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Autophagic activation in porcine oocytes is independent of meiotic progression

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Summary

In this study, we built on our previous research that discovered that autophagy activated the metaphase I stage during porcine oocytes *in vitro* maturation. We investigated the relationship between autophagy and oocyte maturation. First, we confirmed whether autophagy was activated differently by different media (TCM199 and NCSU-23) during maturation. Then, we investigated whether oocyte maturation affected autophagic activation. In addition, we examined whether the inhibition of autophagy affected the nuclear maturation rate of porcine oocytes. As for the main experiment, we measured LC3-II levels using western blotting after inhibition of nuclear maturation via cAMP treatment in an *in vitro* culture to clarify whether nuclear maturation affected autophagy. After autophagy inhibition, we also counted matured oocytes by treating them with wortmannin or a E64d and pepstatin A mixture. Both groups, which had different treatment times of cAMP, showed the same levels of LC3-II, while the maturation rates were about four times higher after cAMP 22 h treatment than that of the 42 h treatment group. This indicated that neither cAMP nor nuclear status affected autophagy. Autophagy inhibition during *in vitro* oocyte maturation with wortmannin treatment reduced oocyte maturation rates by about half, while autophagy inhibition by the E64d and pepstatin A mixture treatment did not significantly affect the oocyte maturation. Therefore, wortmannin itself, or the autophagy induction step, but not the degradation step, is involved in the oocyte maturation of porcine oocytes. Overall, we propose that oocyte maturation does not stand upstream of autophagy activation, but autophagy may exist upstream of oocyte maturation.

Introduction

Autophagy is the evolutionarily well conserved cellular recycling process in which intracellular materials such as expired proteins and organelles are taken up by autophagosomes and materials in autophagosomes are degraded by several lysosomal enzymes. It is known that the autophagy process is activated to sustain cellular metabolism as they prevent the accumulation of expired cellular components and reuse their ingredients after degradation (Mizushima and Komatsu, 2011; Parzych and Klionsky, 2014). Autophagy has various sequential steps (induction, elongation, completion, maturation, and degradation) that are regulated by autophagy-related genes (ATG) (Reggiori and Ungermann, 2017). Autophagy is often activated during dramatic cellular changes, such as embryo development or insect metamorphosis, via increased metabolic stress (Tracy and Baehrecke, 2013; Wu *et al.*, 2013; Reggiori and Ungermann, 2017). Various studies on autophagy during embryo development have been conducted, however not many studies focusing on autophagy during oocyte maturation have been developed, even though oocyte maturation processes consist of significant cellular changes, including nuclear maturation via meiosis and cytoplasmic maturation.

In our previous studies, we reported that autophagy was activated in porcine *in vitro* oocyte maturation (IVM) and was affected by its inhibitor during IVM (Lee *et al.*, 2014b). In this research, we focused on the relationship between porcine oocyte nuclear maturation and autophagy by exploring autophagy activation related to oocyte maturation to examine further our previous results that indicated that autophagy is activated before oocyte nuclear maturation. We did not consider cytoplasmic maturation, as many factors must be tested when investigating the relationship between autophagy and cytoplasmic maturation. Therefore, oocyte maturation in this study indicates only nuclear maturation. We hypothesized that autophagy or oocyte maturation affected one another. We may overcome the limitations of the present porcine *in vitro* maturation method, in which only good quality oocytes undergo maturation, as we understood the effect of autophagy on oocyte maturation. We designed two main experiments regarding autophagy inhibition and nuclear maturation inhibition.

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The autophagic activity was determined through microtubule-associated protein light chain 3 (MAP1 LC3/LC3)-II, a reliable marker for autophagosome membranes. In mammalian animals, the C-terminus of the LC3 protein is cleaved by mammalian Atg4 homologues to form LC3-I (Mizushima and Yoshimori, 2007). The cytosolic LC3-I protein conjugates with phosphatidylethanolamine (PE) via a ubiquitin-like enzymatic reaction to form LC3-II, which subsequently becomes associated with the autophagosome membrane (Kabeya *et al.*, 2000). Therefore, the amount of LC3-II represents the amount of autophagosomes. Therefore, we used LC3-II to quantify autophagosomes, which could be regarded as autophagic activity. This study will contribute to accumulating basic knowledge about the relationship between autophagy and porcine oocyte maturation.

Materials and methods

Samples collection and preparation

Porcine ovaries were recovered from sows at a nearby slaughterhouse and moved to the laboratory in a container within 2 h. Porcine follicular fluid (pFF) containing porcine oocytes was aspirated from antral follicles of diameter 3–6 mm with a 10-ml syringe combined with an 18-gauge needle. To eliminate the effects of medium on autophagy, we examined autophagy activation patterns during the maturation period using different *in vitro* maturation media, TCM199 and NCSU23 media. Both media are used worldwide in porcine *in vitro* maturation (Im *et al.*, 2004; Yang *et al.*, 2020).

In vitro *culture for maturation of porcine oocytes*

Aspirated cell debris and oocytes were briefly washed in phosphate-buffered saline (PBS; Nissui Pharmaceutical, Ueno, Tokyo, Japan) with added 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St Louis, MO, USA), and only good quality cumulus–oocyte complexes (COCs), which had three-layer cumulus cells and a definitive ooplasm, were chosen. After washing with 0.1% PVA in PBS three times, the COCs were incubated in TCM199 medium (Sigma) for 42 h to mature with the conditions of 38.5°C temperature and a 5% CO_2 atmosphere without medium changes (Petters and Wells, 1993). TCM199 medium was supplemented with 0.2% FSH (Sigma), 0.1% EGF (Sigma), and 10% (v/v) pFF. Conversely, the COCs incubated in NCSU-23 medium had the medium changed. NCSU-23 medium was prepared by the addition of 0.5% insulin (Sigma), 50 μM of β-mercaptoethanol (Sigma), 0.6 mM cysteine (Sigma), 10% (v/v) pFF, 10 IU human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan), 10 IU pregnant mare serum gonadotropin (PMSG; serotropin; Teikokuzouki, Tokyo, Japan), and 1 mM dibutyryl cyclic AMP (dbcAMP) (Sigma) for the first culture of 22 h. A change of the NCSU-23 medium at 22 h in COCs culture was prepared with the addition of the same components except for hormones and dbcAMP supplementation. COCs in NCSU-23 medium were cultured for 42 h to maturation with conditions of 38.5°C temperature and a 5% CO_2 atmosphere. At 42 h of culturing, COCs were stripped as cumulus cells were detached from oocytes by gentle vortexing in PB1 medium containing 0.1% hyaluronidase (Sigma) (Quinn *et al.*, 1982).

Extended dbcAMP treatment in NCSU-23 medium

In the main experiment of oocyte maturation inhibition, we inhibited oocyte maturation by dbcAMP, a well known reagent that mimics endogenous cAMP and inhibits nuclear progression during the meiotic process in porcine oocytes (Somfai *et al.*, 2003; Somfai and Hirao, 2017). COCs in the control group were cultured in NCSU-23 medium using the same method as described above. In addition, COCs in the extended dbcAMP treatment group were cultured following the same process as the control group for the first 22 h, after which time the COCs were cultured for another 20 h in NCSU-23 medium with dbcAMP. After culturing, COCs were stripped as cumulus cells were detached from oocytes by gentle vortexing in PB1 medium containing 0.1% hyaluronidase (Sigma). Then, the oocytes were used for western blotting experiments or orcein staining.

Western blotting analysis for quantification of LC3-II

LC3-II protein levels were observed, as this factor is the most reliable autophagy marker (Tanida *et al.*, 2005). Protein preparation from oocytes was performed at each sampling time. At each sampling time, oocytes were stripped by treatment with 0.1% hyaluronidase (Sigma) in PB1 medium, and placed in a 1× sodium dodecyl sulfate (SDS) lysis buffer with 10% 2-mercaptoethanol, 0.5 M Tris−HCl (pH 6.8), and 20% glycerol. Oocyte lysates were cryopreserved in a freezer at −80°C. Extraction was repeated until 99 oocyte lysates were prepared for each group. After the denaturation step by heat, the proteins in lysates were separated by protein size using 12% SDS-PAGE and subsequently transferred to Immobilon transfer membrane (Millipore, Billerica, MA, USA). After the transfer step to a membrane, a blocking step on the protein transferred membrane was carried out using 5% skimmed milk for 1 h at room temperature (RT), and subsequently washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Rabbit anti-LC3 monoclonal antibody (dilution 1:1000; Cell Signaling, Danvers, MA, USA) was added to the membrane overnight at 4°C as the first antibody. Horseradish peroxidase-labelled anti-rabbit IgG (dilution 1:10,000; Invitrogen, Carlsbad, CA, USA) was treated as the second antibody. After three TBS-T washes, peroxidase activity was evaluated by visualizing using the ECL Plus western blotting detection system (GE Healthcare, Piscataway, NJ, USA). Visualized signalling intensity was analyzed using an Image Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Inhibition of autophagy

Two kinds of inhibitors were used for autophagy inhibition: wortmannin and a mixture of E64d and pepstatin A. Wortmannin has been used frequently as an autophagy inhibitor, as it impedes the induction of autophagosomes. Autophagic inhibition by wortmannin is induced by the inhibitory effect of class III phosphatidylinositol 3-kinase (PI3K), which is known to be an affirmative regulator of autophagic induction (Blommaart *et al.*, 1997; Backer, 2008). Conversely, E64d and pepstatin A have been reported to be inhibitors of lysosomal proteases, that is cathepsins in the autophagic degradation step. In the autophagic degradation stage, autolysosomes fused with autophagosomes and lysosomes were degraded by lysosomal proteases, cathepsins (Dunn Jr, 1990). E64d and pepstatin A are inhibitors of some cathepsins among all kinds of cathepsins (Chen M *et al.*, 2018; Oliveira *et al.*, 2015). In the autophagic degradation stage, the LC3-II protein is also degraded by these cathepsins (Kaminskyy and Zhivotovsky, 2012). We aimed to inhibit both the beginning and end of the autophagic process. A 10 mM stock solution of wortmannin was dissolved in dimethyl sulfoxide (DMSO) to inhibit autophagic induction. A stock solution of wortmannin was diluted in the culture medium to a final

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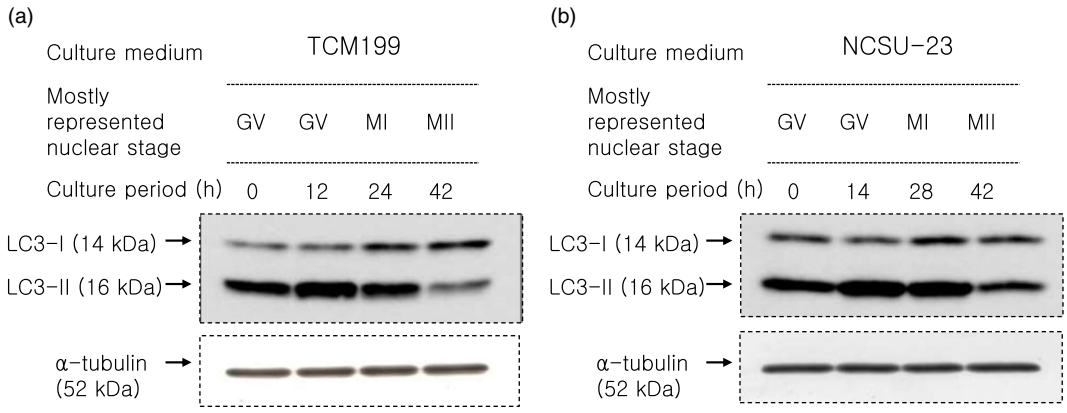

Figure 1. LC3-II levels of oocytes change over time in cultures based on TCM199 or NCSU-23 media. LC3-II levels of oocytes were determined by western blotting in each culture of TCM199 (a) and NCSU-23 (b). The lysate from each group of oocytes was processed and cryopreserved at each sampling times in both cultures. The lysate from, in total, 99 oocytes were loaded in each lane.

concentration for each experiment. To set the same conditions with all examined groups, culture groups were equally treated with 0.5% DMSO. To inhibit autophagic degradation, E64d and pepstatin A were supplemented in the culture medium. A 5 mM E64d stock solution and a 2.6 mM pepstatin A stock solution was dissolved in DMSO and 10% (v/v) acetic acid in DMSO, were dissolved in culture medium to give a final concentration of 29.8 μM E64d and 15.6 μM pepstatin A. In the experimental groups, 0.6% DMSO, including 9.1% (v/v) acetic acid, was applied to the culture medium. To inhibit autophagic initiation and degradation simultaneously, wortmannin and E64d/pepstatin A were added to the NCSU23 culture medium at final concentrations of 50 μM, 29.8 μM, and 15.6 μM, respectively.

Orcein staining of porcine oocytes and observation of metaphase II rate

Denuded oocytes were washed in 0.1% PBS–PVA. Subsequently, the oocytes were fixed on glass slides with ethanol and acetic acid (3:1) for 48 h. The oocytes were then stained with a solution containing 1% orcein (Merck) in 50% acetic acid for 2 h. Subsequently, the oocytes were decolorized with a solution including 20% glycerol and acetic acid. The slides were sealed and detected using a phase contrast microscope. Oocytes representing the germinal vesicle (GV) stage, from germinal vesicle breakdown (GVBD) to metaphase I, from anaphase I to telophase I, and the metaphase II stage were counted and sorted by direct observation under a phase contrast microscope.

Statistical analysis

A comparison of the distribution of oocyte stages between the two treatment groups was performed using Fisher's exact test. Results were regarded as significantly different if P-values were < 0.05.

Results

Similar autophagy dynamics during in vitro maturation in different medium

To clarify whether different media affected autophagy activation patterns, we examined the time course of LC3-II during *in vitro* maturation in TCM199 and NCSU-23 media. Although TCM199 and NCSU-23 media consisted of different base media and different additional ingredients, such as hormones, LC3-II showed the same intensity pattern that increased to a maximum at the GV stage of 12–14 h and decreased to a minimum at 42 h (Figure 1).

Effect of nuclear maturation on autophagy

We examined whether autophagy was affected by nuclear maturation. dbcAMP was used to block GV breakdown. Furthermore, the LC3-II level was not affected by prolonged treatment of dbcAMP until 42 h (Figure 2a). However, the oocyte nuclear maturation rate was reduced significantly when subjected to prolonged treatment by dbcAMP (Figure 2b). Oocyte stages were significantly different ($\chi^2 = 28.084$, $P < 0.001$) between the two treatment groups. Comparing the two treatment groups, the rate of MII stage oocytes was relatively higher when oocytes were treated with 1 mM (IVM-1) + 0 mM (IVM-2) (67.4%) in group I and the rate of GV stage oocytes was higher when oocytes were treated with 1 mM (IVM-1) + 1 mM (IVM-2) (76.7%) in group II (Table 1). This result indicated that autophagy was not affected by dbcAMP treatment and nuclear maturation.

Autophagic inhibition decreased nuclear maturation

To determine whether autophagy participated in oocyte maturation, the MII rate of porcine oocytes was measured after treatment with an inhibitor of autophagosome induction and degradation. The maturation rate in the autophagosome-induction inhibition group using 50 μM of wortmannin was compared to the control group. Oocyte stages were significantly different ($\chi^2 = 30.154$, $P < 0.001$) between the two treatment groups. The rate of MII stage oocytes was lower when oocytes were treated with wortmannin (43.6%) in comparison with the untreated group (78.5%) (Figure 3a; Table 2). In contrast, the maturation rate of the oocytes in the autophagosome-degradation inhibition group using a mixture of 30 μM E64d and 16 μM pepstatin A was 80.2%, similar to that of the control group (82.7%). Oocyte stages were not significantly different ($\chi^2 = 1.888$, $P = 0.676$) between the two treatment groups (Figure 3b; Table 3). In the combined inhibitor experiment of 40 μM of wortmannin and a mixture of 30 μM E64d and 16 μM pepstatin A, oocyte stages were significantly different ($\chi^2 = 8.680$, $P = 0.029$) between the two treatment groups. Comparing the two treatment groups, the rate of MII stage oocytes was lower when oocytes were treated with wortmannin and E64d/pepstatin A (39.6%) in comparison with the untreated (67.9%) (Figure 3c; Table 4). These results showed that two inhibition groups, wortmannin and wortmannin combined with E64d/pepstatin A, reduced the oocyte maturation rate.

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Table 1. The number of oocytes at different stages after 22 h or prolonged 42 h treatment of dbcAMP

dbcAMP concentration	No. (%) of oocytes at GV	No. (%) of oocytes at GVBD–MI	No. (%) of oocytes at Ana–Tel	No. (%) of oocytes at MII stage	Total (%)	χ^2 (*P*)
1 mM (IVM-1) +0 mM (IVM-2)	9 (20.9)	4 (9.3)	1 (2.3)	29 (67.4)	43 (100)	28.084** (<0.001)
1 mM (IVM-1) +1 mM (IVM-2)	33 (76.7)	2 (4.7)	0 (0.0)	8 (18.6)	43 (100)	

*$P < 0.05$, **$P < 0.01$.

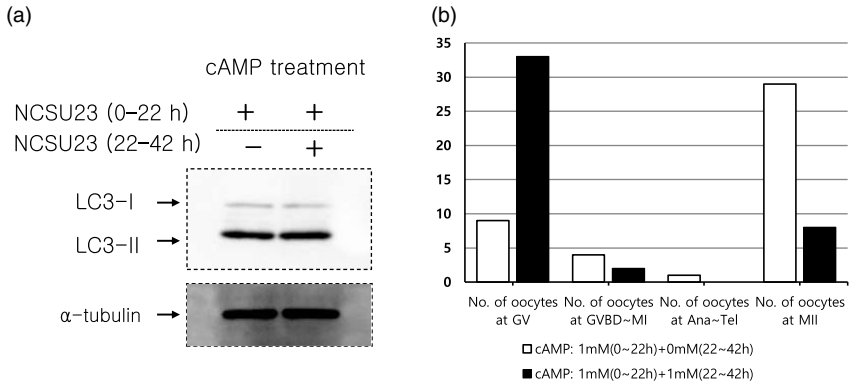

Figure 2. LC3-II level comparison and oocyte status in a 42 h culture against cAMP treatment time. LC3-II quantitation result of oocytes between cAMP treatments of 22 h and 42 h in each culture (a). The lysate from each group of oocytes was processed and cryopreserved after 42 h. The number of oocytes at different stages at 42 h culture between cAMP treatment times of 22 h and 42 h in each culture of total 43 oocytes (b).

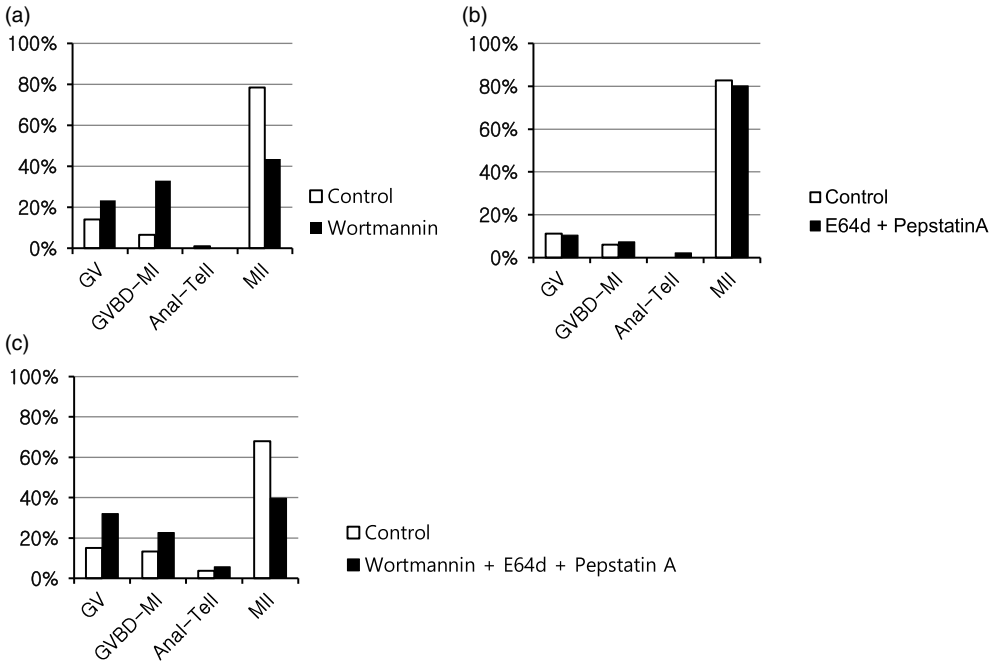

Figure 3. Oocyte status at 42 h in culture with added autophagy inhibitor reagents. Oocyte status at 42 h culture with added wortmannin (a), E64d and pepstatin A mixture (b), Wortmannin and E64d/pepstatin A mixture (c). Each experiment was compared with a control oocyte condition without added reagents.

Discussion

Autophagy is a well conserved response in the cellular degradation and recycling system. One of the major roles of autophagy is as a decider of a cell's survival or death (Wu *et al.*, 2018). Usually, stresses such as cellular signals, nutrient shortage, and oxidative stress induce autophagy (Lee *et al.*, 2014a; Muriach *et al.*, 2014). Although it is still controversial whether autophagy induction is involved in maintaining cellular homeostasis followed by cell survival or apoptosis, in our previous study, we previously verified that autophagy is activated during oocyte maturation before polar body extrusion, which is the most important indicator of nuclear maturation in oocytes (Lee *et al.*, 2014b). Therefore, we investigated whether autophagy activation before polar body extrusion was involved in oocyte maturation and whether the nuclear maturation of oocytes affected autophagy.

Our results of nuclear maturation inhibition revealed that neither dbcAMP nor nuclear maturation affected autophagy. Our results of autophagic inhibition seem to indicate that the target pathway line of wortmannin in autophagic induction was involved in porcine oocyte nuclear maturation. In the group of combined inhibitors, porcine oocyte nuclear maturation was affected by

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Table 2. The number of oocytes at different stages with or without wortmannin treatment at 42 h

Group	No. (%) of oocytes at GV	No. (%) of oocytes at GVBD–MI	No. (%) of oocytes at Ana–Tel	No. (%) of oocytes at MII stage	Total (%)	χ^2 (*P*)
0	13 (14.0)	6 (6.5)	1 (1.1)	73 (78.5)	93 (100)	30.154** (<0.001)
Wortmannin	22 (23.4)	31 (33.0)	0 (0.0)	41 (43.6)	94 (100)	

*$P < 0.05$, **$P < 0.01$.

Table 3. The number of oocytes at different stages with or without E64d and pepstatin A mixture treatment at 42 h

Group	No. (%) of oocytes at GV	No. (%) of oocytes at GVBD–MI	No. (%) of oocytes at Ana–Tel	No. (%) of oocytes at MII stage	Total (%)	χ^2 (*P*)
0	11 (11.2)	6 (6.1)	0 (0.0)	81 (82.7)	98 (100)	1.888 (0.676)
E64d + Pepstatin A	10 (10.4)	7 (7.3)	2 (2.1)	77 (80.2)	96 (100)	

*$P < 0.05$, **$P < 0.01$.

Table 4. The number of oocytes at different stages with or without wortmannin, E64d and pepstatin A mixture treatment at 42 h

Group	No. (%) of oocytes at GV	No. (%) of oocytes at GVBD–MI	No. (%) of oocytes at Ana–Tel	No. (%) of oocytes at MII stage	Total (%)	χ^2 (*P*)
0	8 (15.1)	7 (13.2)	2 (3.8)	36 (67.9)	53 (100)	8.680* (0.029)
Wortmannin + E64d + Pepstatin A	17 (32.1)	12 (22.6)	3 (5.7)	21 (39.6)	53 (100)	

*$P < 0.05$, **$P < 0.01$.

wortmannin, but not by the E64d and pepstatin A mixture, which also had no effect on the nuclear maturation rate in the separate treatment. In this study, we only showed the nuclear maturation rate of porcine oocytes after treatment with autophagic inhibitors, because we have already shown that wortmannin acts as an autophagy inhibitor in porcine oocytes by LC3-II protein quantification (Lee *et al.*, 2014b). Regarding the inhibition of autophagic induction, it is unclear whether it directly affects porcine oocyte nuclear maturation. Wortmannin is a powerful regulator of autophagy (Backer, 2008) as it inhibits PI3K class III activity, which is known to be essential for autophagic induction (Blommaart *et al.*, 1997; Wu *et al.*, 2010). However, wortmannin also temporarily inhibits PI3K class I, which has been reported to be involved in porcine oocyte maturation (Song *et al.*, 2018). Therefore, it is possible that the reduction in nuclear maturation rate observed in this study was due to the inhibition of PI3K class I, not autophagy induction. This question will be resolved in later studies.

Regarding the inhibition of autophagic degradation, our results showed similar nuclear maturation rates in the inhibition and control groups of autophagic degradation. The E64d/pepstatin A mixture was used to inhibit autophagic degradation by inhibiting the cathepsin enzymes in lysosomes, which are related to the degradation of autolysosome components. Among the many types of cathepsins in lysosomes, E64d primarily inhibits cathepsins B and L, and pepstatin A mainly inhibits cathepsins D and E (Hook *et al.*, 2014; Cocchiaro *et al.*, 2016). Therefore, cathepsins are generally involved in the cellular recycling of the autophagy system. In our previous study, we found that, although not all cathepsins were inhibited, there was a higher amount of accumulated autophagosomes and autolysosomes than that of the control group (Lee *et al.*, 2014b). Despite the potential of the E64d/pepstatin A mixture, our results showed that the nuclear maturation rate was almost the same as that of the control group. This indicated that autophagic degradation rarely affected nuclear maturation in porcine oocytes. However, it should be considered that autophagic degradation may be involved in cytoplasmic maturation, which can affect early embryo development after fertilization. Autophagy is activated by cellular stress such as signalling stimulation, nutrient starvation (Mortimore and Pösö, 1987), amino-acid deprivation (Mortimore and Schworer, 1977), and oxidative stress (Han *et al.*, 2012) and may have a role in cellular recycling. In this autophagic cellular recycling, autophagic degradation is the important process that supplies raw materials such as amino acids for early embryo development. The next study should aim to determine whether autophagic degradation affects oocyte cytoplasmic competence and the subsequent embryo development rate.

This study clearly showed that porcine nuclear maturation did not affect autophagic activation. Also, we suggested the possibility that autophagy contributed positively to the porcine oocyte maturation. However, further studies are needed to clarify the exact role of autophagy on porcine oocyte maturation.

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