—Mini Review—

The Signal Transduction of Meiotic Progression in Mammalian Oocytes

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Abstract: *Mammalian oocytes acquire their intrinsic ability in a stepwise manner through ovarian folliculogenesis, ultimately becoming competent to undergo complete oocyte maturation at the final stage of the Graafian follicle. The fully-grown oocyte is tightly surrounded by compact layers of specialized granulosa cells (cumulus cells) known as the cumulus-oocyte complex. Oocyte maturation consists of the nuclear and the cytoplasmic maturation. Dynamic morphological changes such as cumulus expansion, chromosome alignment, and spindle formation are observed during the oocyte maturation. Mounting evidence that oocyte quality profoundly affects fertilization and subsequent embryo development drives the continued search for reliable predictors of oocyte developmental competence. It is necessary to understand the phenomenon and the molecular mechanisms active during oocyte maturation to obtain high-quality oocytes for in vitro maturation. In the present paper, we summarize the actions of the molecules that play key roles in meiotic progression and its control mechanism.*

Key words: *Oocyte, Meiotic maturation, Signal transduction, Spindle formation*

Features of Meiotic and Cytoplasmic Maturation

Two kinds of haploid germ cells, the spermatozoon and the oocyte, resulted from two consecutive divisions, lead to the generation of a new diploid progeny after successful fertilization. After meiosis, the four haploid gametes are unlikely to be genetically identical on account of homologous recombination at the diplotene stage of the first meiotic prophase (prophase I). Interestingly, only one haploid gamete is produced as a result of two asymmetric divisions in female mammals compared with four in males. The oocytes complete meiosis over an extended time of their life span including two meiotic arrests. The first meiotic cell cycle starts either before birth (rodents, humans, cows, and sheep) or shortly after birth (hamsters, ferrets, and dogs). The mechanisms involved in the triggering of meiotic initiation are not understood. However, it has been proposed that a gradual diffusion or reduction of meiosis-initiating or preventing factor in the central-periphery area of the ovary activates meiosis. The oocytes interrupt meiosis at the G2 phase of the cell cycle, the diplotene/dictyate stage of prophase I commonly known as the germinal vesicle (GV) stage, and remain transcriptionally and translationally active until puberty. After sexual maturity, fully-grown oocytes undergo the latter half of meiosis via luteinizing hormone (LH) signaling shortly before ovulation, which is again interrupted at the metaphase II (MII) stage while awaiting fertilization, this is termed oocyte maturation. The MII oocytes acquire fertilizability and complete developmental potential as a result of cellular events, including both nuclear and cytoplasmic alterations [1]. The traits of nuclear maturation, also termed meiotic maturation are as follows. 1) Following the surge of pituitary LH, meiotic resumption is morphologically characterized by GV breakdown (GVBD), chromosome condensation and spindle formation. 2) Organization of the bipolar spindle, separation of homologous chromosomes and exclusion of the polar body take place, described as metaphase I, anaphase I and telophase I, respectively. 3) Transition to meiosis II occurs without an intermediate phase of DNA replication, arrest at the MII stage until fertilization. On the other hand, cytoplasmic maturation involves metabolic and structural change in the individual organelles, accompanied by the synthesis of

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biochemical molecules and their modification, to ensure the competence of precise meiosis, normal fertilization and the subsequent development. Thus, unlike meiosis in the male, the process of oocyte maturation influences the oocyte quality and the normal developmental potential of the embryo.

It is well known that maturation promoting factor (MPF) is a key molecule active in the regulation of the cell cycle progression during meiosis [2]. MPF is activated at GVBD and increases until it reaches a plateau at the end of the first meiotic metaphase. A transient decline in MPF activity takes place during the transition from metaphase I (MI) to MII. MPF is reactivated rapidly to enter MII and is maintained at a high level during the MII arrest. The equilibrium between pre-MPF and MPF is universally controlled at the post-transcriptional level by the balance between the double specificity kinase that phophorylates residues of Thr14 and Tyr15 of cell division control protein 2 (Cdc2) and the dual specificity phosphatase, Cdc25. Cdc2 maintains cyclin B-Cdc2 complexes in the inactive pre-MPF form, and Cdc25 dephosphorylates these inhibitory residues converting pre-MPF into active MPF.

Preservation of Arrest at the GV Stage

Even if the fully-grown oocytes in the antrum or secondary follicles obtain complete maturity potential, they remain at the GV stage, awaiting the LH surge. In comparison with *in vivo* meiotic resumption in response to LH signaling, it is also stimulated by liberation of cumulus-oocytes complexes (COCs) from the follicle into a suitable culture medium [3, 4]. These facts imply that the follicular environment affects the oocyte by maintaining meiotic arrest at the GV stage with an inhibitory factor. It is considered that some factors such as oocyte-maturation inhibitor (OMI) are produced by granulosa cells, accumulate in the follicular fluid and sustain meiotic arrest in preovulatory follicles [5, 6]. However, the biochemical properties of this protein remain unknown. It is also possible that a meiotic inhibitor such as cAMP is transmitted from follicular cells to immature oocytes efficiently through gap junctions. It has been shown that a rise in intracellular cAMP induced by analogues of cAMP, folskolin, and cAMP phosphodiesterase (PDE) inhibitor, such as 3-isobutyl-1-methylxantine (IBMX) and hypoxanthine, block spontaneous *in vitro* maturation [7]. Also, purines such as hypoxanthine in follicular fluid, have been proposed as a candidate for this inhibitory activity [8]. Thus, cAMP is well established as a major inhibitory substance and its inhibitory action has been demonstrated [9]. Recently, an alternative hypothesis has suggested that the oocyte produces its own cAMP through G-protein coupled receptors (GPCRs) in the oocyte-plasma membrane. Mehlmann *et al.* found that maintenance of GV arrest in fully grown oocytes requires the heterotrimetic G protein, Gs, and they identified the oocyte G-protein coupled receptor GPR3 that activates Gs [10]. Gs stimulates adenylyl cyclase (AC) in the oocyte to keep cAMP elevated [11].

Signal Transduction for Meiotic Resumption and Progression

The LH surge induced by increased levels of serum estradiol rapidly acts on each structural cell of Graafian follicles *in vivo*, inducing meiotic maturation of oocytes, expansion of cumulus cells, and differentiation of mural granulosa cells with alternation of the expression of the genes required for ovulation and luteinization. Although the signaling pathways for LH in the oocyte and follicular cells affecting meiotic resumption remain unclear, it most likely involves a decrease in the oocyte cAMP level, cessation of production of meiosis inhibitory molecules by follicular cells and stimulation of synthesis or activation of meiosis inducing/promoting molecules. After meiotic resumption, chromatin is condensed into chromosomes, microtubules organize into the meiotic spindle, and homologous chromosomes separate to emit the first polar body. These post-GVBD maturational processes are precisely controlled by histone modifications, centrosome protein, various protein kinases/phosphatases, spindle checkpoints and cytoskeletons [12–16]. Recently, the understanding of several signaling cascades that lead to meiotic resumption and progression has improved (summarized in Fig. 1).

PKA signaling pathway

The downstream cascade(s) by which an adequate cAMP concentration within the oocyte regulates meiotic arrest has been partially elucidated; ultimately, a decrease in oocyte cAMP level precedes and leads to meiotic resumption accompanied by GVBD. The uncoupling of cumulus cells from the oocytes by interruption of gap junctions accompanied by cumulus expansion may block elevation of intra-oocyte cAMP concentration and permit reinitiation of meiotic maturation [17]. However, it has been found that cell-cell communication in COCs until after GVBD [18].

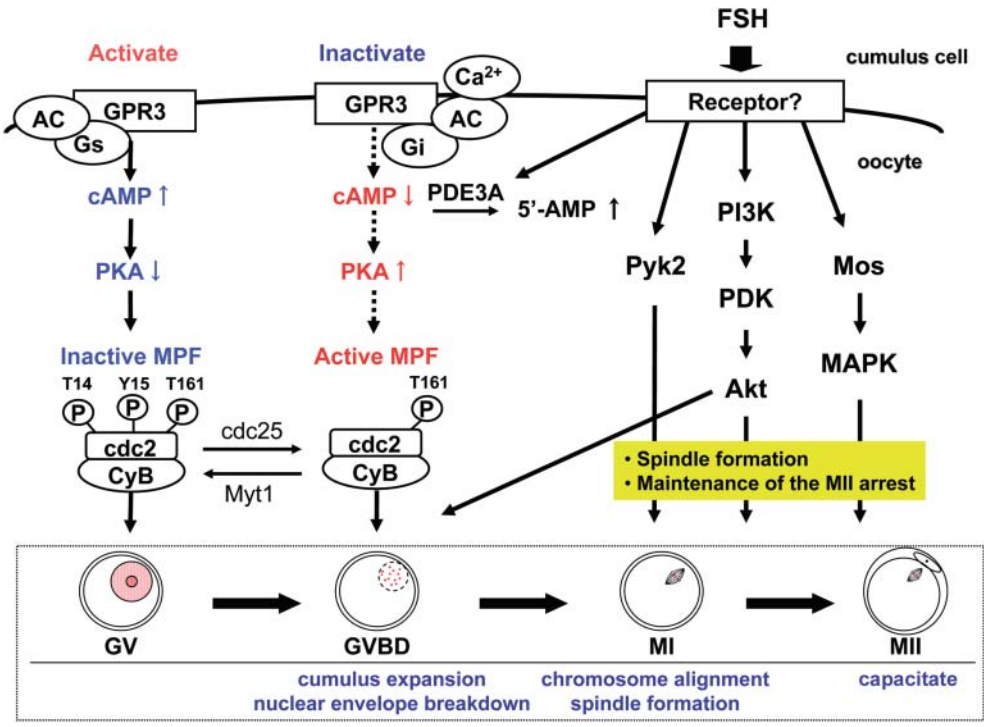

Fig. 1. A schematic diagram of the signaling cascades regulating meiotic progression, such as the PKA, Pyk2, PI3K/Akt, and Mos/MAPK pathways. G-protein coupled receptor, GPR3, has a certain level of constitutive activity and interacts with sphingosine 1-phosphate and sphingosylphosphorylcholine to further activate G protein, Gs, which stimulates adenylyl cyclase (AC) elevating cAMP. The elevated cAMP activates PKA, which finally inactivates MPF by phosphorylation of Cdc2 kinase. The potential stimulation inhibits the coupling of GPR3-Gs-AC, and MPF is activated by dephosphorylation of Cdc2 kinase. The Pyk2, PI3K/Akt, and Mos/MAPK cascades are involved in the transition from MI to MII, especially, spindle formation and maintenance.

Moreover, more recent studies have shown an increase rather than a decrease in cAMP facilitates induction of meiotic maturation [19]. Also, *in vitro* studies have indicated that FSH induces an increase in cAMP in the cumulus cells resulting in an increase in cAMP in the oocyte via diffusion from the somatic cells to the oocyte through gap junctions [20]. Thus, it is hypothesized that a certain concentration of cAMP is maintained in GV oocytes, while a transient increase in cAMP induced by hormonal stimulation is likely to trigger GVBD [21]. The drastic change in cAMP, not its absolute level, may be the important stimulus for reinitiation of meiosis, in the oocyte. The action of cAMP within oocytes is mediated by cAMP-dependent protein kinase (PKA).

Downs and Hunzicker-Dunn have demonstrated that two major isozymes of PKA are involved in opposing functions of meiotic regulation in COCs [22]. They suggest that elevation of type I PKA within the oocyte is related to the maintenance of meiotic arrest while type II PKA mediates cAMP-stimulated cumulus expansion and meiotic resumption. Additionally, cAMP phosphodiesterase is an important enzyme controlling PKA activity. This enzyme is present within oocytes, keeps cAMP levels low and tolerates spontaneous maturation [23]. The accumulation of 5'-AMP, a product of PDE activity, stimulates AMP-activated protein kinase (AMPK), leading to meiotic resumption [24].

PI3K (PI3K/Akt) Akt signaling pathway

Phosphatidylinositol 3-kinase (PI3K) is known to play critical roles in signal transduction processes related to a variety of cellular activities such as cytoskeletal rearrangement, cellular migration, differentiation, protection against apoptosis and mitogenesis [25]. Previously, we reported that PI3K participates in FSH-induced cumulus expansion and meiotic maturation in mouse oocytes, but not spontaneous maturation [26]. Akt also known as protein kinase B, has been identified

as a serine-threonine kinase. The activation of Akt is thought to be a critical step in the PI3K pathway that regulates cell growth and differentiation [27]. In fully-grown mouse oocytes, a decrease in cAMP concentration precedes and is linked to Cdc2 activation [28]. In mouse oocytes, Akt is involved in Cdc2 activation and resumption of meiosis [29]. We examined the distribution of phosphorylated Akt during meiotic maturation. Thr308-phosphorylated Akt was localized in pericentriolar materials (PCM) at MI and MII. In contrast, the distribution of Ser473-phosphorylated Akt was similar to that of microtubules at prometaphase I (PMI) and was localized in spindles at MI and MII [26]. The activity of Akt is related to spindle formation in mouse oocytes. When COCs were treated with PI3K inhibitor (LY294002) in FSH-induced meiotic maturation, the amount of Thr308-phosphorylated Akt decreased to a very low to undetectable level in PMI, MI and MII oocytes. The distribution of Ser473-phosphorylated Akt in LY294002-treated PMI oocytes was similar to that in normal PMI oocytes, whereas aberrant distribution and a very low to undetectable level of expression were seen in LY294002-treated MI and MII oocytes, respectively. These results suggest that Akt participates in gonadotropin-induced meiotic maturation as a downstream effecter of the PI3K pathway in mouse oocytes.

It is interesting that the localizations of two phosphorylated forms of Akt are different. The difference suggests that the role of each active form could be different. To address this issue, individual phosphorylated Akt antibodies were injected into MII oocytes. Both Thr308- and Ser473-phosphorylated Akt antibody caused a shorter spindle to form in MII oocytes. A short spindle in MII oocytes may interrupt the process of fertilization. To address whether the functions of Thr308- and Ser473-phosphorylated Akt are different in MII oocytes for fertilization, *in vitro* fertilization was performed using oocytes treated with inhibition peptide at MII for 3 hours. Although oocytes showed second polar body emission after the injection of the peptide for Thr308-phosphorylated Akt, the chromosomal alignment and microtubular organization were aberrant. In contrast, the injection of the peptide for Ser473-phosphorylated Akt caused a failure of second polar body emission. These results suggest that individual Thr308- and Ser473-phosphorylated Akt activities are involved in fertilization to complete meiosis. Furthermore, these results suggest that the two active forms have different roles, i.e. Ser473-phosphorylated Akt activity is involved in the second polar body emission while Thr308 regulates the organization of microtubules [30].

Mos/MAPK signaling pathway

Mitogen activated protein kinases (MAPKs), also termed extracellular-regulated kinases (ERKs), comprise another family of serine/threonine protein kinases that are involved in meiosis [31]. There are two isoforms, ERK1 (44 kDa) and ERK2 (42 kDa), which are more abundantly expressed in both the oocytes and cumulus cells at the later stage of meiosis [32]. MAPKs are activated by an upstream kinase identified as a dual specific MAPK kinase (MAPKK) or MAPK/ERK kinase (MEK). The activation of MAPK and MEK during oocyte maturation has been reported in several species, including the marine invertebrate, Xenopus, and mammals [33]. The proto-oncogene Mos, which is member a member of the serine/threonine protein kinase family, appears to be required for activation of MAPK. In Xenopus oocytes, it is well established that Mos/MAPKs is crucial for the activation of MPF and GVBD through a progesterone-induced cAMP-PKA decrease [34]. Oocytes from the c-mos knockout mouse show normal levels of MPF activity despite deletion of the MAPK activity and simply undergo GVBD. However, they produce an abnormally large polar body and show irregular spindle formation. Furthermore, some oocytes from c-mos knockout mice that reached the MII stage emitted a second polar body and progressed to the third meiotic metaphase when they were fertilized or stimulated by ethanol. These results demonstrate that the Mos/MAPK pathway is independent of MPF activity and is not essential for GVBD, but plays a critical role in normal spindle and chromosome morphology in mice [35]. It is now well accepted that the Mos/MAPK pathway is responsible for proper spindle formation at MI and MII, repression of DNA replication at the MI-MII transition and maintenance of the MII arrest.

Pyk2 kinase

Pyk2 kinase is a no-receptor tyrosine kinase essential for actin filament organization. It exerts its effect on microfilaments by regulating other proteins such as gelsolin, which is also involved in cytoskeleton regulation [36]. Recently, it has been shown that Pyk2 is expressed in rat oocytes and co-localizes with microfilaments during rat oocyte maturation. Microinjection of Pyk2 antibody perturbs microfilament assembly and inhibits the first polar body extrusion [37].

Moreover, Pyk2 has also been shown to regulate the activity of the Pho-GTPase family. These results could be of great interest since it has recently been shown that Cdc42, a small GTPase belonging to the Rho family, could be involved in spindle positioning during meiotic maturation in mouse oocytes [38]. Indeed, the expression of a dominant-negative mutant of Cdc42 inhibits the first meiotic spindle migration to the cortex and induces unusual spindle lengthening. As a result, asymmetric division does not occur [38]. Thus, the pathway affecting the actin cytoskeleton, which is required for the first meiotic spindle migration, could be regulated by Cdc42. Hence, it is possible that Pyk2 might also regulate actin microfilament organization in mouse oocytes via Cdc42; however, the molecular interactions remain to be investigated.

Conclusion

It is clear that there are a lot of molecules that are essential for the control of meiotic resumption, progression and spindle formation. If each molecular mechanism involved in nuclear and cytoplasmic maturation is clarified it would likely to lead to the improvement of *in vitro* maturation. Especially, there is a possibility that a high-quality oocyte could be selected based on the morphology of the spindle and the chromosome observed by the nuclear maturation. We assume a clarified key molecule to be an index, and are aiming at the establishment of the technology that can select high-quality oocytes under the microscope.

References

1) Eppig, J.J. (1996): Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod. Fertil. Dev., 8, 485–489.
2) Josefsberg, L.B., Galiani, D., Lazar, S., Kaufman, O., Seger, R. and Dekel, N. (2003): Maturation-promoting factor governs mitogen-activated protein kinase activation and interphase suppression during meiosis of rat oocytes. Biol. Reprod., 68, 1282–1290.
3) Edwards, R.G. (1965): Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature, 208, 349–351.
4) Pincus, G. and Enzmann, E.V. (1935): The comparative behavior of mammalian eggs in vivo and in vitro: I. the activation of ovarian eggs. J. Exp. Med., 62, 665–675.
5) Tsafriri, A. and Channing, C.P. (1975): An inhibitory influence of granulosa cells and follicular fluid upon porcine oocyte meiosis in vitro. Endocrinology, 96, 922–927.
6) Sato, E. and Koide, S.S. (1984): A factor from bovine granulosa cells preventing oocyte maturation. Differentiation, 26, 59–62.
7) Cho, W.K., Stern, S. and Biggers, J.D. (1974): Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. J. Exp. Zool., 187, 383–386.
8) Eppig, J.J., Ward-Bailey, P.F. and Coleman, D.L. (1985): Hypoxanthine and adenosine in murine ovarian follicular fluid: concentrations and activity in maintaining oocyte meiotic arrest. Biol. Reprod., 33, 1041–1049.
9) Aberdam, E., Hanski, E. and Dekel, N. (1987): Maintenance of meiotic arrest in isolated rat oocytes by the invasive adenylate cyclase of Bordetella pertussis. Biol. Reprod., 36, 530–535.
10) Mehlmann, L.M., Saeki, Y., Tanaka, S., Brennan, T.J., Evsikov, A.V., Pendola, F.L., Knowles, B.B., Eppig, J.J. and Jaffe, L.A. (2004): The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. Science, 306, 1947–1950.
11) Horner, K., Livera, G., Hinckley, M., Trinh, K., Storm, D. and Conti, M. (2003): Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. Dev. Biol., 258, 385–396.
12) Sun, Q.Y., Lai, L., Park, K.W., Kuhholzer, B., Prather, R.S. and Schatten, H. (2001): Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization in vitro. Biol. Reprod., 64, 879–889.
13) Wang, Q., Yin, S., Ai, J.S., Liang, C.G., Hou, Y., Chen, D.Y., Schatten, H. and Sun, Q.Y. (2006): Histone deacetylation is required for orderly meiosis. Cell Cycle, 5, 766–774.
14) Wang, Q., Ai, J.S., Idowu, O.S., Gu, L., Zhang, Y.Z., Chen, D.Y. and Sun, Q.Y. (2008): The spatial relationship between heterochromatin protein 1 alpha and histone modifications during mouse oocyte meiosis. Cell Cycle, 7, 513–520.
15) Saskova, A., Solc, P., Baran, V., Kubelka, M., Schultz, R.M. and Motlik, J. (2008): Aurora kinase A controls meiosis I progression in mouse oocytes. Cell Cycle, 7, 2368–2376.
16) Solc, P., Saskova, A., Baran, V., Kubelka, M., Schultz, R.M. and Motlik, J. (2008): CDC25A phosphatase controls meiosis I progression in mouse oocytes. Dev. Biol., 317, 260–269.
17) Thomas, R.E., Armstrong, D.T. and Gilchrist, R.B. (2004): Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3',5'-monophosophate levels. Biol. Reprod., 70, 548–556.
18) Motlik, J., Fulka, J. and Flechon, J.E. (1986): Changes in intercellular coupling between pig oocytes and cumulus cells during maturation in vivo and in vitro. J. Reprod. Fertil., 76, 31–37.
19) Mattioli, M., Galeati, G., Barboni, B. and Seren, E. (1994): Concentration of cyclic AMP during the maturation of pig oocytes in vivo and in vitro. J. Reprod. Fertil., 100, 403–409.

20) Webb, R.J., Marshall, F., Swann, K. and Carroll, J. (2002): Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase a in mammalian oocytes. Dev. Biol., 246, 441–454.
21) Dekel, N., Galiani, D. and Sherizly, I. (1988): Dissociation between the inhibitory and the stimulatory action of cAMP on maturation of rat oocytes. Mol. Cell Endocrinol., 56, 115–121.
22) Downs, S.M. and Hunzicker-Dunn, M. (1995): Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus cell complex by site-selective analogs of cyclic adenosine monophosphate. Dev. Biol., 172, 72–85.
23) Bornslaeger, E.A., Mattei, P. and Schultz, R.M. (1986): Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. Dev. Biol., 114, 453–462.
24) Downs, S.M., Hudson, E.R. and Hardie, D.G. (2002): A potential role for AMP-activated protein kinase in meiotic induction in mouse oocytes. Dev. Biol., 245, 200–212.
25) Franke, T.F., Kaplan, D.R. and Cantley, L.C.(1997): PI3K: downstream AKTion blocks apoptosis. Cell, 88, 435–437.
26) Hoshino, Y., Yokoo, M., Yoshida, N., Sasada, H., Matsumoto, H. and Sato, E. (2004): Phosphatidylinositol 3-kinase and Akt participate in the FSH-induced meiotic maturation of mouse oocytes. Mol. Reprod. Dev., 69, 77–86.
27) Neri, L.M., Borgatti, P., Capitani, S. and Martelli, A.M. (2002): The nuclear phosphoinositide 3-kinase/AKT pathway: a new second messenger system. Biochim. Biophys. Acta., 1584, 73–80.
28) Morgan, D.O. (1995): Principles of CDK regulation. Nature, 374, 131–134.
29) Kalous, J., Solc, P., Baran, V., Kubelka, M., Schultz, R.M. and Motlik, J. (2006): PKB/AKT is involved in resumption of meiosis in mouse oocytes. Biol. Cell, 98, 111–123.
30) Hoshino, Y. and Sato, E. (2008): Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. Dev. Biol., 314, 215–223.
31) Yokoo, M. and Sato, E. (2004): Cumulus-oocyte complex interactions during oocyte maturation. Int. Rev. Cytol., 235, 251–291.
32) Verlhac, M.H., de Pennart, H., Maro, B., Cobb, M.H. and Clarke, H.J. (1993): MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. Dev. Biol., 158, 330–340.
33) Wu, J.Q. and Kornbluth, S. (2008): Across the meiotic divide - CSF activity in the post-Emi2/XErp1 era. J. Cell Sci., 121, 3509–3514.
34) Duckworth, B.C., Weaver, J.S. and Ruderman, J.V. (2002): G2 arrest in Xenopus oocytes depends on phosphorylation of cdc25 by protein kinase A. Proc. Natl. Acad. Sci. USA, 99, 16794–16799.
35) Araki, K., Naito, K., Haraguchi, S., Suzuki, R., Yokoyama, M., Inoue, M., Aizawa, S., Toyoda, Y. and Sato, E. (1996): Meiotic abnormalities of c-mos knockout mouse oocytes: activation after first meiosis or entrance into third meiotic metaphase. Biol. Reprod., 55, 1315–1324.
36) Wang, Q., Xie, Y., Du, Q.S., Wu, X.J., Feng, X., Mei, L., McDonald, J.M. and Xiong, W.C. (2003): Regulation of the formation of osteoclastic actin rings by proline-rich tyrosine kinase 2 interacting with gelsolin. J. Cell Biol., 160, 565–575.
37) Meng, X.Q., Zheng, K.G., Yang, Y., Jiang, M.X., Zhang, Y.L., Sun, Q.Y. and Li, Y.L. (2006): Proline-rich tyrosine kinase2 is involved in F-actin organization during in vitro maturation of rat oocyte. Reproduction, 132, 859–867.
38) Na, J. and Zernicka-Goetz, M. (2006): Asymmetric positioning and organization of the meiotic spindle of mouse oocytes requires CDC42 function. Curr. Biol., 16, 1249–1254.