Mechanisms of Development 121 (2004) 1275–1287

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Ubiquitin–proteasome pathway modulates mouse oocyte meiotic maturation and fertilization via regulation of MAPK cascade and cyclin B1 degradation

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Received 6 February 2004; received in revised form 22 April 2004; accepted 11 May 2004

Available online 17 June 2004

Abstract

Degradation of proteins mediated by ubiquitin–proteasome pathway (UPP) plays important roles in the regulation of eukaryotic cell cycle. In this study, the functional roles and regulatory mechanisms of UPP in mouse oocyte meiotic maturation, fertilization, and early embryonic cleavage were studied by drug-treatment, Western blot, antibody microinjection, and confocal microscopy. The meiotic resumption of both cumulus-enclosed oocytes and denuded oocytes was stimulated by two potent, reversible, and cell-permeable proteasome inhibitors, ALLN and MG-132. The metaphase I spindle assembly was prevented, and the distribution of ubiquitin, cyclin B1, and polo-like kinase 1 (Plk1) was also distorted. When UPP was inhibited, mitogen-activated protein kinase (MAPK)/p90rsk phosphorylation was not affected, but the cyclin B1 degradation that occurs during normal metaphase-anaphase transition was not observed. During oocyte activation, the emission of second polar body (PB2) and the pronuclear formation were inhibited by ALLN or MG-132. In oocytes microinjected with ubiquitin antibodies, PB2 emission and pronuclear formation were also inhibited after in vitro fertilization. The expression of cyclin B1 and the phosphorylation of MAPK/p90rsk could still be detected in ALLN or MG-132-treated oocytes even at 8 h after parthenogenetic activation or insemination, which may account for the inhibition of PB2 emission and pronuclear formation. We also for the first time investigated the subcellular localization of ubiquitin protein at different stages of oocyte and early embryo development. Ubiquitin protein was accumulated in the germinal vesicle (GV), the region between the separating homologous chromosomes, the midbody, the pronuclei, and the region between the separating sister chromatids. In conclusion, our results suggest that the UPP plays important roles in oocyte meiosis resumption, spindle assembly, polar body emission, and pronuclear formation, probably by regulating cyclin B1 degradation and MAPK/p90rsk phosphorylation.

Keywords: Gamete biology; Fertilization; Meiosis; Ovum; Signal transduction

1. Introduction

Degradation of proteins mediated by ubiquitin-proteasome pathway (UPP) plays key roles in many aspects of eukaryotic cell cycle progression. Proteins critical to regulation of the cell cycle progression including cyclins, Polo-like kinase (Plk), Cdks, c-mos proto-oncogene products, securin, Cut2 and Ase1, are known to be degraded by UPP at specific cell cycle points (for reviews: Peters, 1999; 2002). Ubiquitin, composed of 76 amino acids, is an 8.45 kDa protein that is highly conserved in nearly all eukaryotes. The proteasome is a large, 26 S, multicatalytic protease complex that degrades polyubiquitinated proteins to small peptides. It is composed of three subcomplexes: a 20 S core particle that carries the catalytic activity and two 19 S regulatory particles, yielding a dumbbell-shaped complex. Degradation of a protein via the UPP involves two discrete and successive steps: (1) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and (2) degradation of the tagged protein by the 26 S proteasome complex with release of free and reusable ubiquitin (Glickman and Ciechanover, 2002).

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doi:10.1016/j.mod.2004.05.007

Anaphase-promoting complex/cyclosome (APC/C) functions as a cell cycle-regulated ubiquitin ligase that mediates protein ubiquitination (Kotani et al., 1998).

Currently, three major cell cycle transitions, entry into S phase, separation of sister chromatids, and exit from mitosis, are known to require the degradation of specific proteins (Kotani et al., 1998). Mitotic entry is regulated by M-phase or maturation promoting factor (MPF), a kinase complex composed of the regulatory subunit cyclin B and the catalytic subunit p34cdc2 kinase. Mitotic (Reimann and Jackson, 2002) or meiotic (Tokumoto et al., 1997) exit requires MPF inactivation, which is achieved by cyclin B destruction via UPP. However, almost all of the data concerning the relationship between cyclin degradation and chromatid separation at exit from M phase are from mitotic cells, and it is not known whether the same mechanisms are involved in segregation of homologous chromosomes during meiosis I and of chromatids during meiosis II.

Fully grown mammalian oocytes are arrested at the diplotene stage of first meiotic prophase, which is also termed germinal vesicle (GV) stage. Basically, three models have been employed in studying the oocyte meiotic resumption in vitro: spontaneous meiosis model, induced meiosis model and follicle-enclosed oocyte meiosis model. In mouse, denuded oocytes or cumulus cell-enclosed oocytes (CEOs) can spontaneously resume meiosis when they are released from the inhibitory environment of follicles. CEOs also mature in vitro under the stimulation of gonadotropin, epidermal growth factor (EGF), or follicular fluid meiosis-activating sterol (FF-MAS) when spontaneous maturation is prevented by meiotic inhibitors, such as hypoxanthine and cAMP-elevating agents (Downs, 1993; Faerge et al., 2001). Furthermore, meiosis in follicle-enclosed oocyte could be induced by LH (Su et al., 2003). At the end of meiotic maturation, oocytes arrested again at metaphase II (MII). Completion of meiosis II, characterized by sister chromatid separation and PB2 emission, is triggered by fertilization. The zygote, formed after sperm penetration, contains haploid male and female pronuclei. The haploid pronuclei gradually move toward each other and prepare for the first mitosis by replicating their DNA. The syngamy occurs, spindle forms, and mitosis is turned on to form 2-cell embryo (Josefsberg et al., 2001).

Protein phosphorylation mediated by protein kinases such as mitogen-activated protein kinase (MAPK), MPF, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and Plk represent a well-known mechanism for modifying protein activity in meiosis (Fan et al., 2003; 2004; Fan and Sun, 2004; Tong et al., 2002). Alongside this posttranslational modification, degradation of some important proteins by UPP has also been shown to participate in activation and inactivation of several signal transduction pathways related to oocyte meiotic processes (Pahl and Baeuerle, 1996). Prevention of GVBD by inhibition of proteasomal proteolytic activity suggested that this protease is involved in reinitiation of meiosis in toad and starfish oocytes (Sawada et al., 1997; Takahashi et al., 1994). Potent inhibition of GVBD was also observed by microinjection of anti-proteasome-α-subunit antibodies. The antibody-injected oocytes failed to activate pre-MPF, since the dephosphorylation of phospho-Tyr15 in cdc2 kinase was not observed even in the presence of maturation-inducing hormone (Takagi Sawada et al., 1997). The enzymatic properties of the proteasome purified from starfish oocytes have been characterized and its catalytic subunit was involved in oocyte maturation (Morinaga et al., 2000; Sawada et al., 1999; Tanaka et al., 2000). Furthermore, the establishment of the elaborate enzymatic system that leads to ubiquitin-mediated proteolysis of cyclin B (Glotzer et al., 1991; Murray et al., 1989) was characterized in clam oocytes entering the first meiotic division (Hershko et al., 1994). In *Xenopus*, cyclin B degradation was initiated by the 26S proteasome upon egg activation (Tokumoto et al., 1997).

The proteasome has recently been detected in human and rat oocytes (Josefsberg et al., 2000; Josefsberg et al., 2001; Wójcik et al., 2000). In rat, proteasomal catalytic activity is absolutely essential for the decrease in MPF activity and completion of the first meiotic division (Josefsberg et al., 2000). However, no systematic studies on the roles of UPP in mammalian species have been reported. Especially, the pathways by which UPP regulates meiotic cell cycle and fertilization and the roles of UPP in the regulation of meiotic resumption are unclear.

In this study, we investigated (1) the subcellular localization of ubiquitin protein at different stages of mouse oocyte meiotic maturation, fertilization, and early embryonic cleavage; (2) the roles of UPP in spontaneous oocyte maturation and hypoxanthine (HX)-maintained meiotic arrest; (3) the function of UPP during parthenogenetic activation and in vitro fertilization as well as its regulation on MAPK/p90rsk and MPF activity.

2. Results

2.1. Subcellular localization of ubiquitin protein during oocyte meiotic maturation

The distribution of ubiquitin protein during mouse oocyte meiotic maturation was shown in Fig. 1. In GV-stage oocytes, the ubiquitin protein was localized in the whole oocyte and accumulation in the GV was observed (Fig. 1A). Shortly after GVBD, the ubiquitin protein was distributed diffusely in the whole oocyte (Fig. 1B). With the development of oocytes, the ubiquitin protein concentrated around the condensed chromosomes (Fig. 1C). At metaphase I, with the organization of chromosomes to the equatorial plate, ubiquitin protein was again diffused

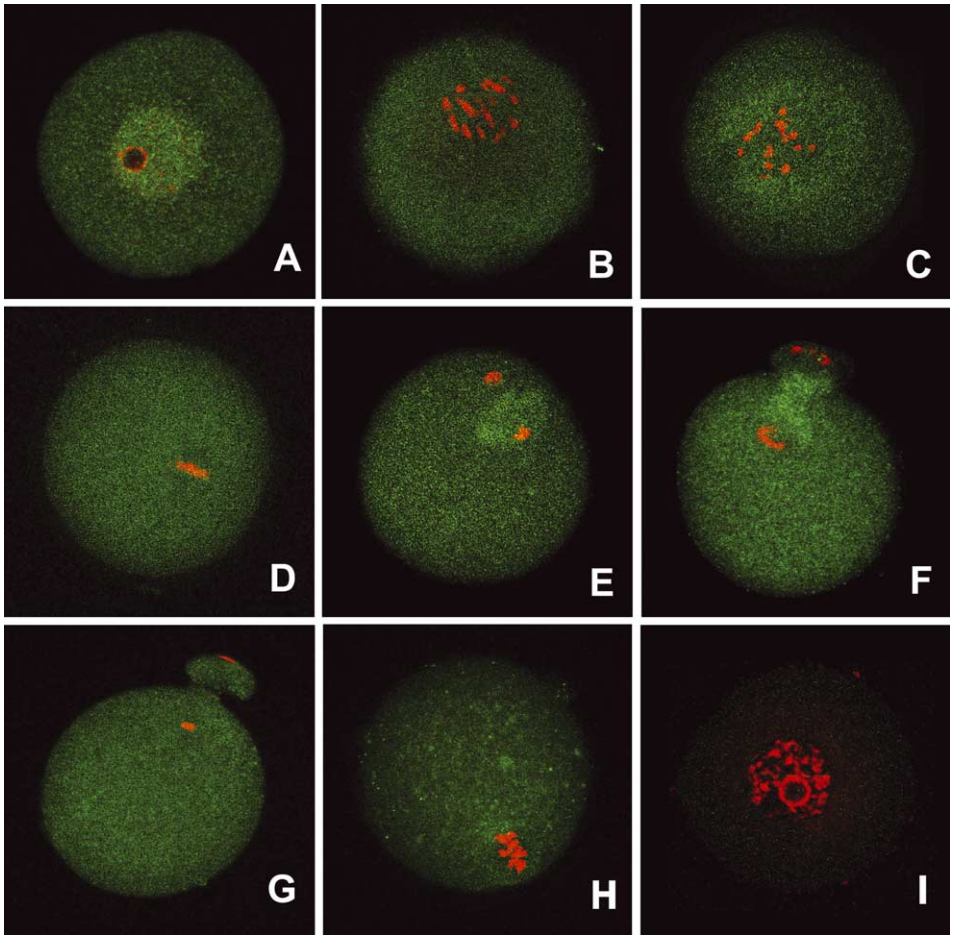

Fig. 1. Subcellular localization of ubiquitin protein during meiotic maturation. Green, ubiquitin; Red, chromatin. At GV stage, the ubiquitin protein was localized in the whole oocyte and mainly accumulated in the GV (A). Shortly after GVBD, the ubiquitin protein was distributed diffusely in the whole oocyte (B). With the development of the oocytes, the ubiquitin protein again concentrated around the condensed chromatin (C). At metaphase I, with the organization of chromosomes to the equatorial plate, the localization of ubiquitin protein was again diffused in the whole oocyte (D). At anaphase I and early telophase I, following the separation of homologous chromosomes, the ubiquitin protein was accumulated in the region between the separating homologous chromosomes, and associated with the midbody between the PB1 and the oocyte (E–F). But at late telophase I and metaphase II, the ubiquitin protein was distributed diffusely in the whole oocyte (G–H). A GV oocyte was used as a negative control (I), in which the polyclonal rabbit anti-human ubiquitin antibody was replaced with rabbit IgG.

in the whole oocyte (Fig. 1D). At anaphase I and early telophase I, the ubiquitin protein was accumulated in the region between the separating homologous chromosomes, and associated with the midbody between the PB1 and the oocyte (Fig. 1E, F). But at late telophase I and MII, the ubiquitin protein was distributed diffusely in the whole oocytes (Fig. 1G, H). A GV-stage oocyte was used as a negative control, in which the polyclonal rabbit anti-human ubiquitin antibody was replaced with rabbit IgG (Fig. 1I).

2.2. Effect of UPP inhibitors on meiotic resumption of oocytes in both spontaneous maturation model and HX-maintained meiotic arrest model

In order to characterize the function of UPP in meiosis, selective proteasomal inhibitors, ALLN and MG-132, were used. As shown in Figs. 2 and 3, the GVBD rates of the DOs and CEOs were increased by ALLN or MG-132 in a dose-dependent manner in both spontaneous maturation model and HX-maintained meiotic arrest model. After DOs had been cultured in M2 medium for 2 h, the GVBD rate in 100 μM ALLN-treated DOs (79 ± 4%) or 50 μM MG-132-treated DOs (83 ± 2%) was significantly higher than that in the inhibitor-free group (67 ± 5%). The GVBD rate in 100 μM ALLN-treated CEOs (96 ± 4%) or 50 μM MG-132-treated CEOs (100%) was also significantly higher than that in the inhibitor-free group (88 ± 2%) (Fig. 2C). In the HX-maintained meiotic arrest system, as shown in Fig. 3A, the GVBD rate in 100 μM ALLN-treated DOs (51 ± 3%) or 50 μM MG-132-treated DOs (35 ± 4%) was also significantly higher than that in inhibitor-free group (15 ± 1%) cultured in HX medium for 24 h. The GVBD rate in 100 μM ALLN-treated CEOs (99 ± 1%) or 50 μM MG-132-treated CEOs (99 ± 2%) was also significantly higher than that in inhibitors-free group (27 ± 1%) cultured in HX medium for 12 h (Fig. 3B).

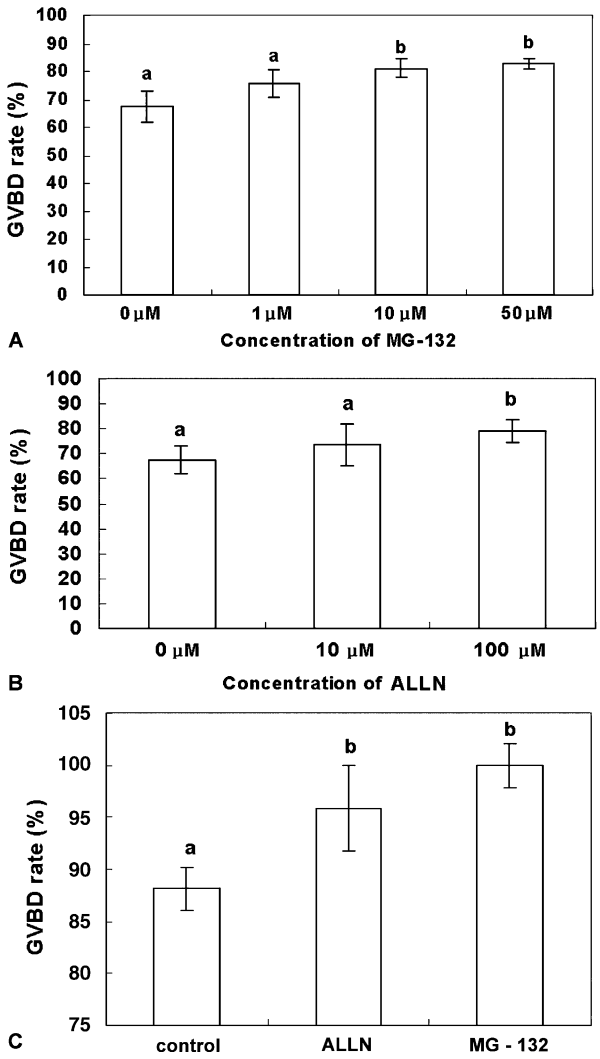

Fig. 2. Effect of ALLN and MG-132 on meiotic resumption of mouse oocytes in spontaneous maturation model. Denuded oocytes (DOs) or cumulus-enclosed oocytes (CEOs) were cultured for 2 h in M2 medium supplemented with or without different concentrations of ALLN or MG-132 to show their effect on GVBD. Data are presented as mean percentage of GVBD ± SEM of three independent experiments. Different superscripts denote statistical difference at a $P < 0.05$ level of significance in the GVBD. The similar demonstrations of statistical analysis are also used in the following figures. A and B represented dose-dependent stimulation of GVBD by 1, 10, 50 μM MG-132 and 10, 100 μM ALLN in mouse DOs, receptively. C represented stimulation of GVBD by 50 μM MG-132 and 100 μM ALLN in mouse CEOs.

2.3. Effect of UPP inhibitors on the spindle assembly and subcellular localization of cyclin B1, Plk1, and ubiquitin during oocyte meiotic maturation

As shown in Fig. 4A, E, α-tubulin concentrated as several dots near condensed chromosomes at prometaphase, while it failed to assemble into a spindle in ALLN or MG-132-treated oocytes. It diffusely distributed in the region around the condensed chromosomes and the cortical region. The condensed chromosomes could not move to the metaphase equatorial plate. The localization of cyclin B1 (Fig. 4B, F),

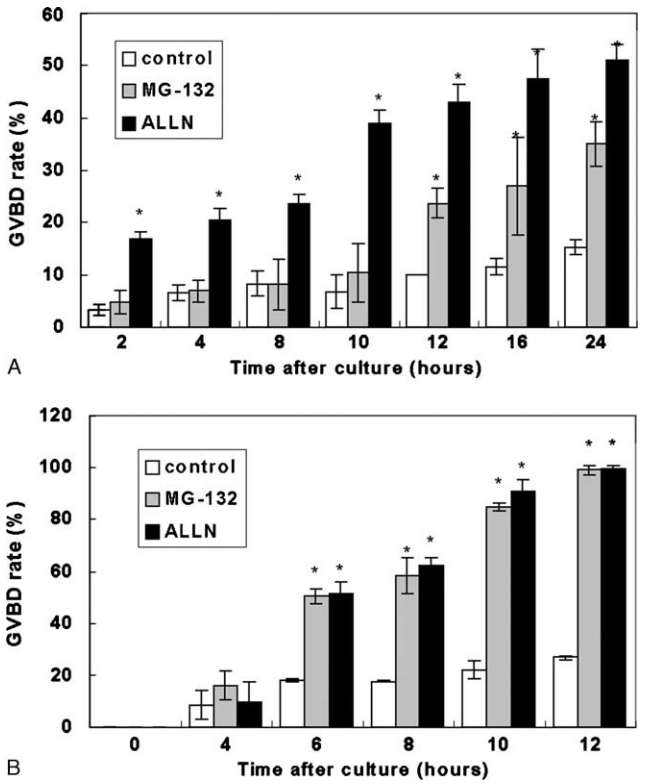

Fig. 3. Effect of ALLN and MG-132 on meiotic resumption of mouse oocytes in HX-maintained meiotic arrest model. Mouse DOs or CEOs were cultured for 24 or 12 h, respectively, in HX-medium supplemented with or without 50 μM MG-132 or 100 μM ALLN to show their effect on GVBD. A and B represented the effect of 50 μM MG-132 and 100 μM ALLN on GVBD of mouse DOs and CEOs, respectively.

ubiquitin (Fig. 4C, G), and Plk1 (Fig. 4D, H) was also affected by ALLN or MG-132 treatment. All concentrated but scattered evenly in the periphery of the condensed chromosomes, with a significant difference from the normal localization of these proteins in oocytes after GVBD (Fig. 4I–L) or at metaphase II (Fig. 4M–P).

2.4. Subcellular localization of ubiquitin protein during parthenogenetic activation and fertilization of oocytes

The distribution of the ubiquitin protein varied at different developmental stages during parthenogenetic activation and fertilization of mouse oocytes. As shown in Fig. 5, the ubiquitin protein was accumulated to the region between the separating sister chromatids and associated with the midbody between the PB2 and the oocytes during the PB2 emission (Fig. 5A, B). Following the completion of meiosis II and the decondensation of chromatin, the ubiquitin protein was redistributed into the cytoplasm (data not shown). A more intensitive staining of ubiquitin protein could also be detected in pronucleus after parthenogenetic activation (Fig. 5C) and in the male and female pronuclei after fertilization (Fig. 5D).

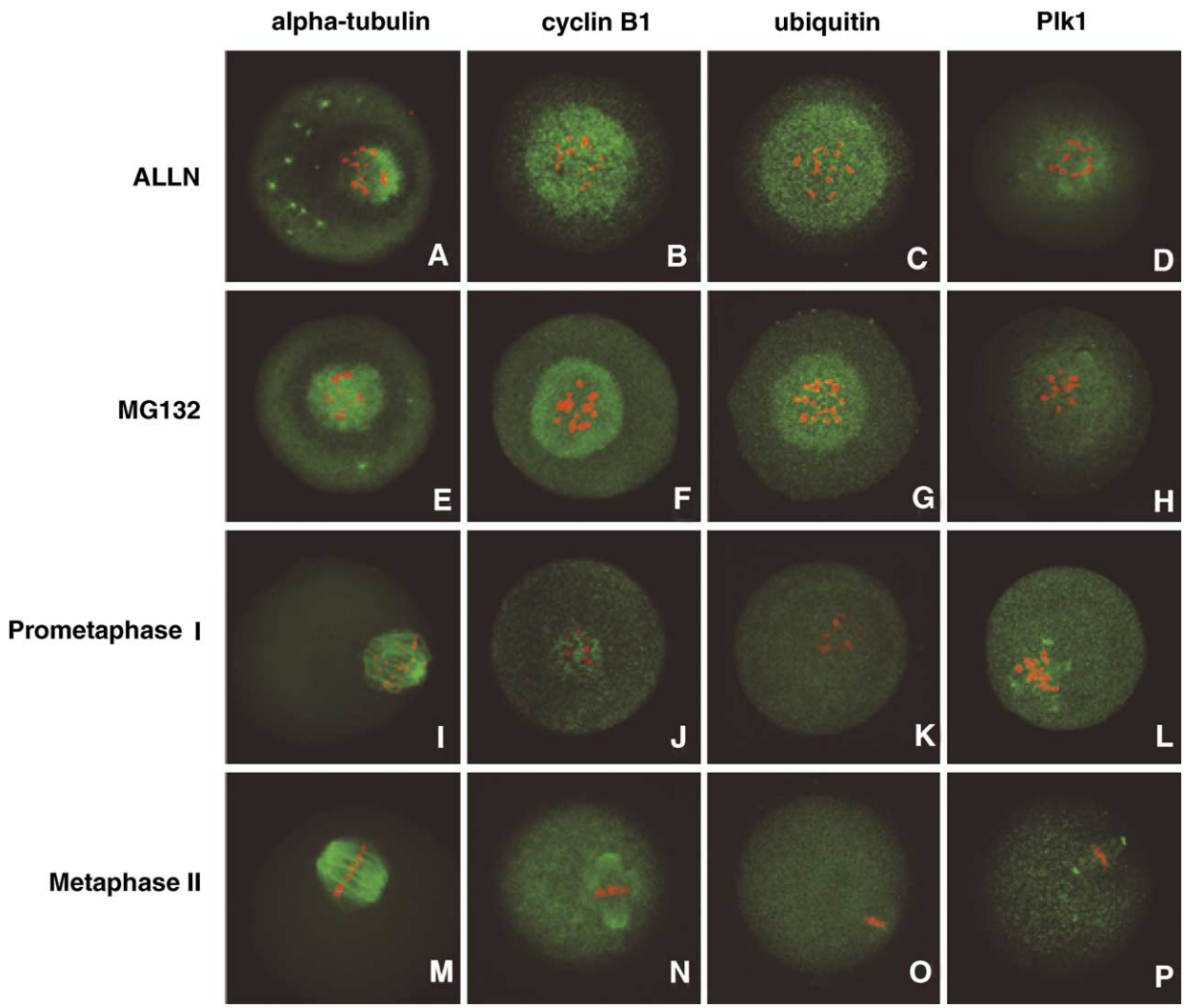

Fig. 4. Effect of ALLN and MG-132 on α-tubulin organization and cyclin B1, ubiquitin, as well as Plk1 localization during meiotic maturation. GV-stage mouse oocytes were cultured for 12 h in M2 medium containing 50 μM MG-132 or 100 μM ALLN, and then collected for detecting α-tubulin, cyclin B1, ubiquitin, and Plk1 by confocal microscopy. α-tubulin could not be assembled into a spindle, but concentrated in the region around the condensed chromosomes and the cortical region (A and E), α-tubulin did not focus as several dots near the condensed chromosomes as in normal maturation culture after GVBD. The condensed chromosomes did not move to the metaphase equatorial plate in the ALLN or MG-132-treated oocytes. The localization of cyclin B1 (B and F), ubiquitin (C and G), and Plk1 (D and H) was also influenced by treatment of oocytes with ALLN or MG-132. They all evenly distributed in the periphery of the condensed chromosomes, with a significant difference from the normal localization of these proteins in mouse oocytes after GVBD (I–L) or at metaphase II (M–P).

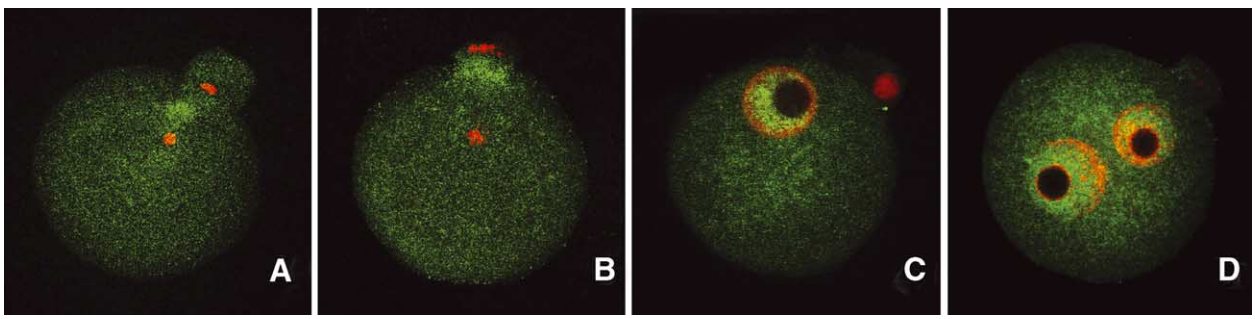

Fig. 5. Subcellular localization of ubiquitin protein during parthenogenetic activation and fertilization of mouse oocytes. During the emission of PB2, the ubiquitin protein was accumulated in the region between the two separating chromosomes and associated with the midbody between the PB2 and the oocyte (A–B). The ubiquitin protein could also be detected a more intensitive staining in one pronuclei (C) after parthenogenetic activation and in the male and female pronuclei (D) after fertilization.

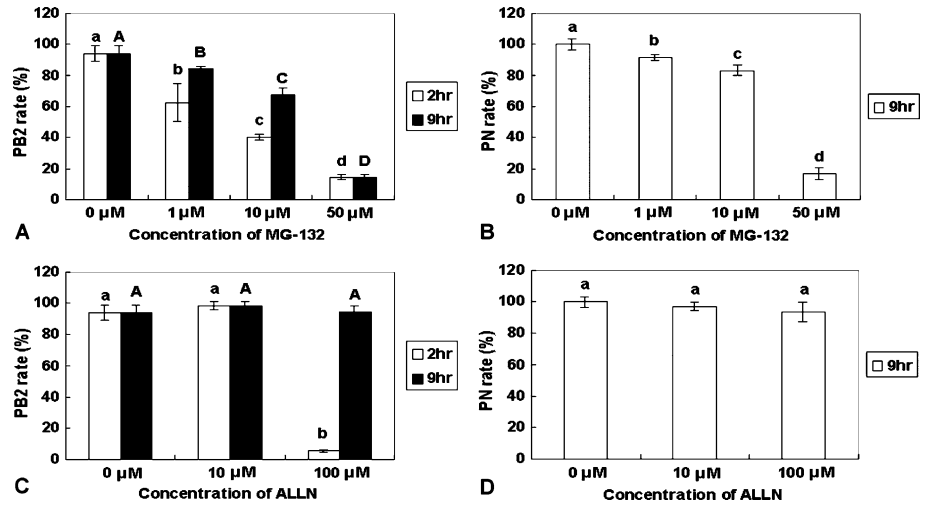

Fig. 6. Effect of ALLN and MG-132 on PB2 emission and pronuclear (PN) formation during A23187-induced mouse oocyte parthenogenetic activation. The MII-arrested mouse oocytes were first pretreated for 30 min in M2 medium with or without 1, 10, 50 μM MG-132 or 10, 100 μM ALLN, and then incubated in M2 medium containing 10 μM calcium ionophore A23187 and different concentrations of MG-132 or ALLN. After that, the oocytes were incubated in M16 medium containing 10 μg/ml CHX and different concentrations of MG-132 or ALLN. The PB2 emission was recorded at 2, 8, and 9 h (A and C represented MG-132 and ALLN groups, respectively), and the PN formation rate was recorded at 9 h after activation (B and D represented MG-132 and ALLN groups, respectively). Data are presented as mean percentage of PB2 or PN ± SEM of three independent experiments. Different superscripts denote statistical difference at a $P < 0.05$ level of significance.

2.5. Effect of UPP inhibitors on PB2 emission and pronuclear formation during parthenogenetic activation and in vitro fertilization

Before performed for parthenogenetic activation or IVF, MII-arrested oocytes were pretreated in M16 medium containing 100 μM ALLN or 50 μM MG-132 for 30 min. As shown in Fig. 6A–D, ALLN and MG-132 inhibited PB2 emission and pronuclear formation in a dose-dependent manner during parthenogenetic activation and IVF of mouse oocytes. The percentages of PB2 emission and pronuclear formation were 94 ± 5 and 96 ± 4%, respectively, in calcium ionophore A23187-activated mouse oocytes (Fig. 6), while the percentages of PB2 emission and the pronuclear formation were only 14 ± 2 and 16 ± 4%, respectively, in 50 μM MG-132-treat oocytes (Fig. 6A, B). To our surprise, 100 μM ALLN (Fig. 6C, D) did not effectively inhibit the PB2 emission and pronuclear formation. The percentages of PB2 emission and pronuclear formation were 95 ± 2 and 93 ± 6%, respectively. But if we prolonged the pretreatment time to 1 h in M2 medium containing 100 μM ALLN or pretreated oocytes for 30 min in M2 medium containing 200 μM ALLN, the percentages of PB2 emission and pronuclear formation were only 24 and 10% ($n = 117$), respectively, significant lower than in the control oocytes.

During IVF, 100 μM ALLN or 50 μM MG-132 completely inhibited the PB2 emission and pronuclear formation (data not shown), while the percentages of PB2 and pronuclear formation were 97% ($n = 176$) in the drug-free oocytes. To eliminate the possibility that the proteasome inhibitor affects IVF via sperm, as a control, MII-arrested oocytes were not subjected to ALLN or MG-132 pretreatment but directly incubated with capacitated sperm in M16 medium containing 100 μM ALLN or 50 μM MG-132. The percentage of PB2 emission was 98% ($n = 122$) at 4 h after insemination.

2.6. Microinjection of ubiquitin antibody affects PB2 emission and pronuclear formation during IVF

As shown in Fig. 7, the results showed that the activation of MII-arrested oocytes was inhibited by microinjection

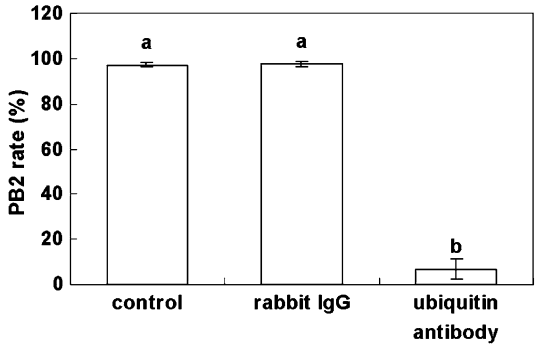

Fig. 7. Effect of ubiquitin antibody microinjection on PB2 emission and pronuclear formation during IVF. The MII-arrested mouse oocytes microinjected with polyclonal rabbit anti-human ubiquitin antibody (200 μg /ml) or rabbit IgG (7 pl per oocyte) were performed for IVF. The PB2 emission (4 h) and pronuclear formation (8 h) were recorded respectively. Data are presented as mean percentage of PB2 ± SEM of three independent experiments. Different superscripts denote statistical difference at a $P < 0.05$ level of significance. The PB2 rate was very low but the pronuclear formation was completely inhibited (data not shown).

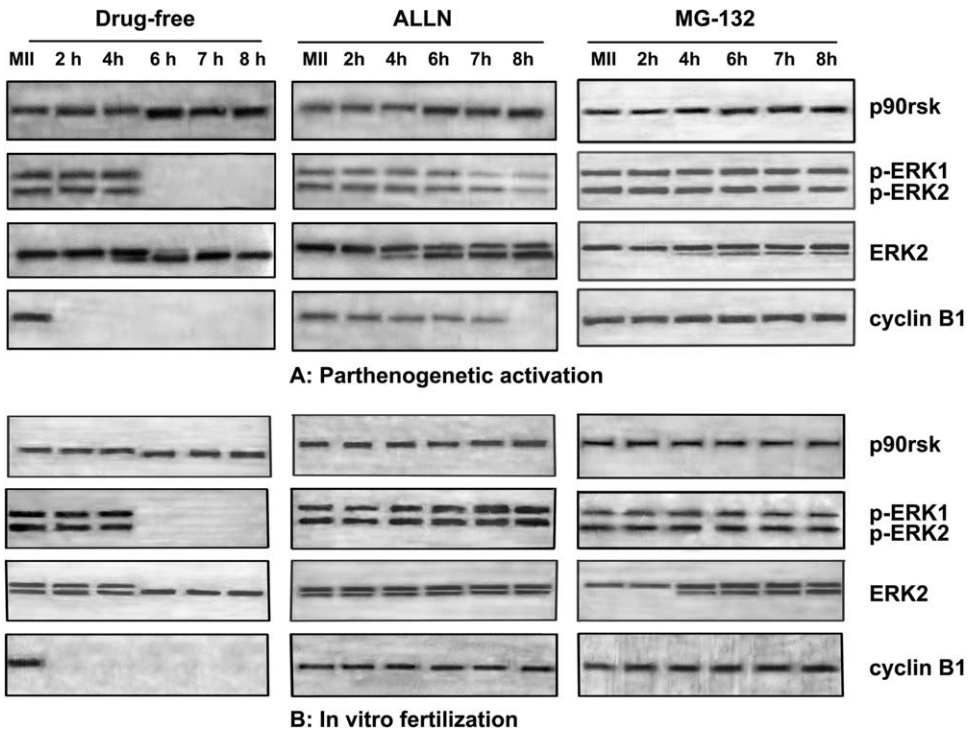

Fig. 8. Effect of ALLN and MG-132 on the expression of cyclin B1 and the phosphorylation of MAPK and p90rsk in mouse oocytes during parthenogenetic activation and IVF. The MII-arrested mouse oocytes were first pretreated for 30 min in M2 medium with or without 50 μM MG-132 or 100 μM ALLN, and then for parthenogenetic activation or IVF. For parthenogenetic activation, the treated oocytes were incubated in M2 medium containing 10 μM calcium ionophore A23187 and 50 μM MG-132 or 100 μM ALLN. After that, the oocytes were incubated in M16 medium containing 10 μg/ml CHX and 50 μM MG-132 or 100 μM ALLN. For IVF, the treated oocytes were incubated for 8 h with capacitated sperm in M16 medium containing 50 μM MG-132 or 100 μM ALLN. Oocytes after parthenogenetic activation were or IVF collected at 0, 2, 4, 6, 7, and 8 h for Western blot. The experiment was repeated three times, including 100 oocytes for each sample. A: Parthenogenetic activation; B: IVF. Left panels, middle panels, and right panels represent the drug-free, ALLN-treated-, and MG-132-treated oocytes, respectively.

of ubiquitin antibody during IVF, the percentage of PB2 emission was only 7 ± 4%, and was significantly lower than that in oocytes microinjected with (98 ± 1%) or without (97 ± 1%) rabbit IgG and then performed for IVF. The pronuclear formation was completely inhibited in oocytes microinjected with ubiquitin antibody (data not shown), which was significantly lower than in the control oocytes (97%, $n = 99$).

2.7. Regulation of UPP inhibitors on MAPK/p90rsk and cyclin B1 during parthenogenetic activation and IVF of mouse oocytes

Before performing parthenogenetic activation or IVF, MII-arrested oocytes were pretreated in M2 medium containing 100 μM ALLN or 50 μM MG-132 for 30 min. As shown in Fig. 8, left panel, cyclin B1 could be detected in the MII-arrested oocytes, while this protein could not be detected after 2 h during parthenogenetic activation or IVF. However, when the UPP was inhibited by 50 μM MG-132 (Fig. 8A, right panel) during parthenogenetic activation, or by 100 μM ALLN (Fig. 8B, middle panel) or 50 μM MG-132 (Fig. 8B, right panel) during IVF, cyclin B degradation did not occur at 8 h after oocyte activation. The expression of cyclin B1 could also be detected at 7 h after parthenogenetic activation in 100 μM ALLN-treated oocytes (Fig. 8A, middle panel). But if we prolonged the pretreatment time to 1 h in M16 medium containing 100 μM ALLN or pretreatment of the oocytes for 30 min in M16 medium containing 200 μM ALLN, and then performed for parthenogenetic activation, the cyclin B1 expression did not change at 9 h after activation (data not shown), which was similar to that in MII-arrested oocytes.

In our system, the pronuclei formed in activated oocytes at 6–8 h after activation (Fig. 6). The MAPK and p90rsk were kept phosphorylated 4 h after activation, but were completely dephosphorylated at 6 h after activation (Fig. 8, left panel). The contents of both kinases kept stable during the whole process of parthenogenetic activation and IVF. In ALLN (Fig. 8A, B, middle panel) or MG-132 (Fig. 8A, B, right panel) treated oocytes, the phosphorylation of MAPK and p90rsk could be detected at 7 or 8 h after parthenogenetic activation (Fig. 8A, middle and right panels) or IVF (Fig. 8B, middle and right panels). The MAPK and p90rsk were partly dephosphorylated at 6 h after parthenogenetic activation in 100 μM ALLN-treated oocytes (Fig. 8A, middle panel). If we prolonged the pretreatment time to 1 h in M16 medium containing 100 μM ALLN or pretreatment of the oocytes for 30 min in M16 medium containing 200 μM ALLN, and then

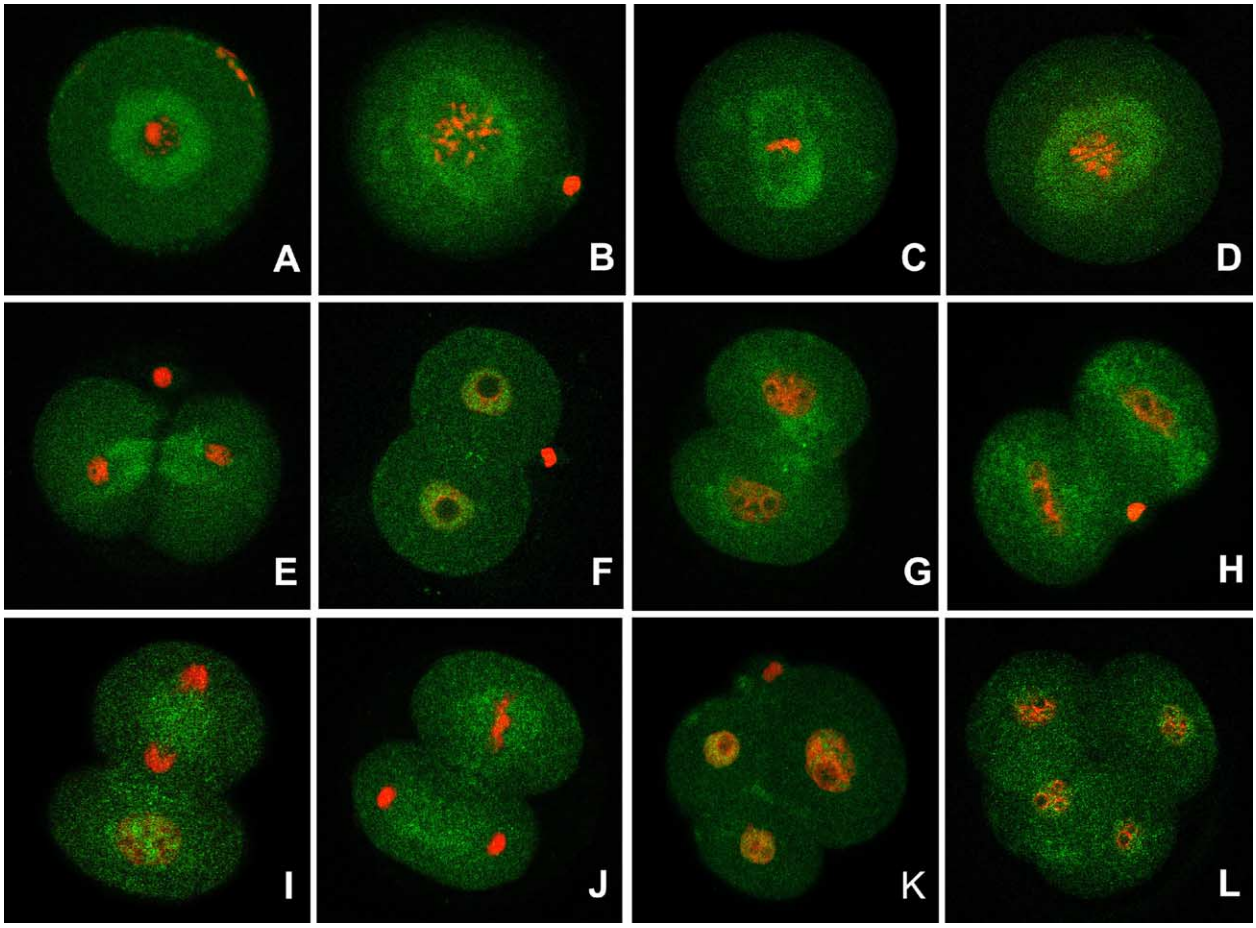

Fig. 9. Subcellular localization of ubiquitin protein during mouse early embryonic mitosis. With the pronuclear envelope breakdown, a significant signal of the ubiquitin protein was detected around the periphery of the chromosomes of 1-cell embryo (A–B). At the metaphase and anaphase of the first mitosis, the ubiquitin protein was accumulated in the periphery of the chromosomes, especially in the periphery of the spindle (C–D). At late anaphase and telophase of embryos (E, I, J), the ubiquitin protein was accumulated in the region between the two separating chromosomes and associated with the midbody between the two separating or separated daughter cells. In 2-cell embryos, the ubiquitin protein mainly accumulated in the nucleus and some staining of the ubiquitin protein could also be detected in the cytoplasm, and changed with the status of the nucleus (F–H). In 2-, 3-, and 4-cell embryos, the localization of ubiquitin protein could be detected in the nucleus (F–L).

performed for parthenogenetic activation, the phosphorylation of MAPK and p90rsk could be detected at 9 h after activation, which was similar to that in MII-arrested oocytes.

2.8. Subcellular localization of ubiquitin during early embryonic cleavage

With the pronuclear envelope breakdown, a significant signal of the ubiquitin protein was detected around the periphery of the chromosomes of 1-cell embryos (Fig. 9A, B). At this stage, its localization changed with the status of chromosomes. At the metaphase and anaphase of the first mitosis, the ubiquitin protein was accumulated in the periphery of the chromosomes, especially around the spindle of embryos (Fig. 9C, D). At late anaphase and telophase, the ubiquitin protein was accumulated in the region between the separating sister chromatids, and associated with the midbody between the two separating or separated cells (Fig. 9E, I, J). In 2-cell embryos, the ubiquitin protein mainly accumulated in the nucleus and some staining could be detected in the cytoplasm, and changed with the status of nucleus (Fig. 9F–H). In 2-, 3-, and 4-cell embryos, the localization of ubiquitin protein could also be detected in the nucleus (Fig. 9F–L).

3. Discussion

Degradation of proteins mediated by UPP plays very important roles in multiple processes during eukaryotic mitosis. Proteins critical to the regulation of the cell cycle progression including cyclins, Cdks, c-mos products, securin, Clb2 and Ase1, are known to be degraded by the UPP (for reviews: Peters, 1999, 2002). Most of these proteins are degraded at specific cell cycle points. Although numerous studies have been conducted in lower eukaryotes (Glotzer et al., 1991; Hershko et al., 1991, 1994;

Murray et al., 1989; Sawada et al., 1997; Suzumori et al., 2003; Takagi Sawada et al., 1997; Takahashi et al., 1994; Tokumoto et al., 1997), our understanding of the roles and the regulatory mechanisms of UPP in mammalian oocyte meiosis, fertilization and early embryonic mitosis was limited.

In this study, we investigated the roles of UPP and its regulation on MAPK/p90rsk and cyclin B1 in mouse oocyte during spontaneous and induced meiotic maturation, using DOs and CEOs. In these two models, different mechanisms are employed in regulating the progression of meiotic cell cycle. The former is thought to be a relatively passive response to the artificial removal from a meiotic inhibiting environment of the follicles, whereas the latter is more active, presumably requiring gonadotropin-triggered production of a meiosis-inducing stimulus that is similar to that in physiological environment in vivo (Bu et al., 2003; Downs et al., 2002; Fan et al., 2004; Ma et al., 2003). In spontaneous maturation model, we found that inhibition of proteasome by ALLN or MG-132 slightly stimulated GVBD in DOs and CEOs. But in lower eukaryotes, such as in toad and starfish oocytes, the inhibition of proteasomal proteolytic activity prevented GVBD, and potent inhibition of GVBD was also observed by microinjection of anti-proteasome-α-subunit antibodies. The antibody-injected starfish oocytes failed to activate pre-MPF, since the dephosphorylation of phospho-Tyr15 in Cdc2 kinase was not observed even in the presence of 1-methyadenine, a maturation-inducing hormone in this species (Takagi Sawada et al., 1997). The difference may result from the different regulatory mechanisms of oocyte meiotic resumption between lower eukaryotes and rodents. Interestingly, others reported that the proteasome inhibitors, lactacystin and MG-132, did not interfere with GVBD of rat oocytes (Josefsberg et al., 2000), but the authors did not show if the inhibitors could stimulate GVBD. To further test our results, we used the HX-maintained meiotic arrest model. GVBD was inhibited by HX, while ALLN or MG-132 could overcome this inhibitory effect of HX and induce GVBD in mouse CEOs and DOs. Our results strongly suggest that the inhibition of protein degradation mediated by UPP does not inhibit GVBD, but on the contrary, stimulates GVBD in mouse oocyte. The accumulation of ubiquitin protein in GV of mouse oocytes as revealed in this study provides further evidence for the UPP involvement in GVBD.

We inferred that this enhanced oocyte GVBD by ALLN or MG-132 could be related to the increased activity of MAPK/p90rsk and/or the accumulation of cyclin B1. ALLN or MG-132 could stimulate oocyte GVBD (80–90%), but the percentage of oocyte GVBD is also very high in the control (70–80%) in spontaneous maturation model. So it is not easy to differentiate the expression level of cyclin B1 and the activity of MAPK/p90rsk between in ALLN or MG-132 treated oocytes and in the control. On the other hand, the percentage of oocyte GVBD is significantly increased after ALLN or MG-132 treatment in HX-maintained meiotic arrest model, which may be related to the activation of MAPK/p90rsk and/or MPF resulted from the accumulation of cyclin B1.

We also found that UPP may involve in spindle assembly. After ALLN or MG-132 treatment, α-tubulin, an essential component of the metaphase spindle, could not be assembled into asters and the metaphase spindle, but concentrated in the region around the condensed chromosomes and the cortical region. The staining Plk1 (a key kinase regulating microtubule assembly), cyclin B1 and ubiquitin all scattered evenly in the periphery of the condensed chromosomes, significantly different from the normal localization of these proteins in mouse oocytes after GVBD. All these abnormalities caused the failure of normal spindle assembly. This result was different to that obtained in rat oocytes, in which MG-132 cause the meiotic arrest at metaphase I (Josefsberg et al., 2000). The difference may be caused by different culture times. They incubated rat oocytes in medium with MG-132 for 24 h, but we incubated mouse oocytes for 12 h. One possibility is that inhibition of proteasome catalytic activity by ALLN or MG-132 could only delay the spindle formation, but could not completely inhibit the spindle formation. Another possibility is that MG-132 may be not effective in inhibiting proteasome after 24 h culture.

The accumulation of ubiquitin protein in the region between the separating chromosomes and in the midbody connecting polar body and the oocyte suggests that UPP is involved in the polar body emission. Others also suggested that the MG-132-induced transient delay of proteasomal activity during mouse oocyte maturation in vitro predisposed oocytes to abnormal chromosome segregation (Mailhes et al., 2002). This is similar to a report that the proteasome is involved in the metaphase-to-anaphase transition of meiosis I in rat oocytes (Josefsberg et al., 2000). An elaborate enzymatic system that leads to ubiquitin-mediated proteolysis of cyclin B (Glotzer et al., 1991; Hershko et al., 1991; Murray et al., 1989) was characterized in clam oocytes entering the first meiotic division (Hershko et al., 1994). It has been found that the degradation of cyclin B1 mediated by UPP is necessary for disjunction of pairs of homologous chromosomes during the first meiotic division in mouse oocytes (Herbert et al., 2003), but not in *Xenopus* oocytes (Peter et al., 2001; Taieb et al., 2001).

The MII arrest of vertebrate oocytes is maintained by the activity of cytostatic factor (CSF) that may include MPF and MOS/MEK/MAPK/p90rsk cascade (Fan and Sun, 2004; Tunquist and Maller, 2003). After mouse oocyte activation, MPF was quickly inactivated by a calcium-induced and APC/C-dependent degradation pathway, which is the prerequisite for metaphase–anaphase transition (Nixon et al., 2002). However, the MAPK/p90rsk phosphorylation and expression remain stable after oocyte activation until the initiation of pronuclear formation (Sun et al., 1999). The prevention of MII-arrested oocyte activation by ALLN or

MG-132 or microinjection of ubiquitin antibody as indicated by inhibition of PB2 emission and pronuclear formation, illustrated the possibility that UPP may become active as a result of mouse oocyte activation, which in turn serves to mediate the degradation of cyclin B1, and thus the inactivation of MPF and MAPK/p90rsk. Our Western blot result showed that the phosphorylation of MAPK and p90rsk were maintained until 8 h after activation and the expression of cyclin B1 could also be detected at 8 h after activation in oocytes cultured in M16 medium containing proteasome inhibitor, strongly suggesting that the inhibition of degradation of some proteins by ALLN or MG-132, especially cyclin B1, may be important for maintaining the activity of MPF and the phosphorylation of MAPK and p90rsk, and eventually maintain the MII arrest of mouse oocytes.

Recently, the idea that other proteins serve as substrates for the proteasome at the metaphase–anaphase transition and that their degradation is absolutely necessary to allow the completion of cell division has been raised. For example, the specific component of cohesin protein complex (separase inhibitor securin) is responsible for holding together the homologous chromosomes (first meiotic division) and sister chromatides (second meiosis and mitosis) in mouse oocyte (Herbert et al., 2003).

The accumulation of ubiquitin protein in pronucleus during parthenogenetic activation and fertilization suggested that this localization was vital for the function of pronucleus, especially for pronuclear envelope breakdown. Others reported that proteasome inhibitor MG-132 could alter the orderly progression of DNA synthesis during S-phase in HeLa cells and lead to rereplication of DNA, suggesting the existence of proteasome-dependent mechanisms regulating the orderly progression of S-phase (Yamaguchi and Dutta, 2000). Moreover, the localization of ubiquitin protein in the region between the two daughter cells during the early embryonic mitosis suggests that UPP maybe related to the cytokinesis and karyokinesis in early embryonic mitosis, as the case shown in meiosis.

Taken together, our results suggest that UPP, probably by regulating the activity of MPF and MAPK cascade, is involved in multiple steps of mouse oocyte meiotic maturation, activation, and early embryonic mitosis, including the initiation of meiotic resumption, spindle assembly, polar body emission, MII arrest release, and pronuclear formation.

4. Materials and methods

4.1. Chemicals and solutions

Two potent, reversible, and cell-permeable proteasome inhibitors, ALLN (*N*-Acetyl-Leu-Leu-Nle-CHO) and MG-132 (*Z*-Leu-Leu-Leu-CHO) were purchased from Calbiochem (La Jolla, CA). ALLN and MG-132 were prepared as 100 mM stocks in dimethyl sulfoxide (DMSO) and stored at −20 °C in a dark box. The stock solutions were diluted with M2, M16 or HX medium prior to use. Polyclonal rabbit anti-human ubiquitin antibody, polyclonal rabbit anti-mouse p90rsk antibody, monoclonal mouse anti-p-ERK1/2 antibody, and polyclonal rabbit anti-human ERK2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma Chemical Co Ltd, (St. Louis, MO) unless noted otherwise.

For handling of oocytes, M2 medium was used. For sperm capacitation, parthenogenetic activation, in vitro fertilization (IVF) and early embryonic culture, M16 medium plus 2.5 mM taurine was used. For HX-maintained meiotic arrest of mouse oocytes, TCM-199 (Gibco, Grand Island, NY) supplemented with 4 mM hypoxanthine, 0.23 mM sodium pyruvate, 2 mM glutamine, 3 mg/ml lyophilized crystallized bovine serum albumin (BSA) (Calbiochem, CA), 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulphate was used. This medium is termed 'HX medium'.

4.2. Oocyte collection, culture and in vitro fertilization

Fully grown GV-intact oocytes were obtained from Kunming mice as previously described by Tong et al. (2002). After washing, GV-arrested oocytes were cultured in M2 or HX medium. All cultures were carried out at 37 °C in a humidified atmosphere of 5% CO2.

For collection of MII-arrested oocytes, female mice were superovulated by intraperitoneal injection with 10 IU of pregnant mares' serum gonadotropin (PMSG) followed 46-48 h later with 10 IU of human chorionic gonadotropin (hCG). Mice were sacrificed and oviducts were removed at 14–16 h after hCG injection. Cumulus masses were collected in M2 medium and the cumulus cells were removed by exposing to 300 IU/ml hyaluronidase.

For IVF, sperm concentration of 1×10^6/ml was used. The motile cauda epididymal sperm had been capacitated for 1 h in M16 medium containing 2.5 mM taurine. ZP-free and MII-arrested oocytes were used to minimize the lag period of sperm–oocyte interaction and achieve a more synchronous time of fertilization. The PB2 and the pronucleus were observed with a phase contrast microscope.

4.3. Zygote collection and culture

In vivo fertilized zygotes were collected at 16–20 h after hCG injection from the oviduct ampullae of superovulated females that had been mated with the same strain of males. After removing cumulus cells with M2 medium containing 300 IU/ml hyaluronidase, zygotes were cultured in M16 medium until use.

4.4. Parthenogenetic activation of oocytes

Parthenogenetic activation of mouse oocytes was performed as previously reported (Tong et al., 2002).

The MII-arrested oocytes were treated with M2 medium containing 10 μM A23187 for 5 min, and then washed thoroughly and incubated in M16 medium containing 10 μg/ml cycloheximide. After 2 and 9 h of culture, the treated oocytes were examined for PB2 emission and pronuclear formation with a phase contrast microscope. The treated oocytes were collected for Western blot analysis at 2, 4, 6, 7 and 8 h after activation.

4.5. Western blot analysis

Oocytes were collected in SDS sample buffer, heated at 100 °C for 4.5 min, and frozen at −20 °C until use. For cyclin B1 and ERK/p90rsk detection, each sample contained 100 and 30 oocytes, respectively. The total proteins were separated by SDS-PAGE with a 4% stacking gel and 10% separating gel at 90 V, 0.5 h and 120 V, 2.5 h respectively and electrophoretically transferred onto nitrocellulose membrane (Gelmen, poresize 0.45 μm) for 2.5 h, 200 mA, at 4 °C. Following the total proteins were transferred onto the nitrocellulose membrane, the membrane was washed in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) twice and blocked in 5% skimmed milk in TBST (TBS containing 0.1% Tween-20) at 4 °C overnight, the membrane was then incubated in TBST containing 1:500 polyclonal rabbit anti-mouse p90rsk antibody and 1:500 monoclonal mouse anti-human p-ERK1/2 antibody at 37 °C for 2 h. After being washed three times in TBST, 10 min each, the membrane was then incubated with HRP-conjugated goat-anti-mouse IgG or 1:1000 goat-anti-rabbit IgG (Jackson Immunoresearch Laboratories, INC, PA) diluted in TBST at 37 °C for 1 h. The membrane was washed three times in TBST, 10 min each, and was processed by using the enhanced chemiluminescence (ECL) detection system according to the manufacture's instructions (Amersham Pharmacia Biotech). As a negative control, the primary antibody was replaced with rabbit IgG or mouse IgG.

For reprobing, the membrane was washed in stripping buffer (100 mM β-mercaptoethanol, 20% SDS, 62.5 mM Tris, pH 6.7) at 50 °C for 30 min to strip off bound antibody after ECL detection. The membrane was reprobed with 1:500 polyclonal rabbit anti-human ERK2 antibody, using the same procedure as described above. All experiments were repeated at least three times.

4.6. Confocal microscopy

For ubiquitin detection, after removal of ZP in acidified Tyrode's solution (pH 2.5), the oocytes or embryos were fixed in 4% paraformaldehyde in PBS for 30 min and then 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). After that, the oocytes or embryos were washed in PBS containing 0.1% Tween 20 for three times, and then incubated with 1:50 polyclonal rabbit anti-human ubiquitin antibody, 1:50 mouse anti-Plk1 antibody or 1:50 polyclonal rabbit anti-human cyclin B1 antibody for 1 h. The oocytes or embryos were rinsed three times and then incubated in 1:100 FITC-conjugated goat anti-rabbit or goat anti-mouse IgG for 1 h according to the primary antibody used, then incubated with 10 μg/ml propidium iodide (Sigma). 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 IgG or mouse IgG. The configuration of spindle was determined by incubating the oocytes in 1: 50 FITC-conjugated anti-α tubulin antibody for 1 h after fixation and permeabilization as described above.

4.7. Microinjection of ubiquitin antibody

Polyclonal rabbit anti-human ubiquitin antibody (200 μg/ml in PBS) was injected into the MII-arrested oocytes as described by Tong et al. (2002). An Eppendoff microinjector was used in this experiment. All microinjections were performed by using a beveled micropipette to minimize damage and were finished within 30 min. A microinjection volume of about 7 pl per oocyte was used in all the experiments. Each experiment consisted of three separate and replicate groups and approximately 50 oocytes were injected each group. The same amount of rabbit IgG was injected into the oocytes as the negative control. After microinjection of ubiquitin antibody, the oocytes were used for IVF or parthenogenetic activation. The PB2 emission and the pronuclear formation were examined at 4 and 8 h after insemination or parthenogenetic activation, respectively.

4.8. Experimental design

Experiments 1–2 were designed to study the roles of UPP in the meiotic maturation of mouse oocytes.

Experiment 1. The subcellular localization of ubiquitin during normal oocyte meiosis in M2 medium and the subcellular localization of ubiquitin, cyclin B1, and Plk1 in oocytes treated with or without proteasome inhibitor ALLN or MG-132 in M2 medium, were examined by confocal microscopy.

Experiment 2. To study the possible roles of UPP in mouse oocyte meiotic resumption, two models, spontaneous maturation and HX-maintained meiotic arrest were used. DOs or CEOs isolated from follicles were cultured in M2 or HX medium with or without different concentrations of proteasome inhibitor, ALLN or MG-132, for 2 h (DOs and CEOs in M2 medium), 12 h (CEOs in HX medium) or 24 h (DOs in HX medium). Then the GVBD of oocytes was examined. As a negative control, oocytes were cultured in M2 or HX medium containing 0.1 or 0.2% DMSO.

The aim of experiments 3–6 was to investigate the roles of UPP in the process of mouse oocyte activation and early embryonic mitosis.

Experiment 3. The subcellular localization of ubiquitin during parthenogenetic activation and fertilization of oocytes were studied by confocal microscopy.

Experiment 4. To determine the involvement of UPP in activation of MII-arrest oocytes and its relationship with MAPK/p90rsk cascade and cyclin B1, MII-arrested oocytes were first pretreated in M2 or M16 medium containing different concentrations of ALLN or MG-132, and then performed for parthenogenetic activation (chemical stimulation) and IVF (physiological stimulation). The proteasome inhibitor, ALLN or MG-132 was persistently included in the medium. The PB2 emission and the pronuclear formation were examined at different time intervals. The treated oocytes were collected for Western blot analysis at 2, 4, 6, 7, and 8 h after activation, and the expression of cyclin B1, ERK2 and p90rsk, as well as the phosphorylation level of ERK1/2 and p90rsk were studied.

Experiment 5. To further determine the roles of UPP in oocyte activation, MII-arrested oocytes were microinjected with polyclonal rabbit anti-human ubiquitin antibodies or rabbit IgG, and then performed for IVF or parthenogenetic activation. The PB2 emission and the pronuclear formation were examined after parthenogenetic activation or IVF at 4 and 8 h, respectively.

Experiment 6. The subcellular localization of ubiquitin at different developmental stages of early embryos was revealed by confocal microscopy.

4.9. Statistical analysis

All percentages from at least three repeated experiments were expressed as mean ± SEM and the number of oocytes observed was labeled in brackets as ($n =$). The rates of GVBD, PB2 emission and pronuclear formation were subjected to arcsin transformation. The transformed data were analyzed by ANOVA followed by Student–Newman–Keuls test. Differences at $P < 0.05$ were considered to be statistically significant.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 30225010, 30170358), Special Funds for Major State Basic Research Project (973) of China (No. G1999055902), and Knowledge Innovation Project of the Chinese Academy of Sciences (KSCX2-SW-303 and KSCX-IOZ-07).

References

Bu, S., Xia, G., Tao, Y., Lei, L., Zhou, B., 2003. Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro. Mol. Cell Endocrinol. 207, 21–30.

Downs, S.M., 1993. Purine control of mouse oocyte maturation: evidence that nonmetabolized hypoxanthine maintains meiotic arrest. Mol. Reprod. Dev. 35, 82–94.

Downs, S.M., Humpherson, P.G., Leese, H.J., 2002. Pyruvate utilization by mouse oocytes is influenced by meiotic status and the cumulus oophorus. Mol. Reprod. Dev. 62, 113–123.

Faerge, I., Terry, B., Kalous, J., Wahl, P., Lessl, M., Ottesen, J.L., Hyttel, P., Grondahl, C., 2001. Resumption of meiosis induced by meiosis-activating sterol has a different signal transduction pathway than spontaneous resumption of meiosis in denuded mouse oocytes cultured in vitro. Biol. Reprod. 65, 1751–1758.

Fan, H.Y., Sun, Q.Y., 2004. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biol. Reprod. 70, 535–547.

Fan, H.Y., Huo, L.J., Meng, X.Q., Zhong, Z.S., Hou, Y., Chen, D.Y., Sun, Q.Y., 2003. Involvement of calcium/calmodulin-dependent protein kinase II (CaMKII) in meiotic maturation and activation of pig oocytes. Biol. Reprod. 69, 1552–1564.

Fan, H.Y., Huo, L.J., Chen, D.Y., Sun, Q.Y., 2004. Protein kinase C and MAPK cascade in mouse cumulus cells: crosstalk and effect on meiotic resumption of oocyte. Biol. Reprod. 70, 1178–1187.

Glickman, M.H., Ciechanover, A., 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.

Glotzer, M., Murray, A.W., Kirschner, M.W., 1991. Cyclin is degraded by the ubiquitin pathway. Nature. 349, 132–138.

Herbert, M., Levasseur, M., Homer, H., Yallop, K., Murdoch, A., McDougall, A., 2003. Homologue disjunction in mouse oocytes requires proteolysis of securin and cyclin B1. Nat. Cell Biol. 5, 1023–1025.

Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R.E., Cohen, L.H., 1991. Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. J. Biol. Chem. 266, 16376–16379.

Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L.H., Luca, F.C., Ruderman, J.V., Eytan, E., 1994. Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. J. Biol. Chem. 269, 4940–4946.

Josefsberg, L.B., Galiani, D., Dantes, A., Amsterdam, A., Dekel, N., 2000. The proteasome is involved in the first metaphase-to-anaphase transition of meiosis in rat oocytes. Biol. Reprod. 62, 1270–1277.

Josefsberg, L.B., Kaufman, O., Galiani, D., Kovo, M., Dekel, N., 2001. Inactivation of M-phase promoting factor at exit from first embryonic mitosis in the rat is independent of cyclin B1 degradation. Biol. Reprod. 64, 871–878.

Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.M., Watanabe, N., Hoog, C., Hieter, P., Todokoro, K., 1998. PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. Mol. Cell. 1, 371–380.

Ma, S., Lan, G., Miao, Y., Wang, Z., Chang, Z., Luo, M., Tan, J., 2003. Hypoxanthine (HX) inhibition of in vitro meiotic resumption in goat oocytes. Mol. Reprod. 66, 306–313.

Mailhes, J.B., Hilliard, C., Lowery, M., London, S.N., 2002. MG-132, an inhibitor of proteasomes and calpains, induced inhibition of oocyte maturation and aneuploidy in mouse oocytes. Cell Chromosome 1, 2–7.

Morinaga, C., Izumi, K., Sawada, H., Takagi Sawada, M., 2000. Activation of maturation promoting factor and 26S proteasome assembly accelerated by a high concentration of 1-methyladenine in starfish oocytes. Biosci. Biotechnol. Biochem. 64, 268–274.

Murray, A.W., Solomon, M.J., Kirschner, M.W., 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature 339, 280–286.

Nixon, V.L., Levasseur, M., McDougall, A., Jones, K.T., 2002. Ca^{2+} oscillations promote APC/C-dependent cyclin B degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. Curr. Biol. 12, 746–750.

Pahl, H.L., Baeuerle, P.A., 1996. Control of gene expression by proteolysis. Curr. Opin. Cell. Biol. 8, 340–347.

Peters, J.M., 1999. Subunits and substrates of the anaphase-promoting complex. Exp. Cell Res. 248, 339–349.

Peters, J.M., 2002. The Anaphase-promoting complex: proteolysis in mitosis and beyond. Mol. Cell. 9, 931–943.

Peter, M., Castro, A., Lorca, T., Le Peuch, C., Magnaghi-Jaulin, L., Doree, M., Labbe, J.C., 2001. The APC is dispensable for first meiotic anaphase in *Xenopus* oocytes. Nat. Cell. Biol. 3, 83–87.

Reimann, J.D., Jackson, P.K., 2002. Emi1 is required for cytostatic factor arrest in vertebrate eggs. Nature 416, 850–854.

Sawada, H., Yokoyama, K., Morinaga, C., Yokosawa, H., Sawada, M.T., 1997. Protease triggers dephosphorylation of cdc2 kinase during starfish oocyte maturation. Biochem. Mol. Biol. Int. 41, 905–911.

Sawada, M.T., Morinaga, C., Izumi, K., Sawada, H., 1999. The 26S proteasome assembly is regulated by a maturation-inducing hormone in starfish oocytes. Biochem. Biophys. Res. Commun. 254, 338–344.

Su, Y.Q., Denegre, J.M., Wigglesworth, K., Pendola, F.L., O'Brien, M.J., Eppig, J.J., 2003. Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte-cumulus cell complex. Dev. Biol. 263, 126–138.

Sun, Q.Y., Rubinstein, S., Breitbart, H., 1999. MAP kinase activity is downregulated by phorbol ester during mouse oocyte maturation and egg activation in vitro. Mol. Reprod. Dev. 52, 310–318.

Suzumori, N., Burns, K.H., Yan, W., Matzuk, M.M., 2003. RFPL4 interacts with oocyte proteins of the ubiquitin–proteasome degradation pathway. Proc. Natl. Acad. Sci. 100, 550–555.

Taieb, F.E., Gross, S.D., Lewellyn, A.L., Maller, J.L., 2001. Activation of the anaphase-promoting complex and degradation of cyclin B is not required for progression from Meiosis I to II in *Xenopus* oocytes. Curr. Biol. 11, 508–513.

Takagi Sawada, M., Kyozuka, K., Morinaga, C., Izumi, K., Sawada, H., 1997. The proteasome is an essential mediator of the activation of pre-MPF during starfish oocyte maturation. Biochem. Biophys. Res. Commun. 236, 40–43.

Takahashi, M., Tokumoto, T., Ishikawa, K., 1994. DFP-sensitive multi-catalytic protease complexes (proteasomes) involved in the control of oocyte maturation in the toad. Bufo japonicus, Mol. Reprod. Dev. 38, 310–317.

Tokumoto, T., Yamashita, M., Tokumoto, M., Katsu, Y., Horiguchi, R., Kajiura, H., Nagahama, Y., 1997. Initiation of cyclin B degradation by the 26S proteasome upon egg activation. J. Cell Biol. 138, 1313–1322.

Tanaka, E., 2000. Takagi Sawada M. Sawada H., Enzymatic properties of the proteasome purified from starfish oocytes and its catalytic subunits involved in oocyte maturation. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 125, 215–223.

Tong, C., Fan, H.Y., Lian, L., Li, S.W., Chen, D.Y., Schatten, H., Sun, Q.Y., 2002. Polo-like kinase-1 is a pivotal regulator of microtubule assembly during mouse oocyte meiotic maturation, fertilization, and early embryonic mitosis. Biol. Reprod. 67, 546–554.

Tunquist, B.J., Maller, J.L., 2003. Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. Genes. Dev. 17, 683–710.

Wójcik, C., Benchaib, M., Lornage, J., Czyba, J.C., Guerin, J.F., 2000. Localization of proteasomes in human oocytes and preimplantation embryos. Mol. Hum. Reprod. 6, 331–336.

Yamaguchi, R., Dutta, A., 2000. Proteasome inhibitors alter the orderly progression of DNA synthesis during S-phase in HeLa cells and lead to rereplication of DNA. Exp. Cell Res. 261, 271–283.