Review Article

Molecular Basis of Meiotic Maturation and Apoptosis of Oocytes, Sperm-Oocyte Interactions and Early Cleavage of Embryos in Mice, Role of Phosphatidylinositol 3-Kinase, Mos, Fas-Fas Ligand, Integrinα6 and MAP Kinase

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Abstract

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The interaction between molecular biology and embryology made an extensive progress in the research on gametogenesis, fertilization and early embryogenesis in mice. In this article, molecules involving in meiotic maturation and apoptosis of oocytes, sperm-oocyte interactions and early cleavage of fertilized embryos in mice are described including our recent following experiments. 1) Phosphatidylinositol 3-kinase and Akt participate in the follicle stimulating hormone-induced meiotic maturation of mouse oocytes. 2) Mos plays a crucial role in normal spindle and chromosome morphology and the reactivation of maturation promoting factor after first meiosis. 3) Follicular atresia is caused by apoptosis and the apoptosis associated with internucleosomal DNA fragmentation is directly regulated by the Fas-Fas ligand system. 4) Integrin α6β1 is involved in sperm-egg binding leading to fusion via direct association of the integrin α6 with sperm. 5) MAP kinase cascade is activated at the M-phase and some MAP kinases other than ERKs are activated during early cleavage of fertilized eggs.

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Introduction

Recently, molecular basis of gametogenesis, fertilization and early embryogenesis has bocome again a subject of interest, because the technologies developed in molecular biology, biochemistry and cytochemistry has been introduced in these field and a number of regulatory protens and genes has been identified in the events of these processes. In this article, recent advances in our study of meiotic maturation and apoptosis of oocytes, sperm-oocyte interactions and early cleavage of fertilized embryos in mice are discussed.

Phosphatidylinositol 3-kinase (PI3K) and Akt in the meiotic maturation

Fully grown mammalian oocytes are arrested in ovarian follicles at the diplotene stage of the first meiotic prophase by meiosis-arresting factors such as cAMP (1) and purines such as hypoxanthine (2). The resumption of meiosis, morphologically identified by germinal vesicle breakdown (GVBD), is triggered in healthy follicles by preovulatory luteinizing hormone (LH) surge. Progression of meiosis beyond GVBD involves chromosome condensation and alignment of the metaphase I spindle, segregation of homologous chromosomes, emission of the polar body (PB1), and metaphase MII spindle formation. GVBD and progression of oocytes to metaphase II is usually referred to as meiotic maturation. At the diplotene stage of the first meiotic prophase, oocytes undergo gonadotropin-independent spontaneous meiotic maturation when they are removed from mature follicles and cultured in vitro in a suitable medium (3). Oocytes also mature in vitro under the stimulation of follicle stimulating hormone (FSH) or other hormones when spontaneous maturation is prevented by meiotic inhibitors such as hypoxanthine and cAMP-elevating agents (2). FSH-induced meiotic maturation

requires the presence of cumulus cells, which are thought to be the source of a putative meiosis-inducing signal (4).

FSH-induced meiotic resumption requires activation of phosphoinositide metabolism and release of intracellular free calcium in mouse cumulus-oocyte complexes (COCs), and both oocyte and somatic cells are potentially sensitive to these messengers (5). It is suggested that following positive stimulation by gonadotropin, inositol triphosphate and possibly calcium may be generated in cumulus cells and subsequently transferred to the oocyte via intracellular communication. PI3K is known to play critical roles in signal transduction processes related to a variety of cellular activities such as cytoskeletal re-arrangement, cellular migration, differentiation, protection against apoptosis and mitogenesis (6). Akt, also known as protein kinase B, was identified as a serine-threonine kinase that functions downstream of PI3K (7). The activation of Akt is thought to be a critical step in the PI3K pathway that regulates cell growth and differentiation. It is also correlated with cell survival in a wide variety of cells, including those of epithelial, mesenchymal, and neuronal origin (8). During meiotic maturation, the inhibition of PI3K activity by LY294002 in cumulus cells interferes with the gonadotoropin-stimulated meiotic resumption of porcine oocytes (9). In rat granulosa cells, FSH increases Akt phopshorylation and activation in a manner that is cAMP-dependent and PI3K-dependent (10). The PI3K/Akt pathway also plays a role in the FSH-stimulated expression of X-linked inhibitor of apoptosis (XIAP), a factor that which suppresses the induction of apoptosis in rat granulosa cells (11). In Xenopus oocytes, PI3K/Akt regulation plays an essential role in the resumption of meiosis (12). However, the role of PI3K and Akt in mammalian oocytes during meiotic maturation is not fully understood. Then, we examined the role of PI3K during meiotic maturation in mouse oocytes, and our results suggest that PI3K participates in FSH-induced cumulus expansion and meiotic maturation in mouse oocytes. When FSH-induced COCs were treated with LY294002, GVBD was inhibited at 100 μM, while PB1 emission was suppressed in a dose-dependent manner. In vitro, cumulus expansion occurs when isolated COCs are treated with FSH (13). We also observed the inhibition of cumulus cell expansion by LY294002. The oocyte meiotic maturation process is initiated by expansion of the cumulus cells and GVBD. Cumulus expansion is also required for normal ovulation rates in vivo (14). The cytoskeletal modification and loss of gap junctions accompanying cumulus expansion in COCs are closely related to oocyte meiotic progression (15). The details of the signal transduction pathway and maturational processes are not yet resolved, but various studies suggest that luteinizing hormone (LH) promotes an increase in cAMP levels within the granulosa cell compartment and a decrease in cAMP in the oocyte, thus indicating the resumption of meiosis as well as cumulus expansion (16). Recently, in mouse, it is reported that gonadotropin induces both the resumption of meiosis and cumulus expansion by mechanisms requiring the activation of mitogen-activated protein kinase (MAPK) in cumulus cells, but this activation cannot occur in the absence of oocytes, and is likely mediated by one or more paracrine factor (17). Thus, oocytes enable the cumulus cells to produce, in response to gonadotropin-induced elevation of granulosa cell cAMP, a return signal that induces the resumption of meiosis (17). In porcine COC, PI3K activity in cumulus cells contributes to the activation of MAPK, and to meiotic progression beyond MI stage (18). Our results suggest that PI3K activity participates in FSH-induced meiotic maturation in mouse COCs. Therefore, PI3K activity in mouse cumulus cells may be contribute to the activation of MAPK and meiotic maturation.

We next examined whether LY294002 affects oocytes directly. Several previous studies have suggested that FSH could stimulate the cumulus cells to generate a meiosis-activating factor, e.g. Ca^{2+} (19) or meiosis-activating sterol (MAS) (20), which positively overcomes the meiotic arrest induced by dbcAMP or hypoxanthine and plays a potent physiological role in triggering gonadotropin-induced meiotic resumption. Since gonadotropin receptors are absent from oocytes, FSH promotes GVBD via an indirect mechanism mediated by the cumulus cells rather than by a direct action on the oocytes (21). Therefore, we examined the effect of LY294002 on denuded oocytes (Dos) cultured in the medium containing FF-MAS. 100 μM LY294002 treatment of DOs inhibited PB1 emission, but not GVBD. These results suggest that PI3K activity in cumulus cells participates in GVBD, while PB1 emission requires PI3K activity in oocytes. In this experiment, we addressed whether LY294002 affects oocytes directly. Therefore, it was necessary to use same culture conditions for both FSH- and FF-MAS-induced experiments in the present study, e.g. volume of droplet overlaid with paraffin liquid, while FF-MAS

may be absorbed by paraffin liquid. Indeed, FF-MAS induced PB1 emission rate was not high (38.0%), while 62.7% of oocytes underwent GVBD. To just examine the effect of LY294002 on FF-MAS induced oocyte meiotic maturation for DO, culture conditions without layer of paraffin liquid may be better to evaluate the PI3K function during meiotic maturation.

Akt was identified as a serine-threonine kinase that functions downstream of PI3K (7). The activation of Akt is thought to be a critical step in the PI3K pathway that regulates cell growth and differentiation. Akt becomes phosphorylated at two residues, Thr^{308} and Ser^{473}, and both are required for full activation (22). Therefore, we examined the distribution of phosphorylated Akt during meiotic maturation in mouse oocytes. Confocal microscopy revealed that Thr^{308} phosphorylated Akt was localized as dots around chromosomes and in the cytoplasm in PMI oocytes. At MI and MII, four intense dots were observed. Merged figures of staining for phosphorylated Akt, microtubules and nuclei showed that Thr^{308} phosphorylated Akt was present in the spindle pole (Fig 1).

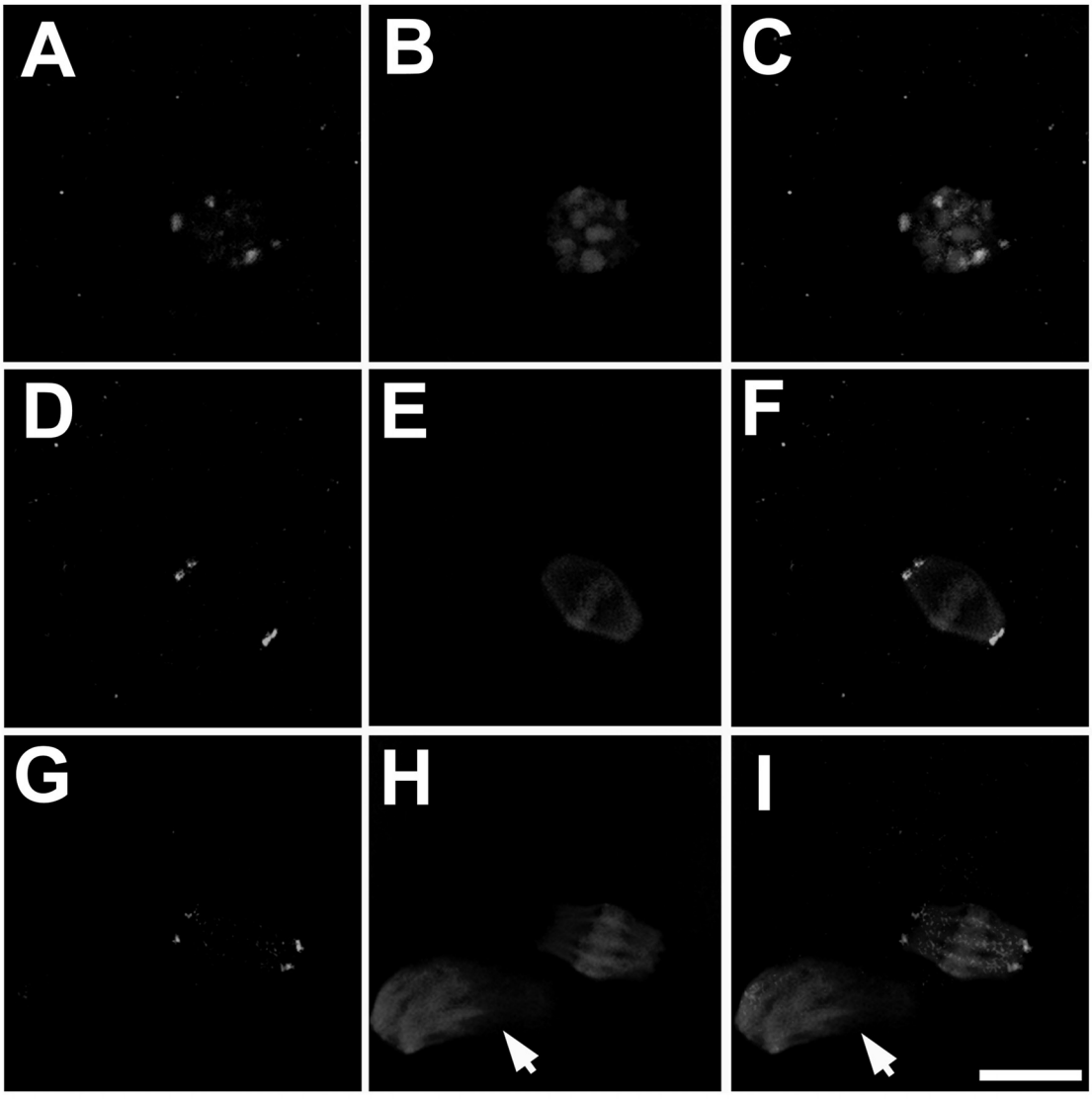

Fig 1: Cellular localization of Thr^{308} phosphorylated Akt during in vitro maturation (A,D,G). COCs were cultured in FSH-induced culture condition. Oocytes at prometaphase I (PMI), MI and MII were collected at 8 to 10 hr, 10 to 12 hr and 18 hr after the start of culture in respectively. The meiotic stages were prometaphase I (A-C), metaphase I (D-F), and metaphase II (G-I). Nuclear status and microtubules were visualized for counter-staining, (B,E,H) and merged with stainig of Thr^{308} phosphorylated Akt (C,F,I). Green, red and blue show Akt, nuclear status and microtubules, respectively. The arrow indicates the first polar body. Bar = 10 µm.

In most animal cells, the microtubule organizing center (MTOC) is composed of a pair of centrioles surrounded by an electron dense material, the PCM, and it is within this material that the MTOC activity is located (23). Mouse MII oocytes as well as MI oocytes and most probably oocytes arrested in meiotic prophase, do not have centrioles (24).

The poles of the meiotic spindle are composed of bands of electron dense PCM (24). Therefore, Thr^{308} phosphorylated Akt was located in PCM at MI and MII. In contrast, the distribution of Ser^{473} phosphorylated Akt was similar to the localization of microtubules at prometaphase I (PMI) and the present in spindle at MI and MII (Fig 2).

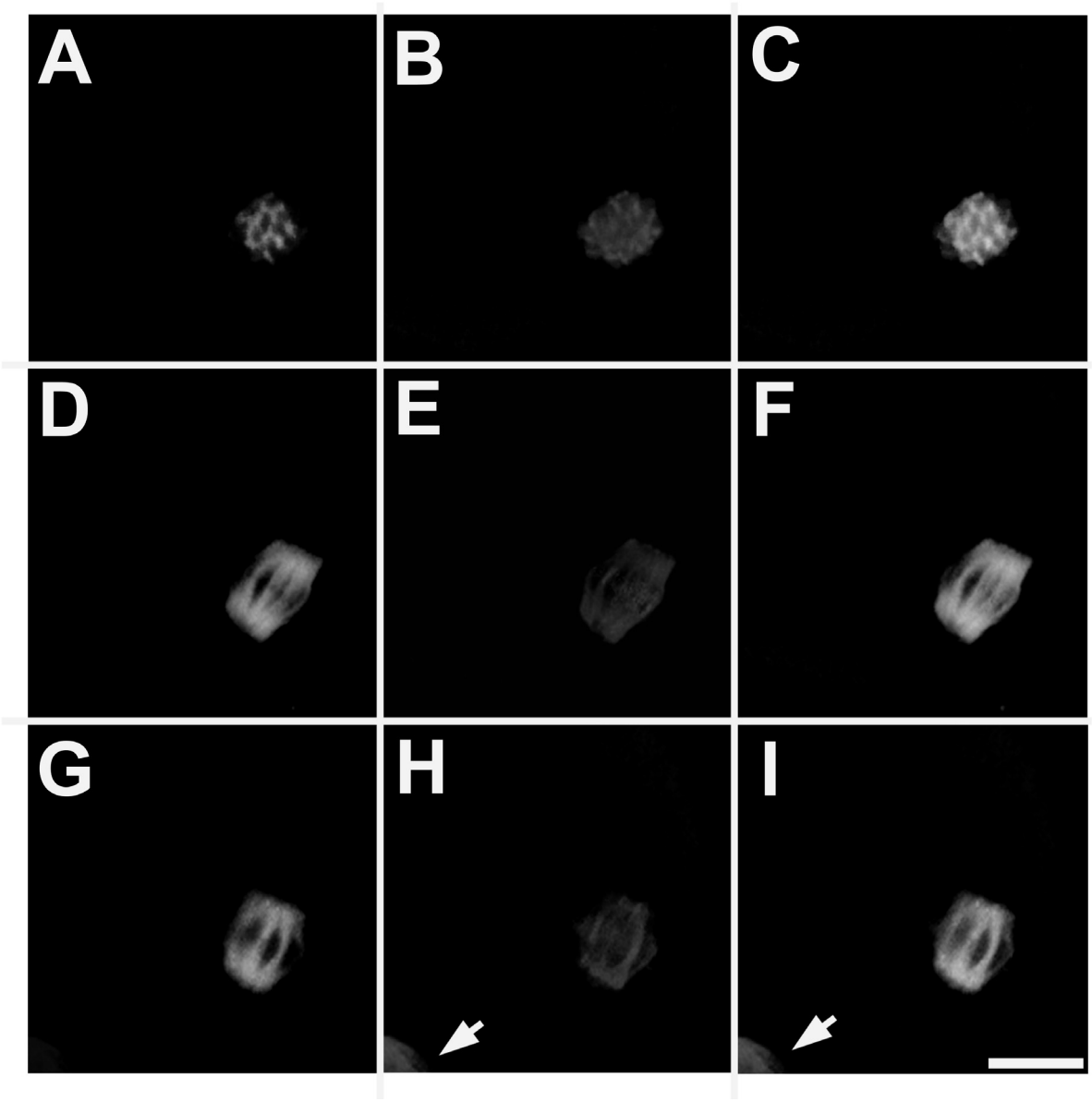

Fig 2: Cellular localization of Ser^{473} phosphorylated Akt during in vitro maturation (A,D,G). COCs were cultured in FSH-induced culture condition. Oocytes at prometaphase I (PMI), MI and MII were collected at 8 to 10 hr, 10 to 12 hr and 18 hr after the start of culture in respectively. The meiotic stages were prometaphase I (A-C), metaphase I (D-F), and metaphase II (G-I). Nuclear status and microtubules were visualized for counter-staining, (B,E,H) and merged with staining of Ser^{473} phosphorylated Akt (C,F,I). Green, red and blue colors show Akt, nuclear status and microtubules, respectively. The arrow indicates the first polar body. Bar = 10 μm.

(This figure has also been printed in full-color at the end of the issue.)

When COCs were treated with LY294002 in FSH-induced meiotic maturation, the amount of Thr^{308} phosphorylated Akt was decreased to very low to undetectable levels in PMI, MI and MII oocytes. The distribution of Ser^{473} phosphorylated Akt in LY294002-treated PMI oocytes was similar to that in normal PMI oocytes, whereas aberrant distribution and very low to undetectable levels of expression were seen in LY294002-treated MI and MII oocytes, respectively. These results suggest that Akt activity participates in FSH-induced meiotic maturation as a downstream effector of the PI3K pathway in mouse oocytes (25).

Akt is best known for its anti-apoptotic effects (26) and plays a role in inhibition of entry into the S phase (27). However, there is very limited information regarding the G2/M phase of the cell cycle. Akt activity is high at the G2/M phase in MDCK renal epithelial cells (28), PANC1 cells, pancreatic carcinoma cell lines, and normal human fibroblasts (29). In MDCK cells, the inhibition of PI3K induces apoptosis and decreases M-phase promoting factor (MPF) activity at G2/M phase (28). In HEK293 cells, Akt controls G2/M cell cycle progression, and activation of Akt can overcome both the p53-independent G2/M cell cycle checkpoint and apoptosis induced by DNA damage (30). It was recently reported that Akt is phosphorylated during mitosis and Thr^{308} phosphorylated Akt is present at the centrosome in HeLa cells (31). Akt also phosphorylates downstream kinase glycogen synthase kinase-3 (GSK-3), which is constitutively active in resting cells. Phospho-GSK-3 is abundant at the centrosome and spindle pole (31). GSK-3 phosphorylation occurs concomitantly with the appearance of phosphorylated and active Akt at the centrosome. Inhibition of GSK-3 promotes defects in microtubule length and chromosomal alignment during prometaphase. However, as PI3K inhibitors do not appear to affect the mitosis-specific phosphorylation of Akt or GSK-3 in HeLa cells, it may be that Akt is activated in a phosphoinositide-independent manner during mitosis (31). In contrast, inhibition of mouse oocyte GSK-3 had no significant influence on viability, morphology, or development to MII, whereas the inhibitor caused abnormal spindle formation and significantly increased incidence of abnormal homologue segregation during first meiotic division (32). These and our present results suggest that PI3K-Akt-GSK-3 pathway may have important role in mouse oocyte meiotic maturation. Furthermore, Akt and GSK-3 are involved in meiosis and mitosis, whereas signal transduction pathway of Akt-GSK on meiotic maturation in mouse oocytes may be different from mitotic division in cells. Although it is unknown whether the localization of phosphorylated Akt has any role in PCM or the spindle at MI and MII during meiotic maturation in mouse oocytes, LY294002 suppressed meiotic maturation and caused a decrease in the level and aberrant distribution of phosphorylated Akt. These results suggest that the PI3K/Akt pathway functions in mouse oocytes during FSH-induced meiotic maturation. In contrast, our results suggest that PI3K activity in cumulus cells participates in GVBD and the expansion of cumulus cells. In cultured rat granulosa cells, FSH increases Akt phopshorylation and activation in a manner that is cAMP-dependent and PI3K-dependent (10). The PI3K/Akt pathway also plays a role in the expression of FSH-stimulated XIAP, a factor which suppresses the induction of apoptosis in rat granulosa cells (11). Therefore, the PI3K/Akt pathway may function in mouse cumulus cells during FSH-induced meiotic maturation.

Taken together, is suggested that spontaneous meiotic maturation in mouse oocytes may not require the PI3K pathway (Fig 3).

It has been reported that inhibitions for phosphoinositide metabolism (5), MAPK (33) block FSH-induced but not spontaneous meiotic resumption in mouse oocytes. It also has been reported that FF-MAS induced maturation of mouse oocytes appear to use different signal transduction pathways compared with spontaneous meiotic maturation (34). These reports suggest that spontaneous maturation probably occurs through a different mechanism. Spontaneous maturation fundamentally involves removal of the inhibitory influence imposed by the follicular environment. However, this model does not reproduce accurately the normal mechanism operating in vivo, and therefore it does not appear to be the most appropriate approach (5). In contrast, systems in which meiotic maturation is induced by gonadotropins or other agents under conditions that prevent spontaneous maturation offer a more physiological model in which reinitiation of meiosis, as it presumably occurs in vivo, is activated by a signal that requires interaction between the oocyte and follicle (5). Therefore, our results suggest that PI3K may be involved in gonadotropin-induced meiotic maturation in vivo (Fig. 3). Because the inhibition of meiotic maturation in our culture condition was not complete by LY294002, there is a possibility the interaction of other pathway with PI3K (25).

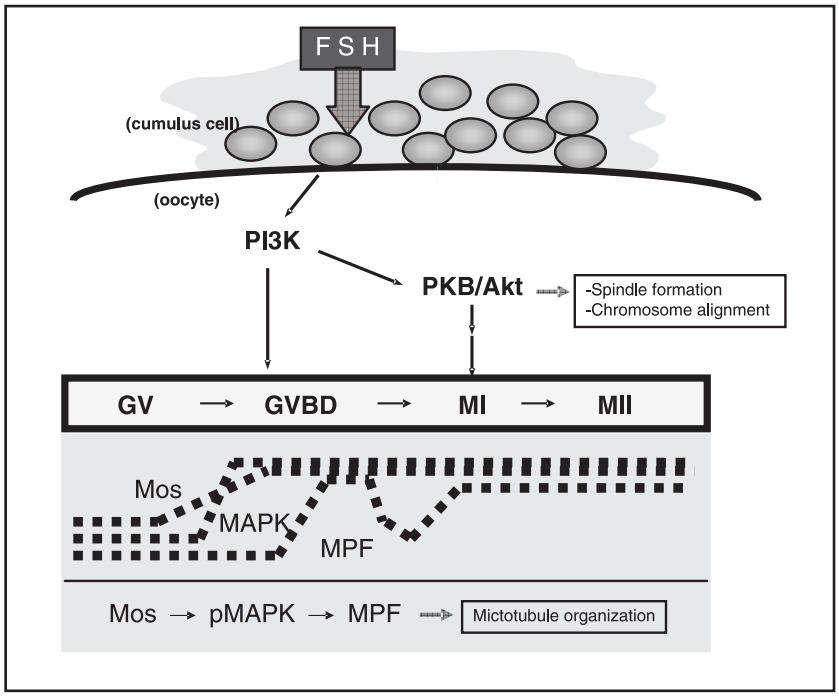

Fig 3: Schematic representation of mechanism of the oocyte maturation.
FSH induces GVBD in cumulus cell-enclosed oocytes. PI3K involved in gonadotropin-induced meiotic maturation. FSH via PI3K also phosphorylates PKB/Akt. Phospho-Akt present in spindle at M-phase and it may be involved in spindle formation and chromosome alignment. The bottom figure shows that the activity changes mos (blue), MAPK (green) and MPF (red) in meiotic maturation. Mos has been shown to be required for MAPK activation and to be involved in microtubule organization.

Mos in meiotic maturation in oocytes in culture

In Xenopus oocytes, the c-mos proto-oncogene product (Mos) is required for activation of the maturation promoting factor (MPF) in G2 arrested oocytes, for reactivation of MPF after metaphase I, for transition to metaphase II without DNA replication, and for maintaining high MPF activity in metaphase II oocytes (35). Mos has a serine-threonine kinase domain (36). In Xenopus oocytes, Mos activates MAPK kinase by direct phosphorylation, and subsequently activates MAPK (37). It has been shown that the above-mentioned Mos functions in Xenopus oocytes are mediated by MAPK activity (38).

In contrast, the function of Mos in mouse oocytes is unclear, although Mos has been thought to play important roles in oocyte maturation because of the exclusive transcription in oocytes (39). When anti-Mos antibody or antisense oligonucleotide was microinjected into immature mouse oocytes, various results were reported. These were: the inhibition of GVBD, the normal induction of GVBD but the inhibition of first polar body emission, and the normal induction of first polar body emission but entrance into interphase instead of second meiosis (40).

Recently c-mos knockout mice were generated by homologous recombination in embryonic stem cells (41). These mutant mice have truncated Mos that has no kinase activity. Oocytes obtained from the c-mos knockout mice have been reported to undergo GVBD and mature normally with a frequent spontaneous parthenogenetic activation, indicating that Mos is not essential for oocyte maturation in the mouse (41). Recently, Mos has been shown to be required for MAPK activation and to be involved in microtubule organization during meiotic maturation in the mouse (42).

We used c-mos knockout mouse oocytes and examined the roles of Mos in mouse oocyte maturation and fertilization in more detail, including whether Mos controls MAPK and MPF activity (43). In sharp contrast to the lack of an effect of Mos on the progression of first meiosis in the mouse, the abnormalities of chromosomes and alpha-tubulin morphologies in the metaphases of homologous mutant oocytes indicate that Mos participates in chromosome condensation and microtubule reorganization. Zhao et al. (44) reported that about 90% of oocytes that received an antibody to Mos did not assemble a meiotic

spindle. Mos has been implicated in the reorganization of the microtubules, which leads to formation of the spindle and the spindle pole (45). Mos overexpression in somatic cells induced meiotic-like alterations in the mitotic spindle (46). Abnormalities in the organization of the microtubules and chromatin were recently reported in c-mos knockout mouse oocytes (42). The morphological abnormalities induced by the loss of Mos in our study are consistent with these previous reports and confirm that Mos plays an important role in the reorganization of microtubules and chromosome condensation.

In our study, MAPK activity was assayed throughout mouse oocyte maturation in wild-type, heterozygous mutant, and homozygous mutant mice. In heterozygous mutant and wild-type oocytes, the fluctuation patterns were in close agreement with those in previous reports: the activity was significantly increased at 4 h of culture, and was maintained at about 5 to 7 times the initial activity during 4-16 h of culture (47). On the other hand, MAPK activity of homozygous mutant oocytes did not significantly fluctuate throughout maturation and was clearly lower than that of wild-type oocytes. It has been shown by SDS-PAGE that mouse MAPK was present as 42- and 44-kDa bands, and the migration rate was decreased when MAPK was activated by phosphorylation (42). The two bands at 42 and 44 kDa were detected in all three genotypes in our study, and a band shift was also observed at 8 h of maturation when MAPK activity was high in heterozygous mutant and wild-type oocytes. In homozygous mutant oocytes, however, no decrease in the migration rate was detected at 8 h of culture when the oocytes were at the first metaphase. Recently, the same result has been reported in a different strain of c-mos knockout mouse oocytes (42). These results indicate that Mos physiologically stimulates MAPK during maturation of murine oocytes as in Xenopus oocytes. MAPK activation is a prerequisite for GVBD in Xenopus oocytes (38). Our results suggest, however, that in murine oocytes, MAPK activation is not essential for GVBD and first polar body emission. In mouse oocytes, MAPK is localized in microtubule-organizing centers (47). Verlhac et al. (48) reported that microtubule and chromatin behavior was controlled by MAPK activity during meiosis in mouse oocytes. We therefore considered that the morphological abnormalities in homozygous mutant oocytes referred to above contributed to this low MAPK activity caused by the loss of Mos. In our study, MPF activity was low in the G2 arresting oocytes and high in the first and the second metaphase oocytes, with a transient decrease at first polar body emission.

There was almost no difference among the oocytes of wild-type, heterozygous mutant, and homozygous mutant mice until first polar body emission, indicating that Mos does not stimulate MPF activity directly and that MPF and MAPK activities are regulated independently. These data suggest that the normal fluctuation in MPF activity can cause the normal process of oocyte maturation in spite of the loss of Mos and MAPK activity, confirming the importance of MPF in oocyte maturation in the mouse. One of the most drastic abnormalities in c-mos knockout mouse oocytes was their entrance into the interphase instead of second meiosis after first polar body emission. In these oocytes, MPF was inactivated to the basal level instead of being reactivated after the decrease in polar body emission. Furuno et al. (49) have reported that suppression of DNA replication during meiotic divisions in Xenopus oocytes is accomplished by the Mos-mediated premature reactivation of cdc2 kinase. Oocytes injected with antisense c-mos oligonucleotides completed the first meiotic division but failed to initiate second meiosis and reformed a nucleus (40). Our results are consistent with these reports, and suggest also that in the mouse Mos plays a crucial role in the reactivation of MPF after the first polar body emission. In our study, however, 56% of oocytes in the homozygous mutant were not activated after first meiosis and reached the second metaphase in spite of the loss of Mos. This indicates that c-mos deletion induces some compensatory factors that reactivate MPF after first meiosis, although there are wide variations in the compensatory efficiency of the oocytes. Verlhac et al. (42) reported that their c-mos knockout oocyte did not require Mos for MPF reactivation after the first meiosis, prompting us to think of the high compensatory activity in their mice. Details of these factors are still unknown, but our results indicate that a compensatory action other than the MAPK cascade may be at work, since MAPK in homozygous mutant oocytes was maintained in an inactive form throughout maturation (50). When matured c-mos knockout oocytes were activated by fertilization or ethanol stimulation, some oocytes were transformed into metaphase III instead of interphase. The phenomenon of metaphase II has already been reported by Kubiak (51). These studies demonstrated that metaphase III frequently appeared at 11-13 h after gonadotropin injection, due to ethanol

stimulation and fertilization, but our and his experimental conditions were different, because all heterozygous oocytes entered the interphase after activation. Under normal conditions in Xenopus oocytes, the stimulus of fertilization destroys cyclin B by the ubiquitin pathway and Mos by calmodulin-dependent protein kinase II or the N-terminal proline-dependent ubiquitin pathway (52), so that the MPF activity is decreased and the cell cycle progresses into the interphase. Under the conditions of our study, however, the MPF activity was maintained not by Mos but presumably by compensatory factors. We therefore present here the following hypothesis to account for metaphase III in c-mos knockout mice. When the metaphase II arrested, the oocytes were penetrated by sperm, degradation of cyclin B and a transient decrease in MPF activity occurred and the cell cycle progressed into the anaphase-telophase. In these oocytes, however, since unknown compensatory factors other than Mos may not be degraded even if sperm penetrate the oocyte, the decreased MPF activity may be restored and the cell cycle then progresses into metaphase III. Verlhac et al. (42) also reported the appearance of metaphase III in their c-mos knockout mice, confirming the presence of Mos-compensatory factors that were not degraded after metaphase II in the c-mos knockout oocytes.

Fas-Fas ligand in apoptosis of cumulus-oocyte complex

The majority of ovarian follicles including oocytes have been revealed to undergo atresia through a mechanism involving apoptotic cell death, but the molecular mechanisms underlying atresia remain to be clarified. Previous studies have reported several physiological markers associated with follicular atresia, including detachment and degeneration of the granulosa cell layer, fragmentation of the basal lamina, reduced DNA synthesis, invariably decreased estrogen production, and decreased gonadotropin binding in ovarian follicles (53). Zeleznik et al. (54) showed the presence of an endonuclease activity in rat granulosa and luteal cells that is modified by the changes in gonadotropin levels and is capable of inducing internucleosomal DNA cleavage. Further evidence has recently been reported indicating that granulosa and thecal cells collected from ovarian atretic follicles display the internucleosomal cleavage of DNA characteristics of cells undergoing apoptosis or programmed cell death (55). It has therefore been clarified that apoptosis is the underlying mechanism of ovarian follicular degeneration during atresia (48). Fas molecule, which is broadly expressed in lymphoid and not-lymphoid tissues, is a member of the tumor necrosis factor/nerve growth factor receptor family inducing the apoptotic cell death (56). On the other hand, Fas ligand (FasL) is a type II transmembrane protein highly homologous to tumor necrosis factor to induce apoptosis in Fas-expressing target cells. Although the expression of FasL has been reported to be restricted predominantly in the activated T cells and in several tissues such as testis, kidney, small intestine and lung (57), further studies have recently shown the expression of FasL in other tissues including ovary. In the immune system, Fas and Fas L are involved in the reduction of immune reactions as well as in T-cell-mediated cytotoxicity (57). In the reproductive system, Fas is expressed not only in the mouse and human oocytes but also in the mouse and human granulosa cells (58). We also examined the contribution of Fas and its ligand (FasL) in the process of follicular atresia by using mouse intraovarian follicles and gonadotropin-hyperovulated eggs (59). Reverse transcriptase/polymerase chain reaction-Southern blot hybridization demonstrated positive expression of Fas in both intraovarian oocytes and hyperovulated eggs. In contrast, expression of FasL was only detected in granulosa cells. These finding were histologically confirmed by in situ hybridization with Fas- and FasL-specific probes. A time-course study showed that Fas mRNA was positive in atretic follicles through day 0 and day 2 of gonadotropin stimulation and negative thereafter. Levels of FasL mRNA were the highest on day 1 and tapered off toward day5 of gonadotropin stimulation. Levels of elongation factor I α mRNA, a constitutive element, were constantly maintained throughout the experimental period. Co-culture of ovulated eggs, intact and zona-free, and granulosa cells demonstrated positive TUNEL staining only in zona-free eggs. Our findings indicate that follicular atresia is caused by apoptosis. This apoptosis, associated with internucleosomal DNA fragmentation, is directly regulated by the Fas/FasL system (50). It is possible that the cell death in the ovarian atresia is modified by other factors, such as Bcl-2 and related proteins (60), acting as the primary regulators of the Fas and FasL system. In fact it has been reported that Fas-induced cell death is partially inhibited by the overexpression of the Bcl-2 gene, and it is completely inhibited by

the coexpression of Bcl-2 and its binding protein BAG-1 (61).
Nevertheless, taken together with our studies, we propose that the system of Fas in oocytes and FasL in granulosa cells is likely to be the direct regulator of undergoing atresia in the diverse species. Furthermore, morphological observations (62) have shown that the zona matrix in mammalian oocytes becomes denser and wider during the development towards tertiary follicles, being traversed by numerous microfilaments with channel-like cytoplasmic protrusions of the follicular granulosa cells.
In the preovulatory stage, the follicular granulosa cells are gradually separated and retracted from zona pellucida. It was therefore strongly suggested that the molecular interactions between Fas in oocytes and FasL in granulosa cells could be interrupted by the mature zona pellucida and the ovulatory oocytes would be freed from follicular atresia through the apoptotic mechanism.

Egg integrins in sperm-egg binding and fusion

The molecular events of sperm-egg binding and fusion have been studied for decades. Several investigators have reported candidates for a ligand on the sperm plasma membrane in mammals; for example, CD46 (63), DE (64), OBF-13 (65), MH61 (66), M29 and M37 (67). These studies were based on immunological assays; a monoclonal or polyclonal antibody inhibited sperm-egg binding and/or fusion. Several lines of evidence support the contention that a candidate ligand on sperm is fertilin α/β. This protein complex, originally called PH-30 antigen, was identified using the monoclonal antibody, PH-30: Fertilin is localized to the postacrosomal region, including the equatorial region, in acrosome-reacted sperm from guinea pigs, bulls and mice (68). The fertilin α and β subunits were characterized as the first members of a novel gene family, the ADAMs (69). The ADAMs contain a disintegrin domain and a metalloproteinase domain (70). Peptide analogs of the disintegrin loop of fertilin β inhibit sperm-egg binding, indicating that fertilin is possibly involved in sperm-egg binding (71). Recently, strong evidence for an involvement of fertilin β in sperm-egg binding was provided by Cho et al (72); mutant sperm lacking fertilin β were found to be severely impaired in binding to zona-free eggs although they could fuse with approximately 50% efficiency. Another possible candidate for a ligand is the ADAM protein, cyritestin. Peptide analogs of the disintegrin domain of cyritestin also strongly inhibit sperm-egg binding (68). These findings led us to propose that integrins are expressed on the egg surface and an egg integrin can function as a sperm receptor. The reason for this assumption is that the disintegrin domains of snake venom metalloproteases bind to the integrin αIIb β 3 (GP IIb/IIIa) (73). By analogy, it is likely that fertilin and cyritestin are recognized by integrin receptors through their disintegrin domains.

Many integrin receptors, such as α2, α3, α5, α6, αv, β 1, β 3 and β 5, have been reported to be expressed in mammalian oocytes at either the mRNA or protein levels (74). The tripeptide, RGD, derived from fibronectin, which is known to be an integrin ligand, has been reported to inhibit sperm-egg binding in hamsters and humans (75). Echistatin, a snake venom disintegrin loop that contains an RGD sequence in its disintegrin loop, was also shown to inhibit sperm-egg binding in hamsters (76). These findings supported the hypothesis that an egg integrin can function as a sperm receptor. Another possible molecular interaction between sperm and eggs was presented by Anderson et al. (63). Human acrosome-reacted sperm express a membrane cofactor protein (MCP), which is a complement component 3-binding and regulatory protein. CR3 (complement receptor 3), which is also referred to as αM β 2 integrin, is expressed on human oocytes. Anderson et al. proposed a model in which the complement component 3 fragment (C3b) mediates gamete membrane apposition and fusion via binding to MCP and CR3. But, this hypothesis has not yet been proven. Mouse egg integrin α6β1 was reported to be a strong candidate for a sperm receptor by the following evidence: 1) The major integrins found to date on the mouse oolemma are α6 β 1 and αvβ3. 2) A function blocking antibody against integrin 6 inhibits sperm-egg binding. 3) A peptide analog of the fertilin β disintegrin loop as well as a function blocking anti-α6 antibody inhibits binding of sperm to α6 transfected cells (77). Evans et al (78) reported that the integrin β 1 is responsible for sperm (fertilin β) binding to eggs based on the observation that an antibody that is reactive with several β1 integrins inhibited binding of sperm or recombinant fertilin protein made in E. coli to the surface of eggs. However, Evans et al. concluded that the α6 subunit was not involved as the anti α6 mAb GoH3 did not inhibit binding of their construct. Conversely, two recent studies have demonstrated that both a disintegrin loop peptide (79) and fertilin β purified from sperm (80) interact with the egg integrin α6β1. Nevertheless, it remains unsolved if egg integrin α6β1 binds directly to a protein (e.g., fertilin β) on the surface of

intact sperm and, if so, in which event the α6 β 1 participates.

We first identified egg molecules with binding-affinity for sperm. The strongest candidate was a protein with an apparent molecular mass of 135 kDa on reducing gels. This protein was identified as integrin α6 subunit by an immunodepletion experiment using the anti-α6 mA (GoH3). Furthermore, when eggs were fertilized, the integrin α6 and β1 subunits were accumulated at the sperm-binding site at an early stage of fertilization. These findings provide the first evidence that the egg integrin α6 binds to the sperm surface and that sperm binding results in clustering of α6β1 integrins on the egg. Zona removal of mouse eggs with proteases results in loss of egg fertilizability (81). Some proteins are reported to be very sensitive to protease treatment, such as with chymotrypsin and trypsin. A 94 kDa protein was modified by both α-chymotrypsin and trypsin (82), and the recovery of a 94-kDa protein after egg incubation coincided with the recovery of egg fertilizability (83). Calarco (84) demonstrated that 97-kDa and 66-kDa proteins were also modified by protease. As well as proteases, the egg surface proteins are thought to be damaged by acid. Interestingly, more biotinylated bands appeared on a gel of acid-treated eggs than of chymotrypsin-treated eggs, although we previously showed that the fertilizability of acid-treated eggs was less than that of chymotrypsin-treated eggs just after zona removal (63). This suggests that the strongest band in chymotrypsin-treated eggs, including 135-kDa protein is a candidate for a sperm receptor. From our previous report, the fertilizability of acidtreated eggs reaches that of chymotrypsin-treated eggs if they are allowed to recover for 3 hr after zona removal (81). Consistent with this result, some bands, including a 135-kDa band, were increased. Therefore, these proteins were assumed to be candidates for a sperm receptor (85).

It has been reported by some laboratories that the integrin α6β1 is involved in sperm-egg interactions (80). However, it is still unclear whether the integrin α6β1 can bind directly to the surface of intact sperm. Therefore, we examined egg molecules with specific affinity for intact sperm. The specific binding of egg-derived molecule(s) is localized at sperm head. This evidence suggests the possible participation of the bound molecule(s) in sperm-egg binding. Only a 135-kDa egg surface protein showed a strong association with sperm, although other molecules were abundantly expressed on the “recovered” eggs. No egg surface proteins bound to sperm when pronase-treated eggs were used. The 135 kDa protein that bound to intact sperm was specifically depleted with the anti-integrin α6 mAb GoH3. Furthermore, GoH3 precipitated a comigrating 135-kDa band from biotinylated egg lysates. These results strongly suggested that the sperm-associated 135-kDa molecule is the integrin α6 subunit, and also that it can adhere specifically to intact sperm. The fact that the α6 integrin subunit appears to be the major egg surface protein that binds to intact sperm suggests that it may be the major sperm receptor on the egg plasma membrane. Involvement of the integrin β1 subunit in binding was reported since a rabbit polyclonal antibody that interacts with the β1 subunit inhibited sperm-egg binding (78). Under our experimental conditions the anti β1 mAb 9EG7 did not precipitate the integrin β1 subunit. And, 9EG7 recognizes its epitope more readily in Mn^{2+}-containing buffers than in Ca^{2+}-containing buffers (86). Therefore, we concluded that 9EG7 could not deplete the integrin α6β1 effectively.

Integrins are the major cell surface components associated with focal adhesion plaques, which are thought to be centers for the interaction of integrins and cytoskeletal proteins and for transmembrane signaling (87). Most of the integrins involved in formation of focal adhesions are members of the β1 and β 3 families (88). Therefore, if the integrin β1 is functional as a sperm receptor, we could hypothesize that focal adhesion-like structures are formed at the site of sperm-egg binding and fusion. The integrin α6 and β1 assembled at the sperm-binding sites. The staining was generally seen on one sperm per egg (data not shown) and the frequency of cluster formation correlated with the extent of sperm-egg fusion, although three or more sperm could bind to an egg. These findings suggest that the integrin α6β1 assembled at the sites where sperm-egg fusion occurred. One possibility is that the integrins clustered only at the sites where sperm binding was mediated via the integrin α6β1. Several proteins are known to mediate the interaction between sperm and eggs (89). Therefore, sperm that did not bind through the integrin α6β1 might not be able to induce the clustering. The other possibility is that modification of sperm proteins during capacitation or acrosome reaction, including pattern of localization, changes in epitope expression or posttranslational modifications, might be necessary to induce clustering even if sperm binding was mediated by the integrin α6β1. Only capacitated sperm could induce aggregation of the integrin α6β1. Some sperm

proteins that trigger integrin clustering may be modified during capacitation and/or acrosome reaction so that they can induce the integrin clustering.

Our immunofluorescence data strongly suggest that not only integrin α6 but also integrin β1 participate in sperm-egg binding and fusion (85). Other integrin molecules or other proteins may also cluster at the sperm-binding sites at the same time. In fact, in our preliminary experiments vinculin, which is an integrin-associated molecule, also assembled at sperm-binding sites. Interestingly, integrin α6β1 disappeared from the site of sperm penetration. These molecules were completely dislocated from the region lying over fertilization cones at 60 min postinsemination or later. According to analysis by scanning electron microscopy, fertilizing sperm are trapped in microvilli (83) and microvilli are dislocated out of the region overlying the fertilization cone (90). Taken together, the distributions of integrin α6 and β1 appear to be related to the distribution of microvilli, and our results strongly suggest that the integrin α6β1 plays an integral role at an early, perhaps only at an early stage of sperm-egg binding and fusion. Both α6 and β1 null mutations in mice are lethal, though the stages at which lethality appear are quite different. The β1 null mutation results in the deformation of the inner cell mass at the early blastocyst stage (91), while α6 null mutants develop to term, but die of severe blistering of the skin (epidermolysis bullosa) and other epithelia (92). Hence, the questions of whether the α6β1 integrin is essential for sperm-egg binding and fusion or whether other integrins or other proteins could serve these functions could not be addressed by the α6 and β1 null mice. Rather, the direct binding of the integrin α6 to intact sperm using a biochemical approach and for the localization of the integrin α6 and β1 subunits at the sperm-binding site using an immunocytochemical approach strongly support the notion that the integrin α6β1 can function as a sperm receptor in mice.

MAP kinase cascade during early cleavage

MAP kinase cascade is one of the main signal transduction pathways expressed ubiquitously in somatic cells, and it transduces many extracellular signals into the nucleus (93). Mitogen stimulations and subsequent activation of the MAP kinase cascade are essential for the proliferation of somatic cells and the removal of mitogens from somatic cell cultures results in their G1/S arrest followed by entrance into the G0-phase (94). The signal transduction pathway of the MAP kinase cascade is thought to be as follows (95). Binding of mitogens, such as insulin and growth factors, to their receptors results in the activation of Ras, a membrane-associated 21-kDa protein belonging to the small GTP-binding protein family, by its dissociation from GDP and association with GTP. Raf-1 protein, a 75-kDa cytosolic serine-threonine kinase, is then activated by transport to the membrane and the subsequent interaction of RasGTP. This process is suggested to involve 14-3-3 (96). The activated Raf-1 phosphorylates serine residues of MAP kinase kinase and activates it. Then the activated MAP kinase kinase activates MAP kinase by phosphorylation of its tyrosine and threonine residues. Phosphorylated MAP kinase translocates into the nucleus and phosphorylates several substrates such as DNA-binding proteins and other protein kinases (95). In mammalian cells, 44-kDa ERK-1 and 42-kDa ERK-2 are known as MAP kinases activated by mitogen signals (97) and 45-kDa MEK-1 and 47-kDa MEK-2 are reported to be MAPkinase kinases locate upstream of the ERKs (98).

In Xenopus oocytes, the activation of ERKs triggers the resumption of meiotic maturation (99). This ERK activation is, however, stimulated not by the Ras/Raf pathway described above but by the c-mos protooncogene product, Mos (100). In mammals, ERK activation during meiosis has also been reported for mouse (47), pig (101), goat (102), rat (103), and bovine (104) oocytes. The Mos-dependent regulation of this ERK activity has been shown in c-mos knockout mouse oocytes (42) and has been suggested in bovine oocytes (105). These studies indicate that the meiotic activation of the MAP kinase cascade depends on meiosis specific Mos production in oocytes. After fertilization, the oocytes escape from meiosis and enter into mitosis. In contrast to somatic cells, mitosis in early embryos is independent of exogenous mitogens, and embryos can undergo cleavage in vitro without the addition of any growth factors or serum. The absence of phosphorylated ERKs during early cleavage has been shown in clam (105) and mouse (106) embryos. Although these reports imply that the MAP kinase cascade is inactivated during embryonic mitosis, at present, the presence and the activation state during early cleavage of the molecules in the

cascade described above has never been reported. In our study, we determined whether these molecules are present and activated in mouse early embryos. The results suggest that M phase-specific activation of the MAP kinase cascade, from Ras to MEK but not the ERKs, occurs during mitosis of mouse early embryos.

We have shown the presence of molecules involved in the MAP kinase cascade--Ras, Raf-1, 14-3-3, MEK-1, and ERKs--in mouse early embryos by immunoblotting. It is well known that exogenous mitogens are not necessary for early embryo culturing, whereas the addition of a mitogen is a prerequisite for cell proliferation in somatic cell cultures. Moreover, the absence of activated ERKs has been reported during early embryo development (107). It seems, therefore, to be widely accepted that the MAP kinase cascade is not activated during early embryo development. Surprisingly, however, a clear shift up of the Raf-1 band in SDS-PAGE was observed at the M-phase of the first and second cleavages, but not at the G2/M phase, in our study. Because a shift up of the Raf-1 band after activation by phosphorylation has been reported (41), our result might show the M-phase-specific activation of Raf-1 during early mouse cleavage (Fig 4) (108).

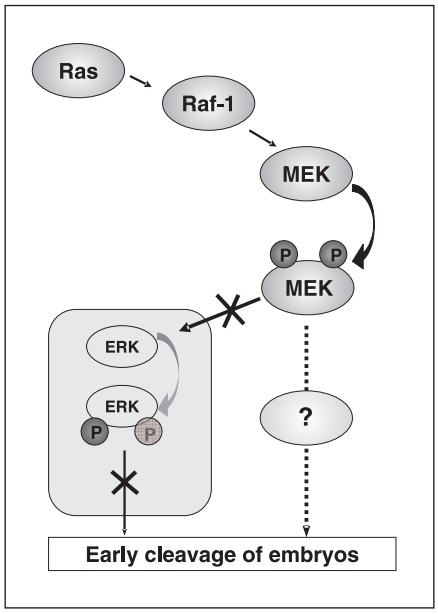

Fig 4: Model for the MAP kinase cascade during early cleavage of embryo. Ras-Raf-1-MAPK cascade activates during early cleavage of embryo. However, the activation of ERK is not necessary.

Recent studies have shown that 14-3-3 associates with inactive Raf-1 in vitro (109) and in NIH3T3 cells (96). It has been suggested that 14-3-3 mediates Ras effects on Raf-1 activation after mitogen stimulation by transferring or anchoring Raf-1 to the membrane and that 14-3-3 dissociates from Raf-1 after its activation (96). Association of 14-3-3 with inactive Raf-1 was shown in G2/M- and M-phase mouse early embryos, and, furthermore, dissociation of 14-3-3 from activated Raf-1 was also detected in the M-phase embryos. These results strongly suggest a role for 14-3-3 in early embryos as a mediator of Ras activity, as in somatic cells, and imply that Ras is also activated at the M-phase in mouse early embryos. Activated Raf-1 phosphorylates and activates MEK in somatic cells (94). This phos-phorylated and activated MEK can be detected by a shift up of the MEK band in SDS-PAGE (110). In our study, as was expected, a shift up of the MEK band was also observed during the period of Raf-1 activation. This indicates that the M-phase-specific activation of the MAP kinase cascade from Ras to MEK occurs in mouse early embryos. In somatic cells, the absence of mitogens results in G1 arrest of the cell cycle, and the cells finally enter into the G0-phase (94). Many studies of the MAP kinase cascade in somatic cells focused on its roles at the G0/G1-phase, and the activation of the MAP kinase cascade at the M-phase has not been reported, although MBP kinase activity has been reported to be higher in the M-phase than in the G1- and S-phases (111). Therefore, the present study might be the first report showing the M-phase-specific activation of the MAP kinase cascade during mitosis. Why the embryonic MAP kinase cascade is activated without mitogen stimulation is unclear. The MAP kinase cascade was activated during the meiotic maturation of mouse oocytes (47), and this activation is regulated not by the mitogen-stimulated Ras/Raf-1 pathway but by the mos proto-oncogene product, Mos, which is synthesized within the oocytes (100). The MAP kinase cascade of the mos-knockout mouse is not activated during oocyte maturation (42). Mos is, however, degraded after fertilization (100), and, therefore, the embryonic MAP kinase cascade should not be regulated by Mos. In fact, Raf-1 was already activated at the meiotic metaphase in our study, as reported

previously (41). It has been reported that early embryos contain mRNAs for many mitogens and their receptors (112) and that the addition of mitogens to the embryo culture improves their developmental rates (113). Embryonic autocrine/paracrine secretion of mitogens has also been suggested (114). It is probable, therefore, that embryos stimulate their own MAP kinase cascade at M-phase by their autocrine/paracrine secretion of mitogens.

It should be noted that phosphorylated forms of ERKs, reported to be active ERKs (47), were not detected at all during early cleavage despite MEK activation. The absence of active ERKs was not due to the failure of the detection methods because the phosphorylated active forms of ERKs were detected in meiotic metaphase oocytes in our study. The absence of active ERKs has been suggested in early cleavage of clam (106) and mouse (107) embryos and also in M-phase-arrested HeLa cells (111), although the activation of upstream molecules of the MAP kinase cascade was not examined in these reports. At present, it is generally believed that ERKs are the only physiological substrate of MEK and conversely that MEK is the only kinase that can phosphorylate both the tyrosine and threonine residues of ERKs physiologically (115). To the best of our knowledge, the activities of MEK and the ERKs follow the same pattern in all reported studies, and our study might be the only report showing the dissociation of MEK and ERK activities (Fig. 4).

In the present study, MBP kinase activity, which reflects the MAP kinase activity (116), was assayed in early embryos; a relatively low but significant activity was detected during the M-phases in early cleavage despite the absence of active ERKs. The high MBP kinase activity at meiotic metaphase may reflect the active ERKs present during this period. The origin of the MBP kinase activity during early cleavage is obscure because MBP can be phosphorylated by kinases other than MAP kinases (106). Heider et al. (111) reported that the MBP kinase activity in the M-phase was high in cultured HeLa cells, although ERKs were not activated during the M-phase. They showed the presence of a 40-kDa novel MBP kinase and suggested that it is a new member of the MAP kinase family. It is well known that the synthesis of many new proteins begins after the fertilization stimulus in mammalian oocytes (117). Therefore, it is probable that the new MAP kinase, which can be activated by the Ras/Raf-1 pathway, is synthesized in early embryos and acts instead of ERKs.

Why do embryos, and perhaps also M-phase somatic cells, have to inhibit the activation of ERKs? The activation of ERKs in the M-phase was reported during meiotic oocyte maturation (103). High ERK activities have been shown to mediate the actions of Mos on oocytes (100): the inhibition of DNA replication between two consecutive meiotic metaphases (118) and the prevention of parthenogenetic activation as cytostatic factor by arresting oocytes at the second metaphase until fertilization (119). Prevention of these inhibitory ERK actions might be one explanation of the suppression of ERKs in the mitotic M-phase. During maturation, MAP kinases are present at the microtubule-organizing center (120) and affect spindle and cytoskeletal functions (121) in addition to their above-mentioned inhibitory actions. Therefore, one possible explanation is a requirement for a new MAP kinase that acts only on the cytoskeletal functions, such as spindle formation, but not as an inhibitor of DNA replication and cytostatic factor during mitosis.

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