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# Regulatory roles of ubiquitin–proteasome pathway in pig oocyte meiotic maturation and fertilization

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**Abstract**

The ubiquitin–proteasome pathway is involved in the degradation of proteins related to cell cycle progression including cyclins. The present study, using two specific proteasome inhibitors, for the first time investigated the roles of ubiquitin–proteasome in cell cycle progression during pig oocyte meiotic maturation and after fertilization. In contrast to its effect in rodent oocytes, proteasome inhibition strongly prevented germinal vesicle breakdown (GVBD). After GVBD, proteasome inhibition disrupted meiotic apparatus organization, cell cycle progression, and first polar body (PB1) extrusion. Sperm penetration into the oocytes was completely inhibited when proteasome inhibitors were added at the beginning of insemination. However, sperm chromatin decondensation and metaphase–interphase transition were not affected when inhibitors were added once sperm penetrated. The results suggest that ubiquin–proteasome complex is one of the critical regulators of meiotic cell cycle, but proteasome inhibitors do not affect major fertilization events when added after sperm penetration into the oocytes in the pig.


*Keywords:* Oocyte; Meiosis; Fertilization; Ubiquitin–proteasome; Pig

## 1. Introduction

Cell cycle progression in eukaryotes is controlled by the synthesis/degradation of some short-lived, phase-specific regulatory proteins and/or protein phosphorylation/dephosphorylation induced by protein kinases/phosphatases. The ubiquitin–proteasome complex is an

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essential component of the proteolytic pathway in eukaryotic cells and is responsible for the degradation of most cellular proteins. The proteasome is a multicatalytic protease that is able to hydrolyze C-terminal peptide bonds to acidic, basic, and hydrophobic amino acid residues. Proteasomes are sorted into two types, 20S and 26S. The 20S proteasome forms the catalytic core of the 26S proteasome. The 26S proteasome is involved in the degradation of ubiquitinated proteins [1]. Proteins critical to the regulation of the cell cycle, such as cyclins, cdk inhibitors or c-*mos* products, are known to be ubiquitinated and degraded by the ubiquitin–proteasome pathway [1].

Fully-grown mammalian oocytes are arrested at the dictyate stage of meiosis I. Oocytes can be induced to resume meiotic cell cycle by a surge of the pituitary gonadotropin in vivo or after being released from the follicle environment, as indicated by germinal vesicle breakdown (GVBD), followed by formation of meiotic apparatus and emission of the first polar body (PB1). Immediately thereafter, oocytes commence the second meiosis and are arrested again at the metaphase of meiosis II (MII). After fertilization or parthenogenetic activation, the second meiosis resumes and the oocyte releases the second polar body (PB2). Then, female and male pronuclei form and get apposed to start mitosis in fertilized eggs.

It has been well established that protein phosphorylation/dephosphorylation induced by protein kinases/phosphatases and the timely production/clearance of cell cycle regulators via proteasomal degradation play important roles in oocyte meiosis progression in toad and starfish oocytes. Inhibition of proteasomal proteolytic activity blocked GVBD [3,4], suggesting that this proteinase is involved in reinitiation of meiosis in these species. Maturation promoting factor (MPF), a complex of p34cdc2 and cyclin B, regulates major events of oocyte maturation and fertilization. Inactivation of this kinase, occurring between the two meiotic divisions and shortly after fertilization, is associated with degradation of cyclin B. In *Xenopus* oocytes, degradation of M-phase cyclin B is mediated by the ubiquitin-dependent proteolytic system [4]. In mammals, we are aware of only two recent reports on the involvement of ubiquitin–proteasome pathway in rodent oocyte maturation. These two studies showed that, in contrast to lower vertebrates, proteasome inhibition did not affect GVBD, but was involved in the first meiosis metaphase-to-anaphase transition in mouse and rat oocytes [5,6]. Oocytes treated with proteasome inhibitors were arrested at MI, but they could complete meiosis after washing out the compound. However, aneuploidy was caused [6].

Pigs are not only important farm animals, but also model animals for biomedical applications, such as human disease processes, drug development, and human organ xenotransplantation [7]. The development of efficient reproductive biotechnology has been increasingly required in this species; however, the efficiency is still extremely low. Although several in vitro culture systems for oocyte maturation, fertilization and embryo development have been established, the outcome is still not satisfactory [8,9]. Thus, it is important to understand the basic aspects of pig oocyte maturation and fertilization. MPF and mitogen-activated protein (MAP) kinases have been shown to be critical regulator of cell cycle in pig oocytes [10–12]. Recently, ubiquitin-dependent proteolysis has been implicated in the elimination of paternal mitochondria in fertilized pig eggs [13,14]. However, the involvement of ubiquitin–proteasome complex in the regulation of major events during oocyte meiotic maturation and fertilization is currently

unknown in this species. In the present study, the possible roles of ubiquitin–proteasome in pig oocyte maturation and fertilization were investigated using two specific inhibitors.

## 2. Materials and methods

### 2.1. Chemicals

Two specific ubiquitin–proteasome pathway inhibitors, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal Z-LLL-CHO (MG-132) and lactacystin, were purchased from Calbiochem Co. (Darmstadt, Germany). MG-132 is a highly specific, reversible inhibitor, while lactacystin is a specific, non-reversible specific inhibitor of ubiquitin–proteasome complex. Stock solutions of both chemicals were prepared with DMSO at the concentration of 10 mM, and kept frozen at −20 °C. The final working solutions of all the above chemicals were diluted soon before usage. In each group, the concentration of DMSO was less than 1% (v/v), and the same concentration of DMSO was added to control groups.

### 2.2. Oocyte collection and in vitro maturation

Ovaries were collected from gilts at a local abbatoir and transported to the laboratory at 37 °C within a thermos containing 0.9% saline. Oocytes were aspirated from antral follicles (approximately 2–6 mm in diameter) with an 18-gauge needle fixed to a 10 ml disposable syringe. After two washes with Medium 199 for use in air [15] and three washes with maturation medium NCSU-37 that contains follicular fluid but not epidermal growth factor [16], oocytes with a compact cumulus and evenly granulated ooplasm were selected for maturation culture. A group of 50 oocytes was cultured in 500 μl maturation medium at 38.7 °C in 5% $CO_2$, 5% $O_2$, 90% $N_2$ and saturated humidity.

### 2.3. In vitro fertilization and parthenogenetic activation

The method of in vitro fertilization was basically based on the method reported by Suzuki et al. [17]. Briefly, after 44 h culture, oocytes with expanded cumulus were washed three times in fertilization medium pig-FM, and then transferred to 80 μl fertilization drops, 20 oocytes per drop. Frozen spermatozoa were thawed in 37 °C water bath for 3 min, washed with M199 plus 12% FCS (pH 7.8) and preincubated for 15 min at 37 °C. Then spermatozoa were added to fertilization medium at a concentration of $5 \times 10^5$ cells/ml. At 4 h post-insemination, oocytes were transferred to NCSU-37 IVC medium, and cultured for 16 h before examination.

### 2.4. Nucleus examination by orcein staining

After maturation culture for a specified time, oocytes were freed of cumulus cells by treatment with 300 IU/ml hyaluronidase and vortexing. The denuded eggs were then washed twice in PBS, mounted on a glass slide, fixed in acetic acid:alcohol (1:3 v/v) for 48 h, stained with 1% orcein and finally examined under a phase-contrast microscope.

### 2.5. Statistical analysis

The data on GV arrest, GVBD, MII and PN formation from three or four repeated experiments were compared among different groups with Chi-square analysis. A value of $P < 0.05$ was considered significant.

### 2.6. Experimental design

#### 2.6.1. Experiment 1

To investigate the effect of ubiquitin–proteasome inhibition on meiosis resumption, either MG-132 (10 and 100 μM) or lactacystin (10 and 100 μM) was added to the maturation medium at the beginning of culture of the cumulus-enclosed GV oocytes. Meiosis progression was evaluated at 26 or 44 h after culture. As a control, cumulus-enclosed oocytes were cultured in drug-free medium and collected for observation at the same period.

#### 2.6.2. Experiment 2

To evaluate the reversibility of the drug's effect, the GV oocytes were cultured in drug-containing medium for the first 24 h, then washed three times, 5 min each, and placed into fresh drug-free medium for another 26 h. Oocytes cultured in drug-free medium or drug-containing medium for 50 h were adopted as controls.

#### 2.6.3. Experiment 3

To evaluate the effect of ubiquitin–proteasome inhibition on further meiosis progression including PB1 emission after GVBD, oocytes that had been cultured in drug-free medium for 26 h were treated with MG-132 or lactacystin for an additional 18 h. In control group, oocytes were cultured in a drug-free medium for 44 h.

#### 2.6.4. Experiment 4

To observe whether ubiquitin–proteasome inhibition affects oocyte MII arrest, oocytes cultured for 44 h were denuded and exposed to MG-132 or lactacystin for 24 h.

#### 2.6.5. Experiment 5

To investigate the effect of ubiquitin–proteasome inhibition on sperm penetration and egg activation events after sperm penetration, ubiquitin–proteasome inhibitors were added to the medium at the beginning of insemination and 4 h after insemination, respectively. PB2 emission and pronucleus formation were observed 20 h after insemination.

## 3. Results

### 3.1. Inhibition of meiosis resumption by ubiquitin–proteasome inhibitors

When COCs were cultured in maturation medium containing 10 or 100 μM MG-132 for 26 h, both cumulus expansion and meiosis resumption were inhibited, while most oocytes

Table 1
Cell cycle progression of cumulus-enclosed pig oocytes after treatment with ubiquitin–proteasome inhibitors for 26 h

| Treatment | No. of oocytes examined | GV (%) | GVBD | Pre-MI to MI | Anaphase–telophase I | MII |
|---|---|---|---|---|---|---|
| Control | 97 | 13 (13.4)[a] | 8 | 75 | 1 | 0 |
| MG (10 μM) | 43 | 36 (83.7)[b] | 0 | 7 | 0 | 0 |
| MG (100 μM) | 35 | 35 (100)[b] | 0 | 0 | 0 | 0 |
| Lac (10 μM) | 55 | 10 (18.2)[a] | 27 | 17 | 0 | 1 |
| Lac (100 μM) | 65 | 54 (81.8)[b] | 11 | 0 | 0 | 0 |

Cumulus-enclosed oocytes were cultured in the maturation medium containing different concentrations of ubiquitin–proteasome inhibitor MG or Lac. Cell cycle progression was evaluated at 26 h of culture after orcein staining. MG, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal Z-LLL-CHO (MG-132); Lac, lactacystin. Within a column, rows without the same superscripts (a and b) are different ($P < 0.05$).

went through GVBD and progressed to pre-metaphase I to metaphase I (MI) stages in control group (Table 1). The GV nucleolus disappeared and chromatin condensed in half of the oocytes (21/44) treated with 10 μM MG-132, but most oocytes (29/35) treated with 100 μM MG-132 contained a nucleolus and no chromatin condensation was observed. Another specific inhibitor (lactacystin at 100 μM) also inhibited GVBD and cumulus expansion as well as nucleolus disruption (40 out of 54 oocytes contained nucleolus), but 10 μM lactacystin affected neither cumulus expansion nor GVBD.

When oocytes were treated with MG-132 or lactacystin for 44 h, strong inhibition of GVBD was still observed in oocytes treated with both concentrations of MG-132 when compared to the control ($P < 0.05$). Lactacystin at 10 μM did not affect oocyte maturation. Although nearly half of the oocytes treated with 100 μM lactacystin underwent GVBD, few progressed to the MII stage (Table 2).

### 3.2. Reversibility of meiosis resumption inhibition induced by MG-132

When COCs that had been cultured with 10 or 100 μM MG-132 for 24 h were transferred to drug-free medium, and cultured for another 26 h, limited expansion of outer cumulus cells was observed in 10 μM MG-132 group, while cumulus cells in 100 μM

Table 2
Inhibition of GVBD after 44-h treatment of oocytes with ubiquitin–proteasome inhibitiors

| Treatment | No. examined | GV (%) | MII (%) |
|---|---|---|---|
| COC (control) | 39 | 4 (10.3)[a] | 33 (84.6)[a] |
| COC (MG 10 μM) | 48 | 36 (75.0)[b] | 0 (0)[b] |
| COC (MG 100 μM) | 29 | 27 (93.1)[b] | 0 (0)[b] |
| COC (Lac 10 μM) | 30 | 4 (13.3)[a] | 26 (86.7)[a] |
| COC (Lac 100 μM) | 32 | 17 (53.1)[b] | 7 (21.9)[c] |

Cumulus-enclosed oocytes were cultured in the maturation medium containing different concentrations of ubiquitin–proteasome inhibitor MG or Lac. Cell cycle progression was evaluated at 44 h of culture after orcein staining. Within a column, rows without the same superscripts (a–c) are different ($P < 0.05$).

Table 3
Reversibility of GVBD inhibition by unbiquitin–proteasome inhibitors

| Treatment | No. of oocytes examined | GVBD (%) | MII(%) |
|---|---|---|---|
| Drug-free | 80 | 69 (86.3)[a] | 57 (71.3)[a] |
| MG (10 μM, 24 h) | 135 | 127 (94.1)[a] | 65 (48.1)[a] |
| MG (100 μM, 24 h) | 99 | 38 (38.4)[b] | 0 (0)[b] |
| MG (10 μM, 50 h) | 73 | 28 (38.4) | 0 (0)[b] |
| MG (100 μM, 50 h) | 87 | 13 (14.9) | 0 (0)[b] |

Cumulus-enclosed oocytes were cultured in the maturation medium containing different concentrations of MG-132 for 24 h, and after three times of through wash, they were cultured in drug-free medium for an additional 26 h. Cell cycle progression was evaluated after orcein staining. Oocytes cultured in drug-free medium or drug-containing medium for 50 h were adopted as controls. Different superscripts (a and b) in the same column imply statistically different ($P < 0.05$).

MG-132 group did not expand at all. In the former group, nearly half of the oocytes completed maturation as indicated by PB2 emission, while two thirds of the oocytes in the latter group were still kept at GV stage, and no oocytes reached MI stage. Instead, a nucleolus still existed in the cytoplasm of GVBD oocytes. As a control, most of the oocytes treated continuously with 10 or 100 μM MG-132 were at GV stage and no oocyte completed maturation even when observed at 50 h (Table 3).

### 3.3. Effect of ubiquitin–proteasome inhibition on cell cycle progression after GVBD

After GVBD, 10 μM MG-132 did not affect progression to MII stages (Fig. 1A), but 100 μM MG-132 or lactacystin blocked oocyte maturation ($P < 0.05$). A large proportion

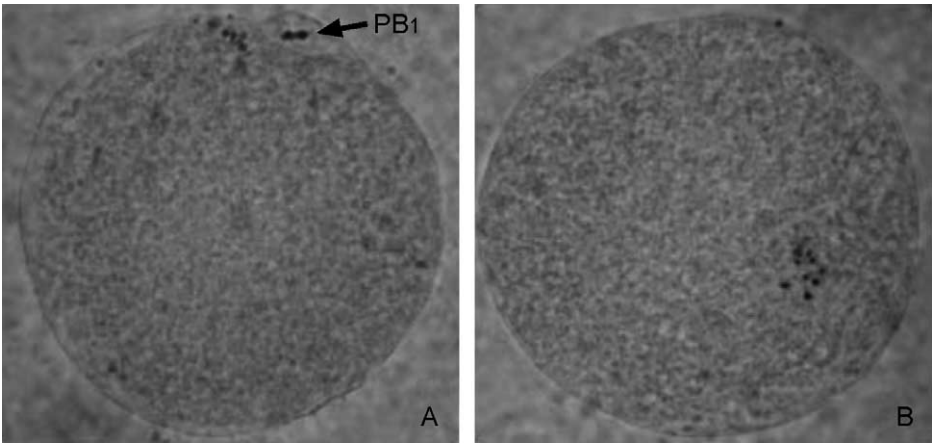


Fig. 1. Configuration of chromosome distribution in oocytes treated with MG-132. Oocytes that had been cultured in drug-free medium for 26 h were treated with 10 or 100 μM MG-132 for an additional 18 h. (A) An oocytes showing extruded first polar body (PB1) and chromosomes aligned on the spindle equator after treatment with 10 μM MG-132; (B) an oocyte showing dispersed chromosomes in the cytoplasm after treatment with 100 μM MG-132.

Table 4
Cell cycle progression of oocytes treated with ubiquitin–proteasome inhibitors 26 h after in vitro culture

| Treatment | No. oocytes | GV | GVBD | Pre-MI to MI | Anaphase–telophase I | MII (%) |
|---|---|---|---|---|---|---|
| Control | 50 | 8 | 4 | 3 | 0 | 35 (70.0)[a] |
| MG (10 μM) | 63 | 1 | 4 | 12 | 2 | 44 (69.8)[a] |
| MG (100 μM) | 56 | 0 | 3 | 36 | 11 | 6 (10.7)[b] |
| Lac (100 μM) | 33 | 4 | 3 | 19 | 3 | 4 (12.1)[b] |

Cumulus-enclosed oocytes were cultured in drug-free maturation medium for 26 h, and then different concentrations of ubiquitin–proteasome inhibitor MG-132 or Lac was added to the culture. Cell cycle progression was evaluated at a total culture time of 44 h after orcein staining. Within a column, rows without the same superscripts (a and b) are different ($P < 0.05$).

of oocytes were arrested at pre-metaphase I to MI stages, and few progressed to MII stage (Table 4). Chromosomes were dispersed in all the arrested oocytes, and movement of chromosomes to cortical region was also inhibited (Fig. 1B).

### 3.4. Effect of ubiquitin–proteasome inhibition on egg activation events

To clarify whether inhibition of ubiquitin–proteasome pathway affects MII arrest, oocytes cultured for 44 h were exposed to 10 or 100 μM MG-132 or 100 μM lactacystin for an additional 24 h. None of the MII oocytes treated with 10 μM MG-132 ($n = 48$), 100 μM MG-132 ($n = 43$) or 100 μM lactacystin ($n = 47$) were activated.

When oocytes were inseminated in the medium containing 100 μM MG-132 ($n = 93$) or lactacystin ($n = 47$), expanded cumulus was digested as in the control group, but sperm penetration was not observed in all the oocytes examined 20 h after culture. When 10 μM MG-132, 100 μM MG-132 or 100 μM lactacystin was added to the medium 4 h after insemination, sperm penetration rate ranged from 67 to 83% among different groups. In the penetrated oocytes, pronucleus was formed in 70–80% of them, and there was no significant difference in PN formation among treated groups and control group (Table 5). The PB2 was extruded in all the penetrated oocytes in 10 μM MG-132 group. Unexpectedly, all penetrated oocytes treated with 100 μM lactacystin and approximately half the penetrated oocytes treated with 100 μM MG-132 extruded the PB2.

Table 5
Effect of ubiquitin–proteasome inhibition on pronucleus formation after sperm penetration

| Groups | No. examined | No. penetrated (%) | PN (−) (%) | PN (+) (%) |
|---|---|---|---|---|
| Control | 69 | 52 (75.4) | 11 (21.2) | 41 (78.8) |
| MG (10 μM) | 68 | 55 (80.1) | 11 (20.0) | 44 (80.0) |
| MG (100 μM) | 92 | 76 (82.6) | 16 (21.1) | 60 (78.9) |
| Lac (100 μM) | 60 | 40 (66.7) | 11 (27.5) | 29 (72.5) |

Oocytes matured for 44 h were inseminated with frozen-thawed spermatozoa. Ubiquitin–proteasome inhibitor MG-132 or lactacystin was added to in vitro development medium 4 h after insemination. Sperm penetration and pronucleus formation were observed 20 h after insemination. PN (−), without pronucleus; PN (+), with pronucleus. There were no significant differences among groups.

## 4. Discussion

In the present study, inhibition of ubiquitin–proteasome pathway blocked the first meiosis resumption in pig oocytes. When cumulus-enclosed pig oocytes were treated with a specific reversible inhibitor MG-132 or a specific irreversible inhibitor lactacystin, both cumulus expansion and GVBD were strongly inhibited when observed at 26 or 44 h of culture. This result was consistent with the reports in starfish and toad oocytes in which proteasome activity is required for GVBD [2,3]. However, proteasome inhibition does not affect GVBD, but arrests oocyte maturation at the MI stage in mouse and rat oocytes [5,6]. Resumption of meiosis requires activation of pre-MPF molecules within oocytes. In starfish oocytes, either proteasome inhibitor MG-115 or proteasome subunit antibody injection blocked GVBD, and it has been suggested that proteasome is an essential mediator of the activation of pre-MPF, and that proteasome triggers the activation of pre-MPF via the dephosphorylation of phospho-Tyr 15 in cdc2 kinase in response to the hormonal stimulus during starfish oocyte maturation [3]. Maturation of pig and rodent oocytes is differently regulated in many aspects. For example, pig oocyte GVBD requires protein synthesis and MAP kinase phosphorylation [12,18,19], whereas mouse and rat oocytes can undergo GVBD in the absence of protein synthesis and MAP kinase activation [18–21]. The present results, together with previous reports, supported the hypothesis that pig oocyte maturation may be regulated by different mechanisms than rodent oocytes, but by similar mechanisms with lower vertebrates.

The inhibition of GVBD by 10 μM MG-132 was reversible. Nearly half of the oocytes arrested at GV stage by 10 μM MG-132 for 24 h could complete maturation after further culture for another 26 h in a drug-free medium. The inhibition of GVBD of pig oocytes by100 μM MG-132 was not reversible, which was consistent with the results obtained in mouse oocytes [6]. Treatment of pig oocytes with an appropriate concentration of MG-132 could be used to synchronize cytoplasmic maturation and nuclear maturation, but it has been proved that even short-term (6 h) exposure of mouse oocytes to MG-132 could cause aneuploidy.

When ubiquitin–proteasome pathway was inhibited after GVBD, further cell cycle progression and PB1 emission were prevented. The concentration of MG-132 blocking meiotic cycle progression was 100 μM, higher than that for blocking GVBD (10 μM). In these oocytes, chromosomes were dispersed, and movement of chromosomes to cortical region was also inhibited. In rat oocytes, proteasome was translocated to the spindle after GVBD, and inhibition of proteasome by 10 μM MG-132 caused irregular segregation of chromosomes and prevented metaphase–anaphase transition [5]. In mice, oocytes treated with 25–50 μg/ml MG-132 also exhibited diffuse chromatin [6]. In mitotic cells, proteasomes are needed for regulating kinetochore structure [22], and sister chromatid cohesion and separation [23]. In addition, MG-132 also represses the activity of calpains, whose activity is involved in the regulation of some cytoskeletons proteins such as α-tubulin, actin and dynamin in starfish oocytes [24]. Therefore, it is possible that inhibition of ubiquitin–proteasome disrupted normal spindle organization and microtubule–chromosome interaction, and thus prevented further cell cycle progression in pig oocytes. Furthermore, inhibition of proteasome results in accumulation of cyclin B and elevated activity of MPF in rat oocytes [5]. This is another possible reason for cell cycle arrest at MI, since MI to

anaphase I transition needs decrease in MPF activity. Further study is needed to clarify whether MPF activity is elevated by ubiquitin–proteasome inhibition in pig oocytes. Meiotic apparatus migration is driven by microfilaments [10], and the failure of chromosome migration to cortical region may be caused by a disruption of actin.

Some treatments kept oocytes at GV stage, and on the other hand induced mature oocytes to undergo interphase entry. For example, PKC activators stimulated both immature and mature oocytes to stay in the interphase of cell cycle [25]. In the present study, ubiquitin–proteasome inhibition prevented GVBD, but did not induce metaphase–interphase transition of mature oocytes. Both inhibitors completely inhibited sperm penetration into the oocytes, although the cumulus was digested as in the control group. We inferred that the unbiquitin–proteasome pathway is required for some steps from sperm acrosome reaction to sperm penetration. Further studies are needed to determine how and where unbiquitin–proteasome affects sperm penetration.

During pig in vitro fertilization, sperm penetration begins at 3 h post-insemination, and by 5 h, decondensing sperm heads and anaphase II plate are observed in half the eggs [26]. Thus, we added inhibitors to the medium at 4 h of insemination, when sperm just penetrated oocytes, to observe the effect of block ubiquitin–proteasome on fertilization events. We expected that inhibition of ubiquitin–proteasome and following inhibition of degradation of ubiquitinated proteins including cyclin B should block the cell cycle progression, since it is widely accepted that inactivation of MPF is associated with cyclin B1 degradation that is brought about by the ubiquitin–proteasome pathway. During egg activation, MPF is inactivated immediately before the transition from metaphase to anaphase by a mechanism involving the degradation of its regulatory subunit, cyclin B. In starfish oocytes, the ATP-dependent proteolytic activity of 26S proteasome initiates the destruction of cyclin B through the first cutting of the $NH_2$ terminus of cyclin B; this cutting allows the cyclin to be ubiquitinated for further destruction by ubiquitin-dependent activity of the 26S proteasome that leads to MPF inactivation [4]. It is reported that degradation of cyclin B1 molecules corresponds to the transition of the pig oocytes from metaphase II arrest to anaphase II/telophase II [27]. We expected that inhibition of ubiquitin–proteasome pathway would block metaphase–interphase transition (PN formation). To our surprise, neither metaphase–interphase transition nor sperm head chromatin decondensation and male pronucleus formation was affected by ubiquitin–proteasome inhibition in pig eggs. Pronuclei were observed in most of the penetrated eggs 20 h after insemination. Therefore, metaphase–interphase transition may not necessarily require degradation of ubiquitinated proteins after sperm penetration of pig oocytes. Since the drug inhibited sperm penetration and thus it was added 4 h after insemination when sperm just penetrated, thus another possibility is that protein ubiquination and degradation happen so fast after sperm penetration and once this occurs, fertilization events including the PN formation are not affected by the inhibitors.

It appeared that the concentration of ubituitin–proteasome inhibitors for GVBD inhibition was lower than that for inhibition of cell cycle progression after GVBD. However, it is difficult to explain why ubiquitin–proteasome inhibitors at a concentration inhibiting PB1 emission did not affect PB2 emission in many oocytes as shown in our experiment. One possible reason is that PB2 is already emitted or cyclin B is already degraded by the time of inhibitors take effect. Alternatively, perhaps the MII meiotic

apparatus (including the interaction between the microtubules and chromosomes) in mature oocytes is more stable than that of MI meiotic apparatus of maturing oocytes.

In summary, ubiquitin–proteasome complex plays important roles in meiotic resumption and completion of the first meiosis as well as sperm penetration during pig fertilization, while metaphase–interphase transition occurred when ubiquitin–proteasome inhibitor was added after sperm penetration. The mechanisms by which the ubiquitin–proteasome affects other molecules like MPF (cyclin B) and MAP kinase cascade in regulating mammalian oocyte cell cycle and how this pathway affects sperm penetration into oocytes require further study.

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## References

[1] Tokumato T. Nature and role of proteasomes in maturation of fish oocytes. Int Rev Cytol 1999;186:261–94.
[2] Takahashi M, Tokumoto T, Ishikawa K. DFP-sensitive multicatalytic protease complexes (proteasomes) involved in the control of oocyte maturation in the toad, *Bufo japonicus*. Mol Reprod Dev 1994;38:310–7.
[3] Sawada M, Kyozuka K, Morinaga C, Izumi K, Sawada H. The proteasome is an essential mediator of the activation of pre-MPF during starfish oocyte maturation. Biochem Biophys Res Commun 1997;236:40–3.
[4] Tokumoto T, Yamashita M, Tokumoto M, Katsu Y, Horiguchi R, Kajiura H, et al. Initiation of cyclin B degradation by the 26S proteasome upon egg activation. J Cell Biol 1997;138:1313–22.
[5] Josefsberg LB, Galiani D, Dantes A, Amsterdam A, Dekel N. The proteasome is involved in the first metaphase-to-anaphase transition of meiosis in rat oocytes. Biol Reprod 2000;62:1270–7.
[6] Mailhes JB, Hilliard C, Lowery M, London SN. MG-132, an inhibitor of proteasomes and calpains, induced inhibition of oocyte maturation and aneuploidy in mouse oocytes. Cell Chromosome 2002;1:2.
[7] Prather RS, Hawley RJ, Carter DB, Lai L, Greenstein JL. Transgenic swine for biomedicine and agriculture. Theriogenology 2003;59:115–23.
[8] Prather RS, Day BN. Practical considerations for the in vitro production of pig embryos. Theriogenology 1998;49:23–32.
[9] Nagai T. The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology 2001;55:1291–301.
[10] Sun QY, Lai L, Park KW, Kühholzer B, Prather RS, Schatten H. Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization in vitro. Biol Reprod 2001;64:879–89.
[11] Kikuchi K, Naito K, Noguchi J, Kaneko H, Tojo H. Maturation/M-phase promoting factor regulates aging of porcine oocytes matured in vitro. Cloning Stem Cells 2002;4:211–22.

[12] Fan HY, Tong C, Lian L, Li SW, Gao WX, Cheng Y, et al. Characterization of ribosomal S6 protein kinase p90rsk during meiotic maturation and fertilization in pig oocytes: mitogen-activated protein kinase-associated activation and localization. Biol Reprod 2003;68:968–77.
[13] Sutovsky P, McCauley TC, Sutovsky M, Day BN. Early degradation of paternal mitochondria in domestic pig (Sus scrofa) is prevented by selective proteasomal inhibitors lactacystin and MG 132. Biol Reprod 2002;68:688–98.
[14] Thompson WE, Ramalho-Santos J, Sutovsky P. Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. Biol Reprod 2003;69:254–60.
[15] Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, et al. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol Reprod 2002;66:1033–41.
[16] Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, et al. Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured in vitro. Biol Reprod 1999;60:336–40.
[17] Suzuki K, Eriksson B, Shimizu H, Nagai T, Rodriguez-Martinez H. Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized in vitro. Int J Androl 2000;23:13–21.
[18] Fulka Jr J, Motlik J, Fulka J, Jilek F. Effect of cycloheximide on nuclear maturation of pig and mouse oocytes. J Reprod Fertil 1986;77:281–5.
[19] Motlik J, Kubelka M. Cell-cycle aspects of growth and maturation of mammalian oocytes. Mol Reprod Dev 1990;27:366–75.
[20] Verlhac MH, de Pennart H, Maro B, Cobb MH, Clarke HJ. MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. Dev Biol 1993;158:330–40.
[21] Lu Q, Smith GD, Chen DY, Yang Z, Han ZM, Schatten H, et al. Phosphorylation of mitogen-activated protein kinase is regulated by protein kinase C, cyclic 3′,5′-adenosine monophosphate, and protein phosphatase modulators during meiosis resumption in rat oocytes. Biol Reprod 2001;64:1444–50.
[22] Paweletz N, Wojcik C, Schroeter D, Finze EM. Are proteasomes involved in the formation of the kinetochore? Chromosome Res 1996;4:436–42.
[23] Zou H, McGarry TJ, Bernal T, Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 1999;285:418–22.
[24] Santella L, Kyozuka K, Hoving S, Munchbach M, Quadroni M, Dainese P, et al. Breakdown of cytoskeletal proteins during meiosis of starfish oocytes and proteolysis induced by calpain. Exp Cell Res 2000;259:117–26.
[25] Sun QY, Rubinstein S, Breitbart H. MAP kinase activity is downregulated by phorbol ester during mouse oocyte maturation and egg activation in vitro. Mol Reprod Dev 1999;52:310–8.
[26] Ding J, Clarke N, Nagai T, Moor RM. Protein and nuclear changes in pigs at fertilization. Mol Reprod Dev 1992;31:287–96.
[27] Miyano T, Dai Y, Lee J, Kano K, Moor RM. Degradation of pig cyclin B1 molecules precedes MAP kinase dephosphorylation during fertilisation of the oocytes. Zygote 2000;8:153–8.