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Cell-cycle-dependent subcellular localization of cyclin B1, phosphorylated cyclin B1 and p34^{cdc2} during oocyte meiotic maturation and fertilization in mouse

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Summary

M phase or maturation promoting factor (MPF), a kinase complex composed of the regulatory cyclin B and the catalytic p34^{cdc2} kinase, plays important roles in meiosis and mitosis. This study was designed to detect and compare the subcellular localization of cyclin B1, phosphorylated cyclin B1 and p34^{cdc2} during oocyte meiotic maturation and fertilization in mouse. We found that all these proteins were concentrated in the germinal vesicle of oocytes. Shortly after germinal vesicle breakdown, all these proteins were accumulated around the condensed chromosomes. With spindle formation at metaphase I, cyclin B1 and phosphorylated cyclin B1 were localized around the condensed chromosomes and concentrated at the spindle poles, while p34^{cdc2} was localized in the spindle region. At the anaphase/telophase transition, phosphorylated cyclin B1 was accumulated in the midbody between the separating chromosomes/chromatids, while p34^{cdc2} was accumulated in the entire spindle except for the midbody region. At metaphase II, both cyclin B1 and p34^{cdc2} were horizontally localized in the region with the aligned chromosomes and the two poles of the spindle, while phosphorylated cyclin B1 was localized in the two poles of spindle and the chromosomes. We could not detect a particular distribution of cyclin B1 in fertilized eggs when the pronuclei were initially formed, but in late pronuclei cyclin B1 was accumulated in the pronuclei. p34^{cdc2} and phosphorylated cyclin B1 were always concentrated in one pronucleus after parthenogenetic activation or in two pronuclei after fertilization. At metaphase of 1-cell embryos, cyclin B1 was accumulated around the condensed chromosomes. Cyclin B1 was accumulated in the nucleus of late 2-cell embryos but not in early 2-cell embryos. Furthermore, we also detected the accumulation of p34^{cdc2} in the nucleus of 2- and 4-cell embryos. All these results show that cyclin B1, phosphorylated cyclin B1 and p34^{cdc2} have similar distributions at some stages but different localizations at other stages during oocyte meiotic maturation and fertilization, suggesting that they may play a common role in some events but different roles in other events during oocyte maturation and fertilization.

Keywords: Fertilization, Kinase, Meiosis, Mouse, Ovum

Introduction

Fully grown mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, which is also termed the germinal vesicle (GV) stage. GV-stage arrested oocytes can spontaneously resume meiosis when they are released from the inhibitory environment of follicles. However, these oocytes arrest again at metaphase II of meiosis. Completion of meiosis, characterized by sister chromatid separation and extrusion of the second polar body (PB2), is triggered by

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fertilization. The zygote contains a haploid male and a haploid female pronucleus (PN). The haploid pronuclei gradually move towards each other and prepare for the first mitosis. Soon after combination of the male and female chromosomes and mitotic spindle formation, mitosis proceeds through the 1-cell zygote to form the 2-cell embryo (Josefsberg *et al*., 2001).

M phase or maturation promoting factor (MPF), a kinase complex composed of the regulatory cyclin B and the catalytic $p34^{cdc2}$ kinase, plays important roles in meiosis and mitosis (Jones, 2004; Masui, 2001). Mitotic or meiotic entry is triggered by MPF activation, while mitotic (Reimann & Jackson, 2002) or meiotic (Tokumoto *et al*., 1997) exit requires MPF inactivation, which is caused by cyclin B destruction via the ubiquitin proteasome pathway. The tyrosine/threonine protein kinase activity of MPF is regulated by several factors including phosphorylation/dephosphorylation of specific residues (Krek & Nigg, 1991; Solomon *et al*., 1990). The $p34^{cdc2}$ kinase activity is negatively regulated by phosphorylation at Thr14 by the Myt1 kinase and at Tyr15 by the Wee1/Mik1 or Myt1 protein kinases (Gould & Nurse, 1989; Kornbluth *et al*., 1994; Mueller *et al*., 1995; Parker *et al*., 1992; Russell & Nurse, 1987), and positively regulated by phosphorylation at Thr161 by $p34^{cdc2}$-activating kinase (Fesquet *et al*., 1993; Poon *et al*., 1993; Gould *et al*., 1991; Solomon *et al*., 1992). Dephosphorylation of Thr14 and Tyr15 is controlled by the cdc25 phosphatase (Gautier & Maller, 1991; Honda *et al*., 1993; Millar *et al*., 1991), and in conjunction with Thr161 phosphorylation, is required for full MPF activity. Furthermore, other factors such as its subunit concentrations and its intracellular localization may also be important for its activity (Masui, 2001; Ohashi *et al*., 2001). Therefore, variations in these parameters would modify the activity of MPF.

It is well known that the synthesis and degradation of cyclin B is important for regulating the activity of MPF, and cyclin B1 and $p34^{cdc2}$ protein have been detected in mammalian oocytes, but the subcellular localization of cyclin B1 and $p34^{cdc2}$ were only briefly reported in pig (Casas *et al*., 1999), goat (Hue *et al*., 1997) and mouse (Chesnel & Eppig, 1995; Kanatsu-Shinohara *et al*., 2000; Mitra & Schultz, 1996) oocytes. Cyclin B1 has also been detected in the nucleus of 2-cell mouse embryo, which was regulated by $p34^{cdc2}$ kinase (Ohashi *et al*., 2001).

Furthermore, some researchers have reported that phosphorylation of cyclin B1 is important for its entry into the nucleus and the activation of MPF in the nucleus (Li *et al*., 1995, 1997; Yang *et al*., 2001), and it is also proposed that cyclin B1 phosphorylation is required for cdc25c and MPF activation *in vivo* (Peter *et al*., 2002), but the subcellular localization of phosphorylated cyclin B1 during oocyte meiosis has not been studied.

In this study, we systematically detected the subcellular localization of cyclin B1, phosphorylated cyclin B1 and p34 at different developmental stages during oocyte meiotic maturation and fertilization in mouse. Our results showed that these three proteins have similar distributions at some developmental stages but a different localization at other stages.

Materials and methods

Chemicals and solutions

Polyclonal rabbit anti-human cyclin B1 antibody (sc-752), polyclonal mouse anti-human phosphorylated cyclin B1 antibody (sc-20253-R) and monoclonal mouse anti-human $p34^{cdc2}$ antibody (sc-154) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals or components of media were embryo culture or cell culture grade and were obtained from Sigma (St Louis, MO) unless noted otherwise.

Mouse oocyte and zygote collection

Fully grown germinal vesicle-intact oocytes were obtained as previously described by Tong *et al*. (2002) and maintained in M2 medium supplemented with 60 μg/ml penicillin and 50 μg/ml streptomycin, and cultured in M2 medium. All cultures were carried out at 37 °C in a humidified atmosphere of 5% CO_2, and oocytes at different stages were collected for confocal microscopy.

For the collection of metaphase II-arrested eggs, females were superovulated by intraperitoneal injection using 10 IU of pregnant mare's serum gonadotropin (PMSG) followed 46–48 h later with 10 IU of human chorionic gonadotropin (hCG). Mice were killed and oviducts were removed at 14–16 h after hCG injection. Using a pair of fine forceps to tear the oviducts, cumulus masses were collected in M2 medium. To remove the cumulus cells, eggs were briefly exposed to 300 IU/ml hyaluronidase followed by three washes in M2 medium.

In vitro fertilization was performed using 1×10^6/ml motile cauda epididymal sperm, which had been previously capacitated in M16 medium with 2.5 mM taurine for 1 h. The eggs were collected at different stages for confocal microscopy.

In vivo fertilized zygotes were collected 16 h post-hCG from the oviduct ampullae of superovulated females that had been mated with males of the same strain. After removing cumulus cells with 300 IU/ml hyaluronidase in M2 medium, zygotes were cultured in M16 medium until use. The eggs were collected at different stages for confocal microscopy.

Parthenogenetic activation of mouse oocytes

Parthenogenetic activation of mouse oocytes was performed as we previously reported (Tong *et al*., 2002). The denuded eggs were treated with 10 μM A23187 for 5 min, and then incubated in M16 medium containing 10 μg/ml CHX. The activated eggs were collected at different stages for confocal microscopy.

Confocal microscopy of mouse oocytes

After removal of zonae pellucidae in acidified Tyrode's solution (pH 2.5), oocytes or embryos at the desired stages were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and permeabilized for 30 min in the incubation buffer (0.5% Triton X-100 in 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3), then washed in PBS with 0.1% Tween 20 three times and incubated with polyclonal rabbit anti-human cyclin B1 antibody, polyclonal mouse anti-human p34^{cdc2} antibody or polyclonal mouse anti-human phosphorylated cyclin B1 antibody diluted 1:100 for 1 h. The oocytes or embryos were rinsed three times and incubated for 1 h with 1:100 fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or goat anti-mouse IgG according to the primary antibody, followed by staining with 10 μg/ml propidium iodide. Finally, the oocytes or embryos were mounted on glass slides and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems). As a negative control, the first antibody was replaced with rabbit or mouse IgG.

Results

Subcellular localization of cyclin B1 during mouse oocyte meiotic maturation

The distribution of cyclin B1 during mouse oocyte meiotic maturation is shown in Fig. 1 The specimens were stained with PI to visualize the DNA and confirm the stage of meiotic maturation. The localization of cyclin B1 varied at different developmental stages. In competent GV-stage oocytes the cyclin B1 was mainly localized in the germinal vesicle (Fig. 1A), but was localized over the whole oocyte in incompetent GV-stage oocytes (Fig. 1B). Shortly after germinal vesicle breakdown (GVBD), the cyclin B1 accumulated around the condensed chromosomes (Fig. 1C, D). At metaphase I, with the organization of chromosomes to the equatorial plate, the localization of cyclin B1 was concentrated around the aligned chromosomes (Fig. 1E), putatively the position of the metaphase I spindle and at the two poles of the spindle. At metaphase II, the cyclin B1 was accumulated at the two poles of the spindle. Some staining of cyclin B1 was also observed in cytoplasm around the chromosomes (Fig. 1F).

Subcellular localization of cyclin B1 in mouse fertilized eggs and early embryos

As shown in Fig. 2, at the early female and male pronuclear stage, cyclin B1 was evenly distributed in the whole cytoplasm (Fig. 2A). With the development of the pronuclei, at the middle (Fig. 2B) and the later (Fig. 2C) pronuclear stage, cyclin B1 accumulated in the female and male pronuclei. After pronuclear envelope breakdown, staining of cyclin B1 was observed around the condensed chromosomes (Fig. 2D). In 1-cell metaphase embryos, cyclin B1 also concentrated around the aligned chromosomes (Fig. 2E). In early 2-cell embryos cyclin B1 was diffused throughout the whole cytoplasm (Fig. 2F), but in later 2-cell embryos cyclin B1 accumulated in the nucleus (Fig. 2G). A GV-stage oocyte was used as a negative control, in which the polyclonal rabbit anti-human cyclin B1 antibody was replaced with rabbit IgG.

Subcellular localization of phosphorylated cyclin B1 during mouse oocyte meiotic maturation

The distribution of phosphorylated cyclin B1 during mouse oocyte meiotic maturation is shown in Fig. 3. In GV-stage oocytes, the cyclin B1 was mainly localized in the GV (Fig. 3A). Shortly after GVBD, the cyclin B1 accumulated around the condensed chromosomes, and was focused as several dots near to the chromosomes (Fig. 3B). With the organization of the chromosomes to the equatorial plate at metaphase I, the phosphorylated cyclin B1 migrated to and formed a loop around the poles of the spindles; some staining of the phosphorylated cyclin B1 was also observed around the aligned chromosomes (Fig. 3C). At anaphase and telophase I, the phosphorylated cyclin B1 migrated to the midbody between the separating homologous chromosomes (Fig. 3D, E). At metaphase II, the phosphorylated cyclin B1 was focused as one dot at the poles of the spindles, and some staining of phosphorylated cyclin B1 was also associated with the aligned chromosomes (Fig. 3F).

Subcellular localization of phosphorylated cyclin B1 in mouse fertilized eggs and early embryos

As shown in Fig. 4, at anaphase II the phosphorylated cyclin B1 was accumulated at the midbody between the separating sister chromatids (Fig. 4A). After parthenogenetic activation the phosphorylated cyclin B1 was concentrated in one pronucleus (Fig. 4B), and after fertilization in the female and male pronuclei (Fig. 4C). We also detected the accumulation of phosphorylated cyclin B1 in the nucleus in 2-cell embryos (Fig. 4D).

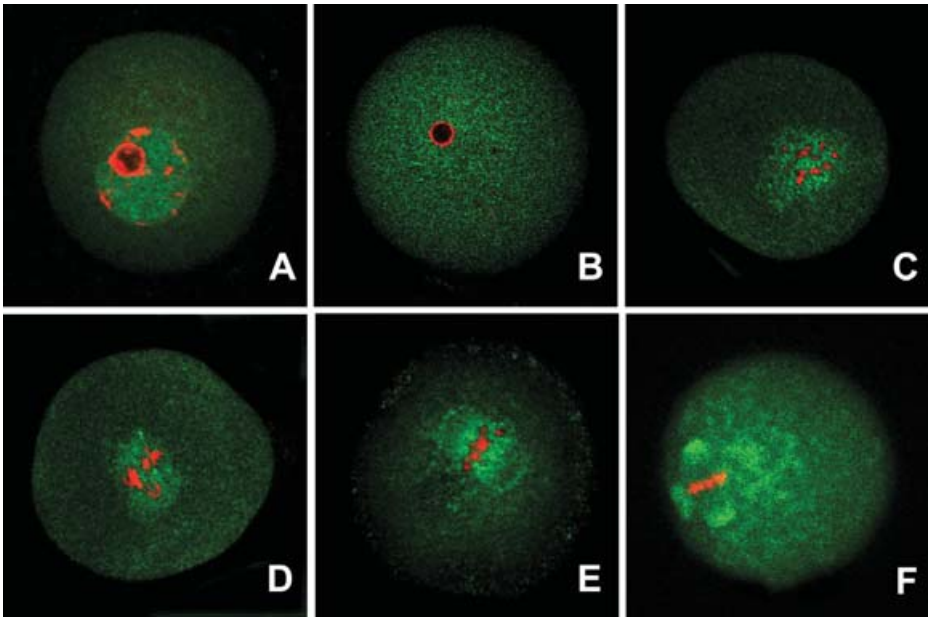

Figure 1 Subcellular localization of cyclin B1 during mouse oocyte meiotic maturation. Green, cyclin B1; red, chromatin; orange–yellow, overlap of green and red. Cyclin B1 was mainly localized in the germinal vesicle of the competent GV-stage oocytes (*A*), but not the incompetent GV-stage oocyte (*B*). Shortly after GVBD, the cyclin B1 accumulated around the condensed chromosomes (*C*, *D*), and with the assembly of the metaphase I spindle the localization of cyclin B1 was concentrated around the aligned chromosomes (*E*), putatively the position of the metaphase I spindle and at the two poles of the spindle. At metaphase II, the cyclin B1 was accumulated at the two poles of the spindle. Some staining of cyclin B1 was also observed in cytoplasm around the chromosomes (*F*).

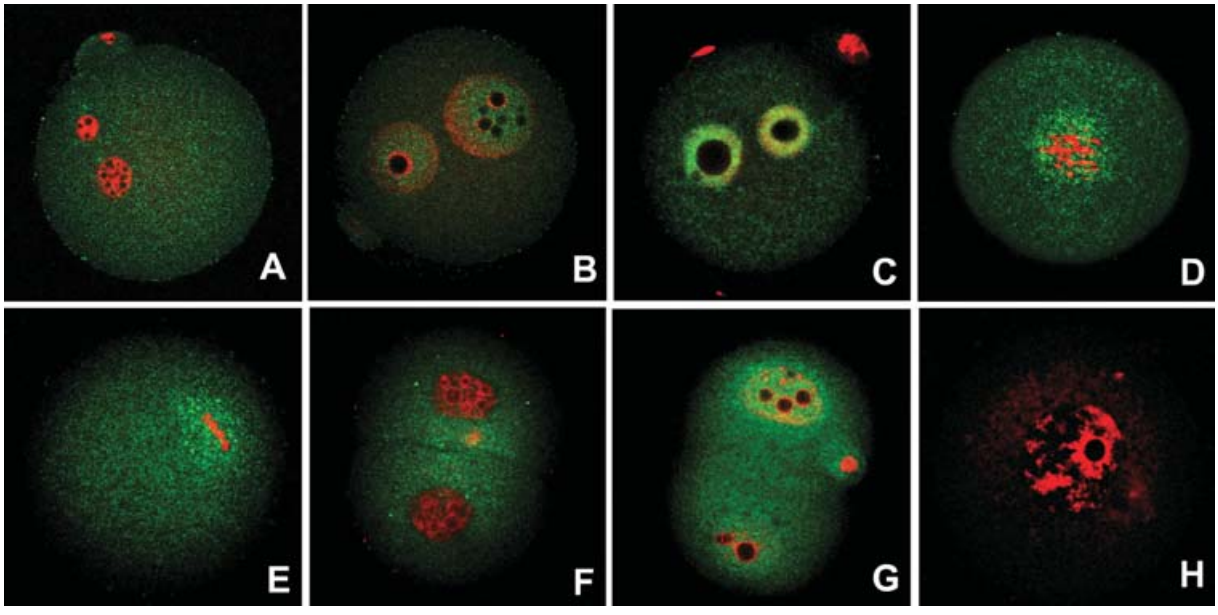

Figure 2 Subcellular localization of cyclin B1 in mouse fertilized oocytes and early embryos. At the early pronuclear stage, cyclin B1 was evenly distributed in the whole cytoplasm (*A*), while at the middle (*B*) and later (*C*) pronuclear stages cyclin B1 accumulated in the female and male pronuclei. After pronuclear envelope breakdown, the staining of cyclin B1 was observed around the condensed chromosomes (*D*). At the 1-cell embryo metaphase, cyclin B1 also concentrated around the aligned chromosomes (*E*). Cyclin B1 was accumulated in the nucleus of late 2-cell embryos (*G*) but not early 2-cell embryos (*F*). A GV-stage oocyte was used as a negative control, in which the polyclonal rabbit anti-human cyclin B1 antibody was replaced with rabbit IgG (*H*).

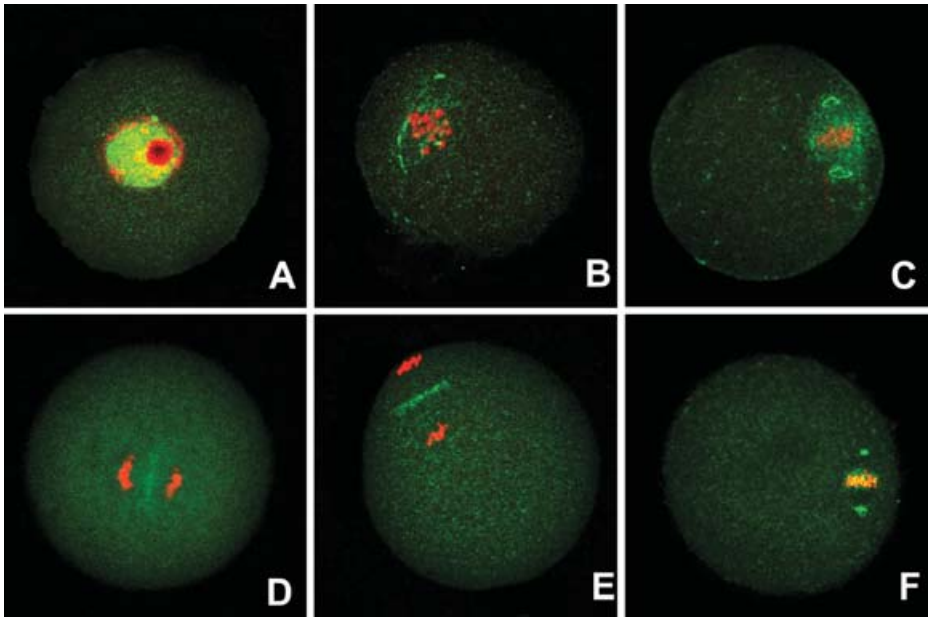

Figure 3 Subcellular localization of phosphorylated cyclin B1 during mouse oocyte meiotic maturation. The cyclin B1 was mainly localized in the germinal vesicle of GV-stage oocytes (*A*). Shortly after GVBD, the cyclin B1 accumulated around the condensed chromosomes, and focused as several dots near the chromosomes (*B*). With the spindle assembly at metaphase I, the phosphorylated cyclin B1 migrated to and formed a loop around the poles of the spindles; some staining of the phosphorylated cyclin B1 was also seen around the aligned chromosomes (*C*). At anaphase and telophase I, the phosphorylated cyclin B1 migrated to the midbody between the separating homologous chromosomes (*D*, *E*). At metaphase II, the phosphorylated cyclin B1 was focused as one dot at the poles of the spindles; some staining of phosphorylated cyclin B1 was also associated with the aligned chromosomes (*F*).

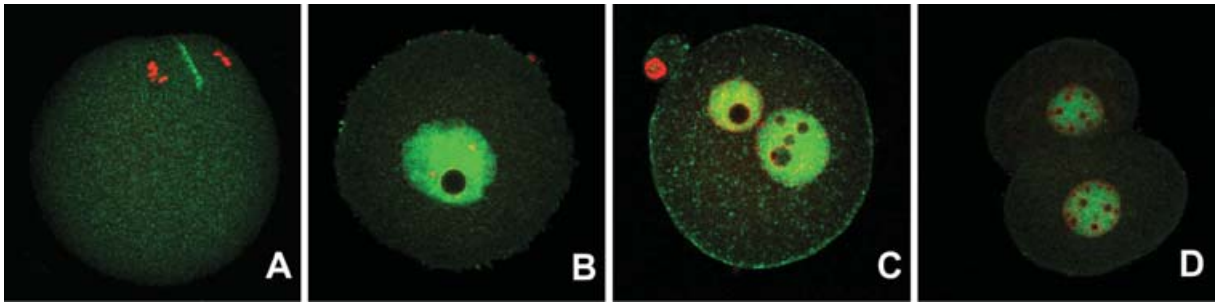

Figure 4 Subcellular localization of phosphorylated cyclin B1 in mouse fertilized oocytes and early embryos. At anaphase II, the phosphorylated cyclin B1 was accumulated at the midbody between the separating sister chromatids (*A*). After parthenogenetic activation phosphorylated cyclin B1 concentrated in one pronucleus (*B*), and after fertilization in the female and male pronuclei (*C*). The accumulation of phosphorylated cyclin B1 in the nucleus of the 2-cell embryo was also detected (*D*).

Subcellular localization of p34^{cdc2} during mouse oocyte meiotic maturation

The distribution of p34^{cdc2} during mouse oocyte meiotic maturation is shown in Fig. 5. At GV stage, p34^{cdc2} was concentrated in the germinal vesicle (Fig. 5A). After GVBD, p34^{cdc2} accumulated around the condensed chromosomes at prometaphase (Fig. 5B). With the organization of the metaphase I spindle, the localization of p34^{cdc2} was concentrated in the spindle (Fig. 5C). At anaphase I, when the first polar body was being extruded, p34^{cdc2} was accumulated between the separating chromosomes but not in the midbody (Fig. 5D, E). At metaphase II, the staining of p34^{cdc2} was detected around the aligned chromosomes and in the spindle (Fig. 5F).

Subcellular localization of p34^{cdc2} in mouse fertilized eggs and early embryos

As shown in Fig. 6, at anaphase II and telophase II, when the second polar body was being extruded, p34^{cdc2} was mainly accumulated between the separating chromatids but not in the midbody (Fig. 6*A*–*D*).

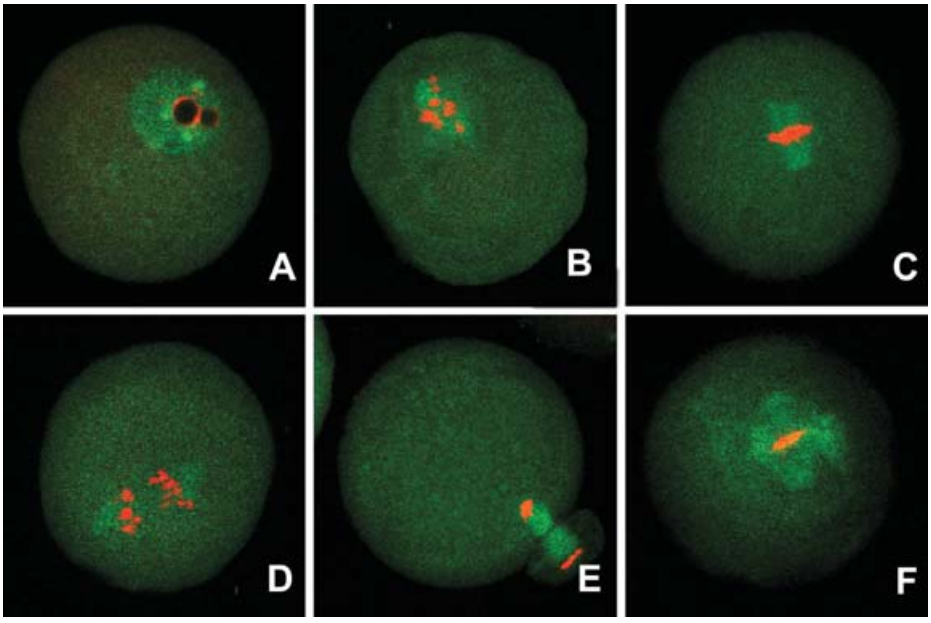

Figure 5 Subcellular localization of $p34^{cdc2}$ during mouse oocyte meiotic maturation. $p34^{cdc2}$ was concentrated in the germinal vesicle of GV-stage oocytes (*A*), while it accumulated around the condensed chromosomes at prometaphase (*B*). With spindle assembly at metaphase I, $p34^{cdc2}$ was concentrated in the spindle (*C*). At anaphase I, when the first polar body was being extruded, $p34^{cdc2}$ accumulated between the separating chromosomes (*D*, *E*). At metaphase II, the staining of $p34^{cdc2}$ was detected around the aligned chromosomes and in the spindle (*F*).

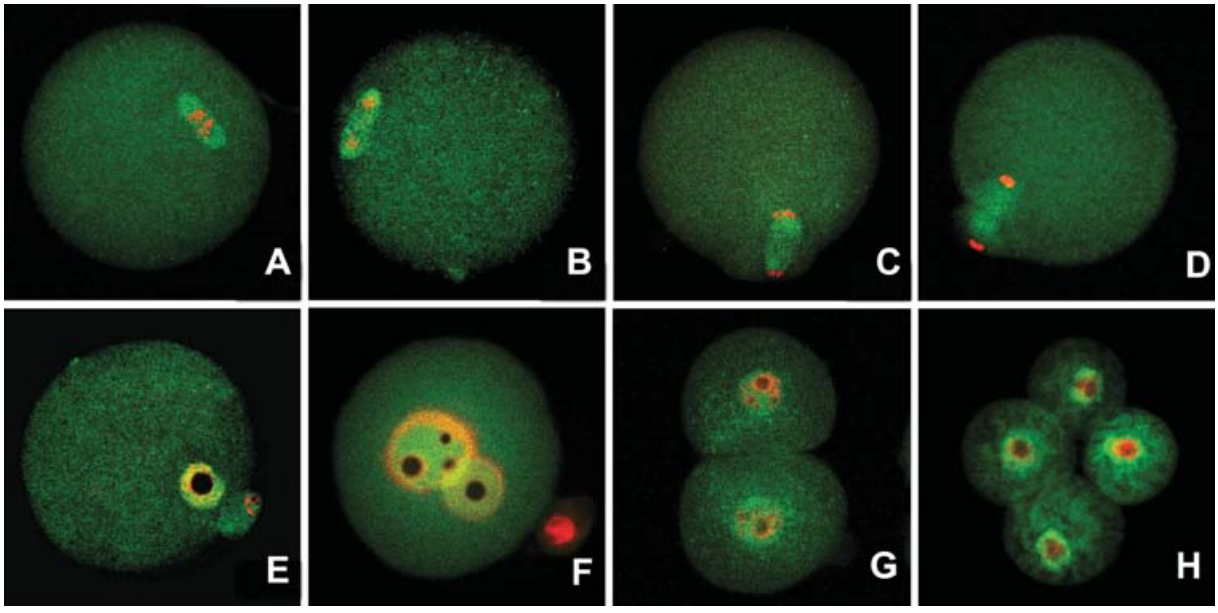

Figure 6 Subcellular localization of $p34^{cdc2}$ in mouse fertilized oocytes and early embryos. At anaphase II and telophase II, $p34^{cdc2}$ was mainly accumulated between the separating chromatids (*A*–*D*). The staining of $p34^{cdc2}$ could be detected in the one pronucleus after parthenogenetic activation (*E*) and in the female and male pronuclei (*F*) after fertilization. In 2- or 4-cell embryos, $p34^{cdc2}$ was concentrated in the nuclei (*G*, *H*).

The staining of $p34^{cdc2}$ could be detected in one pronucleus after parthenogenetic activation (Fig. 6*E*) and the female and male pronuclei after fertilization (Fig. 6*F*). In 2- or 4-cell embryos, p34*cdc2* was concentrated in the nuclei (Fig. 6*G*, *H*).

Discussion

It is well known that MPF, a kinase complex composed of the regulatory cyclin B and the catalytic $p34^{cdc2}$ kinase, is important for the regulation of mitosis and

meiosis (Jones, 2004; Masui, 2001). In this study, we detected and compared the subcellular localization of cyclin B1, phosphorylated cyclin B1 and p34^{cdc2} during oocyte meiotic maturation and fertilization in mouse. Our results showed that these three proteins have similar distribution patterns at some stages but different localizations at other stages during oocyte meiotic maturation and fertilization, suggesting that they may play a common role in some events but different roles in other events during oocyte maturation and fertilization.

It has been reported that cyclin B1 is associated with the germinal vesicle and the condensed chromosomes in pig (Casas *et al*., 1999), goat (Hue *et al*., 1997) and mouse (Chesnel & Eppig, 1995; Kanatsu-Shinohara *et al*., 2000; Mitra & Schultz, 1996) oocytes. Cyclin B1 was also detected in the nucleus of 2-cell mouse embryo, which was regulated by p34^{cdc2} kinase (Ohashi *et al*., 2001). We showed that both cyclin B1 and phosphorylated cyclin B1 exist in the germinal vesicles of competent oocytes but not incompetent oocytes, providing further evidence for the hypothesis that MPF activation is required for GVBD.

We showed that all these proteins were accumulated around the condensed chromosomes after GVBD. With the spindle formation, cyclin B1 and phosphorylated cyclin B1 were localized around the condensed chromosomes and concentrated to the spindle poles. The p34^{cdc2} had a similar localization at metaphase I and metaphase II, which implied that MPF plays important roles in organizing and maintaining the meiotic spindle at these stages.

Cyclin B1 localizes in the cytoplasm during the S and the G2 phases in starfish oocytes (Ookata *et al*., 1992), HeLa cells (Pines & Hunter, 1991) and *Xenopus* oocytes (Li *et al*., 1995, 1997). However, just before mitosis, a proportion of the cyclin B1 accumulates in the nucleus and localizes there until nuclear envelope breakdown occurs at prometaphase (Yang *et al*., 1998; Ohashi *et al*., 2001). Simultaneously with p34^{cdc2} dephosphorylation, cyclin B becomes phosphorylated as described in a variety of models such as yeast (Booher *et al*., 1989), starfish oocytes (Pondaven *et al*., 1990), sea urchin eggs (Meijer *et al*., 1989), goldfish oocytes (Yamashita *et al*., 1995), *Xenopus* oocytes (Gautier & Maller, 1991) and human cells (Pines & Hunter, 1989). The residues phosphorylated in cyclin B1 have been identified in *Xenopus*: Ser-2, Ser-94, Ser-96, Ser-101 and Ser-113 (Izumi & Maller, 1991; Li *et al*., 1995). Mutational studies have suggested that cyclin B phosphorylation is required neither for the p34^{cdc2} kinase activity, p34^{cdc2} binding, nor for cyclin B destruction in anaphase (Izumi & Maller, 1991; Li *et al*., 1995; Yamashita *et al*., 1995). More recently, Li *et al*. (1997) showed that if the residues were mutated to non-phosphorylatable residues, the p34^{cdc2}–cyclin B complex does not migrate from the cytoplasm to the nucleus and therefore loses its MPF activity. In *Xenopus*, mutation of these sites to alanine abolished the ability of the cyclin to promote oocyte maturation (Li *et al*., 1995, 1997). Biological activity was restored to this mutant when a nuclear localization sequence was fused to the amino terminus of the protein, suggesting that phosphorylation of these residues might be important for nuclear accumulation of cyclin B1 during oocyte maturation (Yang *et al*., 1998). It can be proposed that p34^{cdc2}–cyclin B1 kinase activity is controlled not only by phosphorylation/dephosphorylation of p34^{cdc2} but also by the nuclear localization of cyclin B1 regulated by its phosphorylation. It can be concluded that activation of p34^{cdc2}–cyclin B (or MPF) at the prophase/metaphase transition proceeds in two steps: dephosphorylation of p34^{cdc2} and phosphorylation of cyclin B (Borgne *et al*., 1999). Our results show for the first time that the phosphorylated cyclin B1 always exists and has spatiotemporal localization in mouse oocyte during meiotic maturation and fertilization. However, it is not well known what affects the phosphorylation of cyclin B1 and its migration to nucleus. It has been reported that cyclin F regulates the nuclear localization of cyclin B1 through a cyclin–cyclin interaction (Kong *et al*., 2000). Phosphorylation of the cyclin B1 cytoplasmic retention sequence by mitogen-activated protein kinase and Plx has also been reported (Walsh *et al*., 2003; Toyoshima-Morimoto *et al*., 2001). Furthermore, it is reported that cyclin B is mostly phosphorylated by its own associated p34^{cdc2} subunit at the prophase/metaphase transition in starfish oocyte (Borgne *et al*., 1999), in sea urchin eggs (Meijer *et al*., 1989) and in *Xenopus* oocytes (Gautier *et al*., 1990). Our results showed that the localization of phosphorylated cyclin B1 is similar to that of cyclin B1 during oocyte meiotic maturation and fertilization. But, to our surprise, phosphorylated cyclin B1 also has a specific localization at the metaphase/anaphase transition, and even at telophase the importance of the localization is to be further studied. Furthermore, we also observed that phosphorylated cyclin B1 and p34^{cdc2} have different localizations at these stages, the former being localized in the midbody and the latter in the region between the separating chromosomes/chromatids but not in the midbody. The inconsistency of the localization is to be further explored.

Acknowledgements

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