprobing, the blots were stripped of bound antibodies by washing in stripping buffer (100 mmol/L β-mercaptoethanol; 20% SDS, 62.5 mmol/L Tris pH6.7) at 50℃ for 30 min. The membrane was reprobed with polyclonal anti-ERK_2 diluted 1∶300 using the same procedure as described above. The relative p-ERK2 quantity in different development stages was determined by the relative p-ERK2 intensity obtained by densitometric scan of the band.

(ⅶ) Imunofluorescent cytochemistry. Immunofluoresent staining was performed in 50 μL droplets in 35 mm four-wall culture dishes. Denuded oocytes were stripped off zona pellucida in 0.5% pronase, fixed by 4% paraformaldehyde in PBS for 1 h at room temperature, permeabilized in 0.1% Triton X-100 at 37℃ for 5 min, and blocked by 3% BSA in PBS (blocking solution) at 37℃ for 1 h. Then they were cultured in 1∶50 anti-ERK_2 antibody in blocking solution at 37℃ for 1.5 h or at 4℃ overnight. After being washed in M199, they were cultured in 1∶300 FITC-labeled IgG in blocking solution for 1 h at room temperature. At the end, they were cultured in 10 μg/mL PI for 15 min to localize DNA. After mounting on a slide, oocytes were observed with confocal microscopy.

2 Results

(ⅰ) Porcine oocyte development after *in vitro* culture, *in vitro* fertilization and electrical activation. Table 1 shows that porcine oocytes were at GV stage from 0 to 20 h of culture, and at GVBD stage at 24 h, at MI stage from 30 to 36 h, at MII stage from 40 to 44 h. Thus, porcine oocytes normally matured in this culture system.

Table 2 shows that fertilization rates of porcine oocytes were 87.5% and 64.1% when fresh semen and frozen semen were used, respectively. Thus, fresh semen was used in Western blotting experiments. Pronucleus rate was 94.1% when oocytes were electrically activated, which was similar with that when fresh semen was used for insemination.

Table 1   Porcine oocyte maturation after *in vitro* culture

Time/h	GV stage	GVBD stage	MI stage	MII stage	Degenerated
0	65 (94.2%)	4 (5.8%)			
12	43 (75.4%)	11 (19.3%)	3 (5.3%)		
20	35 (71.4%)	6 (12.3%)	5 (10.2%)	2 (4.1%)	1 (2.0%)
24	17 (27.4%)	29 (46.8%)	14 (22.6)	2 (3.2%)	
30	5 (7.8%)	20 (31.3%)	27 (42.2%)	9 (14.1%)	3 (4.7%)
36	2 (3.6%)	7 (12.5%)	30 (53.6%)	16 (28.6%)	1 (1.8%)
40		3 (4.8%)	17 (27.4%)	39 (62.9%)	3 (4.8%)
44		1 (2.0%)	8 (16.3%)	38 (77.6%)	2 (4.1%)

Table 2   Pronuclear formation of porcine oocytes at 12 h after IVF or at 18 h after electric activation

	Fresh semen	Frozen semen	Electric activation
1 PN	74/104 (71.2%)	40/64 (62.5%)	15/85 (17.6%)
2 PN	6/104 (5.8%)	1/64 (1.6%)	7/85 (8.2%)
≥3 PN	11/104 (10.6%)	0/64 (0%)	58/85 (68.2%)
Total	91/104 (87.5%)	41/64 (64.1%)	80/85 (94.1%)

（ⅱ）Phosphorylation/dephosphorylation of MAPK in porcine oocytes after IVM, IVF and electric activation as revealed by Western blotting. Fig. 1 shows that MAPK was not phosphorylated at GV stage; phosphorylation of MAPK began at 20 h of culture, reached a peak level at 30 h, but decreased at 36 h, and then increased to the peak level again from 40 to 60 h. Fig. 2 shows that phosphorylation of MAPK kept at a high level at 10 h after electric activation; there was nearly no phosphorylated MAPK at 18 or 20 h after electric activation; phosphorylation reached to a high level from 22 to 28 h. Fig. 3
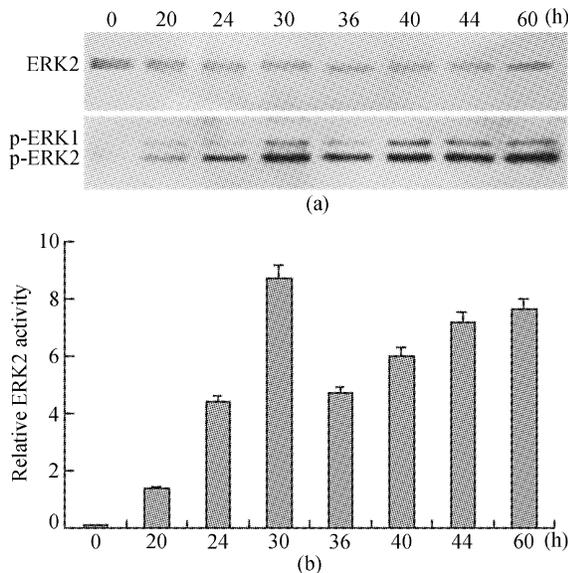
Fig. 1. (a) Expression and phosphorylation of MAPK in porcine oocytes during IVM. This blot represents one of the 3 repeated experiments with similar results; (b) Relative ERK2 activity changes during IVM. Relative ERK2 activity was normalized in each of gels of an experiment by using the total amount of ERK2 in the upper sets of lanes.
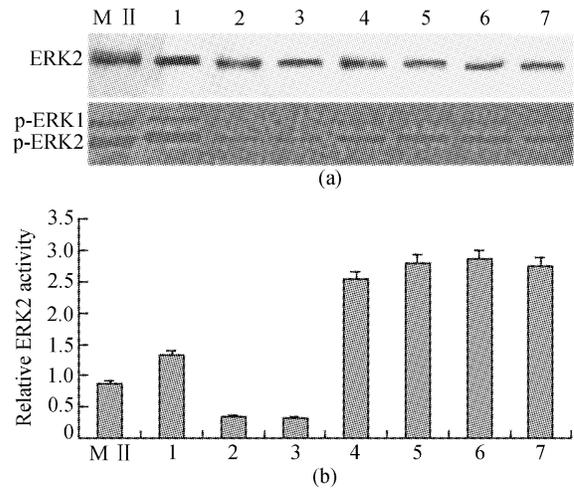
Fig. 2. (a) A Expression and phosphorylation/dephosphorylation of MAPK in porcine oocytes after electric activation (EA). This blot represents one of the 3 repeated experiments with similar results. (b) Relative ERK2 activity after electric activation. Lanes 1—7 represent 10, 18, 20, 22, 24, 26 and 28 h after EA, respectively.
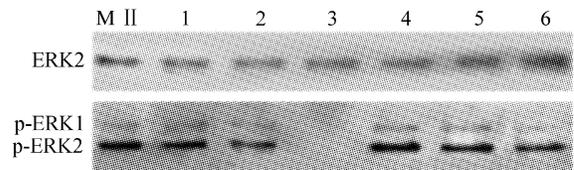
Fig. 3. Expression and phosphorylation of MAPK in porcine oocytes after IVF. This blot represents one of the three repeated experiments with similar results. Lanes 1—6 represent 0, 6, 12, 16, 18 and 22 h after IVF, respectively.

shows that phosphorylation of MAPK decreased at 6 h after IVF, disappeared at 12 h after IVF, while a high level phosphorylation was detected from 16 to 22 h after IVF.

(iii) Translocation of ERK_2 in porcine oocytes during IVM. Plate Ⅰ-1, 3, 5 show that ERK_2 was localized in the peripheral cytoplasm at GV stage and there was no ERK_2 distribution in the germinal vesicle. Plate Ⅰ-2, 4, 6 show that ERK_2 translocated to the inner cytoplasm and the nuclear region at MII stage.

3 Discussion

Researches were conducted to clarify the function of MAPK cascade in mammalian oocyte maturation and fertilization[6,7,9,18—20]. It is shown that MAPK plays important roles in regulation of oocyte meiotic maturation, MII block, fertilization and pronucleus formation, especially the organization of microtubule and nuclear membrane breakdown. There was no phosphorylated MAPK at the GV stage, a little phosphorylated MAPK around GVBD, and much phosphorylated MAPK at or after the MI stage. In this note, we showed that, along with the phosphorylation, MAPK translocated to the inner cytoplasm including the nuclear area during porcine oocyte maturation, suggesting that MAPK is probably related with organization of microtubules. Phosphorylation of MAPK decreased at 36 h of culture when oocytes changed from MI to MII, suggesting that the MI-MII transition may be related with inactivation of MAPK. But such a result has not been reported in other mammalian oocytes, even in bovine oocytes which take a long time to mature[18]. The exact role of this change still need further investigation.

Mammalian oocytes are arrested at the MII stage until fertilization or parthenogenetic activation. MII arrest in mammalian oocytes is kept by cytostatic factor (CSF)[21]. Mos, as an indispensable component of CSF, keeps a high level activity of MAPK and MPF (maturation promoting factor). Mos stabilizes MPF and arrests oocytes at the MII stage by the way of phosphorylation of MAPK. Mouse oocytes whose gene of mos has been knocked out (mos$^{-/-}$) fail to arrest at MII stage, but spontaneously enter parthenogenesis[22,23]. In our experiments, phosphorylation of MAPK kept high even at 60 h of culture when oocyte was still at the MII stage, showing that MAPK is closely related to MII arrest. To the contrary, phosphorylation of MAPK decreased at the MI-MII transition. This proved on the other hand that phosphorylation of MAPK played important roles in MII arrest.

MAPK is highly phosphorylated in MII mouse oocytes. MAPK phosphorylation even goes higher 1 or 2 h after fertilization, but decreases to the level in MII oocytes at 4 h after fertilization. Then MAPK phosphorylation decreases and disappears at 7—8 h after fertilization[6,24]. Correspondingly, pronucleus does not form in mouse oocyte at 4 h, while along with pronuclear formation, MAPK phosphorylation decreases. When pronucleus is formed 8 h after fertilization, MAPK is completely dephosphorylated. Dephosphorylation of MAPK is closely related with pronuclear formation in mouse fertilized eggs. In this note, we reported that phosphorylation of MAPK decreased in porcine oocytes at 6 h after fertilization and disappeared 12 h after fertilization, at the time when pronucleus is formed. This result is consistent with that obtained in mouse oocytes. This shows that dephosphorylation of MAPK is closely related with pronuclear formation in porcine oocytes. In addition, dephosphorylation of MAPK in oocytes that had been electrically activated also proved this conclusion. Phosphorylation of MAPK markedly decreased at 18 or 20 h after electric activation when pronucleus is observed. After pronucleus formation in fertilized or electrically activated oocytes, phosphorylation of MAPK increased. This is possibly related with the initiation of the first mitosis. The exact role of this increase in phosphorylation of MAPK needs to be further clarified.

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