Mos and the Mitogen-Activated Protein Kinase Do Not Show Cytostatic Factor Activity in Early Mouse Embryos

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Abstract. Mos and the mitogen-activated protein kinase (MAPK) cascade have been established as crucial regulators of second meiotic metaphase arrest, the so-called CSF arrest, in mammalian oocytes. They are also thought to play a role in regulating mitotic metaphase arrest of early mammalian embryos. In the present study, we examined whether mitotic arrest is induced in early mouse embryos by activation of extracellular signal-regulated kinases (ERKs), which are major MAPKs in mouse eggs, and their substrate, p90Ribosomal S6 kinase (RSK), as reported in Xenopus embryos. Wild-type Mos (wt-Mos), degradation-resistant Mos mutant (P2G-Mos) or constitutive active mutant of MAPK/ERK kinase, MEK (SDSE-MEK), was expressed in early mouse embryos by injecting the respective expression vectors into the pronucleus of fertilized eggs, and the developmental rates were then examined up to 72 h after insemination. Expression of P2G-Mos and SDSE-MEK succeeded in activating ERKs and RSK in developing mouse embryos, while wt-Mos failed to activate them in spite of expression of mos mRNA, indicating that the wt-Mos protein is unstable in early mouse embryos. Although the activated levels of ERKs and RSK in the vector-injected embryos were comparable to those of meiotically arrested mouse oocytes, their developmental rates were identical to those of the control embryos. These results suggest that activation of MAPK and RSK does not induce mitotic arrest in early mouse embryos. The present study indicates that there are large physiological differences between early mouse embryos and mouse oocytes and that CSF arrest of mouse eggs in mitosis should be discussed separately from that in meiosis.

Key words: Cell cycle, Cytostatic factor (CSF), MEK, Metaphase arrest, Mouse, p90Ribosomal S6 kinase (RSK)

Unfertilized vertebrate oocytes arrest their meiosis in the second meiotic metaphase (MII), resume meiosis in response to a fertilization stimulus and proceed to early development. In developing frog embryos, metaphase arrest has been induced by injection of MII oocyte cytoplasm, and this indicates that the frog MII oocyte contains cytostatic factor (CSF), which arrests the cell cycle at metaphase not only in meiotic oocytes but also in mitotic embryos [1]. Many studies on CSF have been conducted in Xenopus, and Mos, an oocyte-specific mitogen-activated protein kinase (MAPK) kinase kinase, has been shown to play crucial roles in CSF activity. Injection of mos mRNA or Mos protein into 2-cell embryos induces mitotic arrest, and removal of Mos from MII oocyte cytoplasm results in loss of this activity [2, 3]. The same activity has been reported in constitutive active MAPK and Raf-1, a MAPK cascade activator, indicating that this CSF activity of Mos is mediated by the MAPK cascade [3, 4]. In addition, mediation of CSF activity by p90Ribosomal S6 kinase (RSK), a substrate of
MAPK, has been shown by injection of its constitutive active mutant mRNA into 2-cell embryos [5] and by loss of CSF activity from the MII oocyte by immunodepletion of RSK [6].

In mouse oocytes, the requirement of Mos for meiotic arrest has been demonstrated by failure of MII arrest in mos-deficient mouse oocytes [7, 8] and in oocytes with suppressed Mos expression by an RNA interference (RNAi) technique [9]. It is also thought that, in mice, Mos/MAPK plays a role not only in meiotic but also mitotic arrest, since partial inhibition of development has been reported in mouse embryos injected with Mos or a constitutive active mutant of MAPK/ERK kinase (MEK), an activator of MAPK [10]. In this report, however, constitutive active MEK was less effective than Mos in mitotic arrest, and MEK was suggested to be lacking some of the functions specific for Mos [10], although Mos and MEK showed the same level of CSF activity in Xenopus embryos [3]. Moreover, the Mos-injected mouse embryos seemed to be arrested in interphase, not in M-phase [10]. It has been reported recently that injection of RSK into 2-cell mouse embryos failed to arrest their development [11], in spite of the suggested involvement of RSK in CSF activity in mouse embryos [12]. These reports prompted us to reconsider the effect of Mos/MAPK/RSK on mitotic arrest of developing mouse embryos, even though they act as CSF on the meiotic arrest of oocytes.

In the present study, we attempted to activate the MAPK cascade and RSK constitutively by injecting constitutive active MEK, wild-type Mos or degradation-resistant Mos expression vectors into mouse embryos. Their developmental states were analyzed for 72 h, and the effects of MAPK and RSK activation on mitotic arrest of the mouse embryos were examined.

Materials and Methods

Animals and in vitro fertilization

For superovulation, 7–10 week-old BDF1 female mice (SLC Japan, Shizuoka, Japan) were intraperitoneally injected with 7.5 IU of equine chorionic gonadotropin (eCG; Serotropin®, ASKA pharmaceutical, Tokyo, Japan), followed 48 h later by injection with 7.5 IU of human chorionic gonadotropin (hCG; Gonatropin®, ASKA pharmaceutical). The superovulated oocytes were collected from the ampullae of the oviducts of each mouse 16 h after hCG injection and placed in a fertilization drop (400 µl) of modified HTF (mHTF) [13]. For MII oocyte collection, cumulus-oocyte complexes were treated for 5 min with hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) added to the fertilization drop [final concentration 0.05% (w/v)], and cumulus cells were removed by pipetting. Sperm were obtained from the cauda epididymides of 10–15 week-old male BDF1 mice (SLC Japan) and were capacitated for 1 h in a drop (400 µl) of mHTF medium. In vitro fertilization was performed by adding preincubated spermatozoa to the fertilization drop containing the oocytes at a final concentration of 1–2 × 10⁵ spermatozoa/ml. The animal experiments were carried out according to the guidelines for animal experiments of the University of Tokyo.

Embryo culture

Cumulus cells surrounding fertilized eggs were removed by pipetting at 3 h post-insemination. Cumulus-free eggs were then rinsed 3 times in modified KSOM (mKSOM) [14], placed in a culture drop (100 µl) of mKSOM, and cultured at 37°C in 5% CO₂ in air up to 72 h. The eggs were examined at 6 h post-insemination, and only those showing second polar body extrusions or the appearance of two pronuclei were judged to have been fertilized; only these eggs were cultured further.

Plasmid construction

Dr. Okabe (Osaka University, Osaka, Japan) kindly provided pCX(pCAGGS)-enhanced green fluorescent protein (EGFP), which was used for expression control. Mouse MEK1 cDNA was a gift from Dr. H. Greulich (Harvard University, Boston, MA, USA). The constitutive active mutant of mouse MEK1 (SDSE-MEK) [15] was prepared by mutating 218S into D and 222S into E using site-directed mutagenesis (Stratagene, La Jolla, CA, USA). As mos gene is constructed of only one exon, the mouse mos gene was cloned from the 129 strain mouse genome by PCR using the following primer set: 5’-GAATTCACCATGCGCTTGCTTAAG-3’ and 5’-GATCTTCAGCCTATGCCC-3’. The degradation-resistant mutant of Mos (P2G-Mos) was prepared by mutating the second amino acid from proline to glycine using the following PCR primer set: 5’-GAATTCACCATGCGCTTGCTTAAG-3’ and 5’-GATCTTCAGCCTATGCCC-3’. cDNAs
of SDSE-MEK, wild-type Mos and P2G-Mos cDNAs were subcloned into the pCAGGS vector kindly provided by Dr. Miyazaki (Osaka University) [16].

Microinjection and observation of EGFP fluorescence

One of the expression vectors for SDSE-MEK, wild-type Mos or P2G-Mos was co-injected with the EGFP expression vector solution into the male pronuclei of fertilized eggs 6–8 h after insemination using a WR-50 microinjection apparatus (Narishige, Tokyo, Japan). Each vector was dissolved in injection buffer (10 mM Tris-HCl and 0.15 mM EDTA, pH 7.5) at a concentration of 30 ng/µl and was mixed with the same volume of EGFP vector solution (30 ng/µl). Therefore, the final concentration of each vector was 15 ng/µl, and about 2 µl of the DNA solution mixture was injected into each embryo. Some embryos were injected with EGFP expression vector alone (diluted to 15 ng/µl). Zygotes were kept in M2 medium (M7167; Sigma) during the microinjection procedure.

EGFP fluorescence was examined at 24, 48 and 72 h post-insemination by phase contrast microscopy (Diaphot 200; Nikon, Tokyo, Japan). Embryos expressing EGFP illumination as shown in Fig. 1A were used for evaluation of MAPK states and developmental rates.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNAs of 100 oocytes or embryos were isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). They were then reverse-transcribed into cDNAs using SuperScript III (Invitrogen) with random hexamers (Takara Bio, Kyoto, Japan) according to the manufacturer’s instructions. PCR was performed using a thermal cycler (Bio-Rad, Hercules, CA, USA) and either the mos-specific primers 5’-CATCCAGC-GGAGTTCTGG-3’ and 5’-GAACCTGCAGCT-TCTGGGAG-3’ or the hypoxanthine-guanine phosphoribosyl transferase (HPRT)-specific primers 5’-GGTGAGATGATCTCTCAAC-3’ and 5’-AAC-TTGGCGCTCATCTTAGGC-3’.

Western blotting

Western blotting was performed as described previously [17]. Twenty embryos were rinsed in PBS containing 0.4% (w/v) PVP and placed in 2 µl Laemmli buffer [18]. The collected embryos were heated for 5 min at 100 C and stored at –80 C until use. Detection of extracellular signal-regulated kinase (ERK) 1 and 2, major MAPKs in mouse oocytes, and RSK1 was performed using the anti-ERK (sc-94; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-RSK1 (sc-231-G; Santa Cruz Biotechnology) antibodies, respectively. To visualize the protein-bound antibodies, horseradish peroxidase-conjugated anti-rabbit IgG (AP132P; Chemicon International, Temecula, CA, USA) and anti-goat IgG (305-035-003, Jackson ImmunoResearch Laboratories, West Baltimore Pike West Grove, PA, USA) antibodies were used as a second layer, respectively, followed by the detection procedure using an ECL or ECL Plus detection kit (Amersham Pharmasia, Buckinghamshire, UK).

MAP kinase activity assay

MAP kinase activity was assayed in accordance with a previous report [19]. Ten embryos were placed in each assay tube and incubated with myelin basic protein (MBP; 10 mg/ml; M-1891, Sigma) and 32P-labeled ATP (0.4 mCi/ml, ICN Radiochemicals, Costa Mesa, CA, USA) at 37 C for 1 h. The labeled MBPs were detected by autoradiography, and the bands were quantified using the Scion Image software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

The results were evaluated using the Student’s t-test and Chi square test. Values of P<0.05 were considered to be statistically significant.

Results

The effects of SDSE-MEK expression on the MAPK/RSK states and development of mouse early embryos

EGFP illumination first became visible about 2 h after injection of EGFP expression vector (8–10 h after insemination), and about 90% of the injected embryos expressed EGFP illumination 24 h after insemination (Fig. 1A). The embryos without EGFP illumination were omitted from further analyses. Phosphorylated forms of ERK1 and 2, major MAPKs in mouse oocytes, and hyperphosphorylated forms of RSK were detected in MII oocytes, but not in developing embryos as reported previously [20, 21]. In contrast, clear phosphorylation was observed at both 24 and 48 h of culture in...
embryos injected with the SDSE-MEK expression vector (Fig. 1B). In support of the idea that these phosphorylated ERKs were activated forms, SDSE-MEK expression induced a change in MAPK activities in developing embryos from a low level to a high level comparable to that in meiotically arrested MII oocytes (Fig. 1C).

The developmental rates of the EGFP- and SDSE-MEK-expressing embryos are shown along with those of the control embryos expressing only EGFP in Fig. 2A, and examples of the SDSE-MEK-expressing embryos developed to each stage with EGFP illumination are shown in Fig. 2B. The developmental rates of the control embryos decreased gradually over the culture period. In a previous study, it was reported that high-level EGFP expression had deteriorative effects on embryo development [22]. In addition, exposure of embryos to UV light every 24 h might have contributed to embryo damage. A decrease was also observed in the developmental rates of the SDSE-MEK-expressing embryos, and this decrease was not significantly different from that in the control embryos.

**The effects of Mos or P2G-Mos expression on the MAPK/RSK states and development of mouse early embryos**

Injection of wild-type Mos expression vector into 1-cell mouse embryos induced mos mRNA expression 24 h after insemination, whereas mos mRNA expression was not detected in the embryos without injection (Fig. 3A). However, the phosphorylated forms of ERKs were not observed in the Mos expression vector-injected embryos at 24 h of culture, in spite of the expression of mos mRNA. Although a faint band of phosphorylated RSK was present, no hyperphosphorylated RSK, an activated form of RSK [23], was detected in these embryos (Fig. 3B). In contrast, clear phosphorylation of ERKs and hyperphosphorylation of RSK were observed following injection of P2G-Mos expression vector, a degradation-resistant mutant of Mos (Fig. 3B).

As shown in Fig. 4, the embryos injected with the wild-type Mos expression vector developed to the morula stage at the same rate as the control embryos expressing only EGFP, which confirmed the absence of activated forms of ERKs and RSK. Importantly, the developmental rate of the P2G-
MOS AND MAPK ARE NOT CSF IN MOUSE EMBRYOS

Discussion

In the present study, we attempted to determine whether mitotic arrest is induced by activation of MAPK and RSK in early mouse embryos, as has been reported in *Xenopus* embryos [2–4], since previous studies have indicated that their effects are insufficient to affect mitotic arrest in the mouse [10, 12] and some studies have reported conflicting results [11, 12]. For this purpose, we attempted to activate ERK and RSK in early mouse embryos, which have been reported to lack their activations [20, 21]. The expression of constitutive-active MEK and degradation-resistant Mos [24, 25] succeeded in activating ERKs and RSK in developing mouse embryos to levels comparable with those of meiotically arrested MII oocytes. Although these MAPK/

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**Fig. 2.** Development of SDSE-MEK-expressing embryos. (A) The embryos were injected with expression vector solution for EGFP alone (hatched) or SDSE-MEK plus EGFP (black) and cultured up to 72 h. The rates of embryo development to the 2-cell stage, 4-cell stage and morula stage at 24, 48 and 72 h, respectively, are shown relative to 1-cell embryos just after injection (8 h). The different letters indicate significant differences between the results as evaluated by Chi square test \( P<0.05 \). (B) Embryos injected with the SDSE-MEK and EGFP expression vectors and developed to each stage with EGFP illumination are shown. The left and right columns represent phase-contrast and fluorescence microscopy, respectively.

**Fig. 3.** Effects of injection of Mos or P2G-Mos expression vectors into fertilized eggs on MAPK/RSK activities during early mouse embryonic development. (A) The expression of Mos and HPRT mRNAs was examined by RT-PCR. Non-injected MII oocytes (0 h) or 24 h-cultured embryos with or without Mos expression vector injection were analyzed. (B) The phosphorylation states of ERK (upper panel) and RSK (lower panel) are shown for embryos injected with the indicated vectors 6–8 h after insemination and in vitro cultured for 24 h. Every 20th oocyte or embryo was analyzed by Western blotting with anti-ERK or anti-RSK1 antibodies. Arrowheads and asterisks indicate the specific bands of the dephosphorylated and phosphorylated form of each protein, respectively.

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Mos-expressing embryos, which contained activated ERKs and RSK, was also not significantly different from that of the control embryos, as in the case of the SDSE-MEK-expressing embryos.
RSK-activated embryos were cultured up to 72 h, their developmental rates were identical to those of the control embryos, which only expressed EGFP. This result indicates that activation of MAPK and RSK did not induce mitotic arrest in the early mouse embryos.

In meiotic arrest, the involvement of MAPK activation has been established also in mouse oocytes [7–9], and therefore, CSF arrest of mouse eggs should be discussed separately in meiosis and in mitosis. This fact suggests that there are large physiological differences between early mouse embryos and mouse oocytes, a clear difference from the case of *Xenopus*. Generally, almost no transcription occurs during oocyte meiosis, and the cell cycle is regulated by translation and post-translational modification of maternal factors [26]. *Xenopus* embryos contain many maternal mRNAs and proteins, and their cell cycle depends completely on these maternal factors, as in the case of oocytes, until the start of transcription at the midblastula transition [27]. This indicates the similarity of the intracellular conditions of *Xenopus* embryos and oocytes. In contrast, many maternal mRNAs and proteins are degraded at the 2-cell stage in mouse embryos, and their transcription begins at this stage [28, 29]. Therefore, the cell cycle of early mouse embryos probably depends on embryonic factors other than maternal factors that cause the difference between meiotic and mitotic arrest.

In *Xenopus*, the involvement of Cdk2 in MII arrest has been reported previously [30, 31]. Recently, early mitotic inhibitor 1 (Emi1) and emi-related protein 1 (Erp1)/Emi2, inhibitors of the anaphase-promoting complex, have been suggested to be necessary for meiotic and mitotic arrest in *Xenopus* and mouse eggs [12, 32–35]. In addition, a direct link of the Mos/MAPK pathway to Erp1/Emi2 has been also reported in meiotic arrest of *Xenopus* oocytes [36, 37]. As the activation states of ERKs and RSK were indistinguishable for the arrested mouse oocytes and the present non-arrested mouse embryos expressing MEK and Mos mutants, differences in the Emi1, Emi2 or Cdk2 state might have contributed to the differences between the mouse oocytes and early embryos, i.e., the differences between meiotic and mitotic arrest.

In the present study, the expression of wild-type Mos failed to activate ERKs and RSK in developing mouse embryos, in spite of expression of *mos* mRNA. We co-injected Mos expression vector with a protein expression marker, the EGFP expression vector, and performed analyses with only EGFP-illuminating embryos, which have previously been reported to exhibit protein synthesis from the co-injected vector [38]. Taking into consideration the rapid degradation of Mos after fertilization [39, 40], Mos might be unstable during early development of mouse embryos. This consideration is supported by the activation of ERKs and RSK in the degradation-resistant Mos-expressing mouse embryos. Several previous reports have indicated the involvement of Mos in the mitotic arrest of mouse embryos [10, 11]. These reports, however, used wild-type Mos, not a degradation-resistant mutant, and the activation states of ERKs and RSK were not studied. Furthermore, the arresting embryos seemed to be in interphase, not to be in the M-phase [10]. Taken together, these results might suggest that the mitotic arrest observed in these reports was not due to the effect of Mos itself, but rather to artificial effects such as injection and handling stress, although the possibility that differences in the mouse strains and culture systems also played a role cannot be excluded.

In regard to RSK, the requirement of RSK activity for CSF arrest has been well established in *Xenopus*
eggs [5, 6]. The effects of RSK on CSF arrest have also been reported in mouse embryos [12]. Recently, oocytes from RSK-knockout mice have been shown to present a normal MII arrest, and injection of constitutively active mutant forms of RSK have failed to induce meiotic and mitotic arrest in mos-knockout mouse oocytes, demonstrating that RSK was not involved in CSF arrest of the mouse oocytes [11]. Our results agreed well with this recent report in the mouse, although they did not agree with the results of previous reports in Xenopus [5, 6]. In Xenopus, spindle checkpoint factors, such as Bub1, Mad1 and Mad2, have been reported to be involved in meiotic arrest [31, 41]. In contrast, the dispensability of these factors in meiotic arrest has been shown in mouse oocytes by the expression of their dominant negative mutants [42]. Considering these and the present results together, it is possible that there are substantial differences between mice and Xenopus, not only in terms of early embryonic physiology, but also in the mechanisms of meiotic arrest itself. Further studies are required to examine this possibility.

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References


