Ph.D. Thesis

Verification of Autophagy in Porcine Oocyte and Study of Its Role during Maturation

(ブタ卵成熟過程におけるオートファジーの検証とその役割)

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Chapter I

Introduction

1.1. In vitro maturation system in porcine oocyte

Ever since in vitro maturation systems have been established for oocytes, embryonic research using cloning and transgenic technique has been accelerated because it has been possible to obtain larger numbers of mature oocytes. In particular, compared to the in vivo collection of mature oocytes from large livestock animals such as cattle and pigs, in vitro oocyte maturation systems have reduced the cost and time-consuming lab works for researchers in acquiring mature oocytes. However, it is a problem that only few cultured oocytes successfully form embryos after fertilization. In vitro matured oocytes normally display worse developmental competence than those matured in vivo [1, 2]. One of the reasons for this low developmental potential is poor oocyte quality in vitro [3]. Among the factors that could affect oocyte quality, environmentals factor such as nutrients, amino acids, oxidation and pattern of hormonal stimulation are major factors that may cause in vivo and in vitro differences. Therefore, we turned our focus on controlling of environmental factors such as nutrients, amino acids, oxidation and hormones in order to acquire better quality oocytes.

Oocyte maturation is the process whereby oocytes arrested at the diplotene stage proceed to complete division. They subsequently reach the MII stage, at which the first polarbody is extruded [4]. In order to resume and complete successful maturation, oocytes receive various nutrients and hormonal stimulation

from blood. Oocytes finally break their own homeostasis and reach cellular renovation by large stimulation like LH surge, that is the break-down of diplotene stage which is called the resumption of meiotic progression [5, 6].

Recent studies of oocytes are trying to dissolve the mechanism of the resumption of meiotic progression by investigating previously mentioned environmental factors, which affect resumption of oocyte maturation and oocyte quality. However, it is practically impossible to determine every single factor and control all of them simultaneously to get more competent oocytes. Therefore, we considered notion of autophagy as a mediator for various stimulating factors because autophagy is basically a cellular response to stimulating stress and has a dynamic recycling system which degrades intracellular proteins and organelles and produces new building materials and energy for cellular renovation and homeostasis [7]. In addition, it has been reported that autophagy has a relationship with an environmental factors such as nutrients, amino acids, oxidation, and hormones which are major factors that may cause in vivo and in vitro differences [8-11].

1.2. Autophagy

Autophagy is basically a cellular response to stress such as nutrient starvation [12], amino-acid deprivation [13], and oxidative stress [14]. Autophagy is

an evolutionarily conserved process in which intracellular proteins and organelles are sequestered in autophagosomes and subsequently degraded by lysosomal enzymes in order to recycle cellular components to sustain metabolism and to prevent the accumulation of damaged proteins and organelles [15, 16]. Autophagy has a dynamic process, consisting of several sequential stages (initiation, elongation, completion, maturation, and degradation) controlled by a group of autophagy-related genes (Atg genes) [17].

So far, it has been discovered that autophagy is essential for drastic cellular renovation such as embryogenesis and insect metamorphosis [18, 19]. However, with regard to mammalian oocytes during maturation, although maturation is a drastic renovation to the oocyte itself, only a few studies on autophagy in cumulus cells have been performed and none have been conducted on oocytes [20-22]. In this study, we attempted to verify autophagy in porcine oocyte.

Microtubule-associated protein light chain 3 (LC3)-II is a promising marker of autophagosomes. In mammals, the C-terminus of the LC3 protein is cleaved by mammalian Atg4 homologues to form LC3-I [23]. The cytosolic LC3-I protein conjugates with phosphatidylethanolamine(PE) via an ubiquitin-like enzymatic reaction to become LC3-II, which subsequently becomes associated with the autophagosomal membrane [24]. Although the molecular weight of LC3-II is larger

than that of LC3-I due to the addition of PE, LC3-II migrates faster than LC3-I in SDS-PAGE probably because of its extreme hydrophobicity [25]. It has been known that the measurement of LC3-II by immunoblotting is a simple and quantitative method for evaluating the autophagic activity of mammalian cells rather than any other methods [26]. In this study, we used LC3 observation for detecting autophagosome and evaluating autophagic activity.

1.3. Connexin43 (Cx43)

Referring to LC3 localization data, we hypothesized that autophagy is related to something located near the plasma membrane. Thus, we turned our attention to the relationship between autophagy and gap junctions. Mammalian follicular oocytes are surrounded by cumulus cells and they adhere to cumulus cells physically by gap junctions. Molecular exchanges and signal transportation between oocytes and cumulus cells are achieved through gap junction channels [27]. Gap junction channels are assembled from a ubiquitously expressed class of four-pass trans-membrane proteins termed connexins, with connexin-43 (Cx43) being the most abundantly expressed connexin [28]. In autophagy research on somatic cells, it has been revealed that internalized gap junctions, including Cx43, are degraded by autophagy [29-31]. Therefore, in the present study, we attempted to determine if internalized Cx43 is also degraded by autophagy in porcine

oocytes.

1.4. Objective

In this study, we attempted to verify autophagy in porcine oocyte and examine its function during maturation, in order to evaluate its potentiality as a regulator of oocyte mautraiton and to prevent unintentional mistake which we drive oocytes to cell death due to ignorance for autophagy. Examination below was conducted.

1. Localization of autophagosome and evaluation of autophagic activity in porcine oocyte during IVM.
2. Examining the reason for autophagy activation (autophagic pathway) and its possible fuction in porcine oocyte (possible target for autophagic degradation).
3. Effect of autophagy for oocyte maturation.

Chapter II

Verification of autophagy in porcine oocyte maturation

Introduction

In pig embryo research, autophagy during in vitro development has been reported [32]. However, autophagy prior to fertilization has not been studied in porcine oocyte in vitro maturation systems. Indeed, there are no data even indicating the presence of autophagosomes during this stage in vitro.

Therefore, in this chapter, we investigated autophagosomes in porcine oocytes during in vitro maturation. We used western blotting and immunostaining of LC3-II, a marker of autophagosomes, to identify autophagosomes. We also performed a quantitative analysis of LC3-II protein in a time course of in vitro maturation.

Microtubule-associated protein light chain 3 (LC3)-II is a promising marker of autophagosomes. In mammals, the C-terminus of the LC3 protein is cleaved by mammalian Atg4 homologues to form LC3-I [23]. The cytosolic LC3-I protein conjugates with phosphatidylethanolamine(PE) via an ubiquitin-like enzymatic reaction to become LC3-II, which subsequently becomes associated with the autophagosomal membrane [24]. Accordingly, the amount of LC3-II corresponds to the amount of autophagosomes.

Two kinds of inhibitors were treated in vitro cultures for confirming the occurrence of autophagic induction and degradation during porcine oocyte

maturation.

To investigate autophagic induction, we treated in vitro cultures with wortmannin. Wortmannin, which plays a role in blocking the induction of autophagosomes, has been widely used as an autophagy inhibitor based on its inhibitory effect on class III phosphatidylinositol 3-kinase (PI3K) activity [33, 34] which is known to be a positive regulator of autophagic induction [35]. We cultured porcine oocytes with wortmannin for 14 h, after which LC3-II protein was evaluated to examine autophagic induction.

We also treated oocytes with E64d and pepstatinA, which are inhibitors of lysosomal proteases. In the autophagic degradation process, autolysosomes are formed by the fusion of autophagosomes and lysosomes immediately after the completion of autophagosome [36]. The autolysosome is subsequently degraded by lysosomal proteases. The lysosomal protease inhibitors E64d and PepstatinA hamper autophagic degradation by inhibiting lysosomal proteases. In autophagic degradation, the LC3-II protein is also degraded by lysosomal proteases during autophagic degradation [37]. To investigate autophagic degradation during in vitro culture of porcine oocytes, we cultured these with E64d and Pepstatin A for 42 h, after which we evaluated LC3-II protein levels.

Collectively, we provide evidence for autophagy during the in vitro maturation of porcine oocytes by documenting the presence, start, and end of

autophagosomes by using the autophagic marker LC3-II and autophagic inhibitors.

2.1. LC3 detection by immunohistochemistry in the porcine ovary

Materials and methods

Immunohistochemistry of LC3 in porcine ovary

Porcine ovaries were fixed with 10% formaldehyde neutral buffer solution (Nacalai Tesque, Kyoto, Japan), embedded in paraffin wax and sectioned. Cross-sectioned samples (5 μM) of ovary tissue were placed on glass slides and dried at room temperature. The samples were then deparaffinized and incubated with HistoVT One (Nacalai Tesque) at 90°C for 30 min. After washing, Samples were treated with Blocking One (Nacalai Tesque) at 4°C for 1 h. Slide samples were incubated with rabbit anti-LC3 monoclonal antibody (dilution 1:200; Cell signaling, Danvers, MA) overnight at 4°C and then incubated with Alexa Fluor 488-labeled anti-rabbit secondary antibody (dilution 1:500; Invitrogen, Carlsbad, CA) and 20 μg/ml propidium iodide (PI) (Sigma Chemical, St Louis, MO) at 4°C for 2 h. After washing with PBS, the samples were viewed using a LSM700 confocal scanning laser microscope (Zeiss, Feldbach, Switzerland).

Results

Immunohistochemistry was used to examine porcine ovary tissue by using LC3 monoclonal antibody to observe various sizes of follicles. LC3 signal was detected in near the plasma membrane and in cumulus cells as well (Fig. 1). In the magnified picture, ring- and dot- shaped LC3 signals were observed and most of them was shown with dot- shaped LC3. Arrows indicate ring- and dot- shaped LC3 signals (Fig. 1D). Green signal and red signal represent LC3 and nucleus, respectively. In autophagy study, each of dot-, cup- and ring- shaped signal was dominantly detected depending on a kind of cells or their circumstances. We obtained picture with strong gain setting of our confocal microscope because signal intensity was different between cumulus cells and oocyte. We had no choice but we detect samples under relatively strong gain setting in order to detect signal not only in oocyte but also cumulus cell. Although it is difficult to distinguish ring- shaped LC3 because of high gain rate, dot- shape of LC3 can be clearly distinguished in the magnified picture (Fig. 1D).

2.2. LC3 dectection by immunocytochemistry in porcine oocytes during in vitro maturation

Materials and methods

In vitro maturation of oocytes

Porcine oocytes were collected from gilts at a local slaughterhouse and transported to the laboratory in a container within 2 h after extraction. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3-6 mm) with a 10 ml syringe attached to an 18 gauge needle. Compact cumulus-oocyte complexes (COCs) with uniform ooplasm ere selected in phosphate buffered saline (PBS; Nissui Pharmaceutical, Ueno, Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St Louis, MO). After washing 3 times in 0.1% PBS-PVA, the COCs were cultured in NCSU-23 medium [38] supplemented with 50 μM β-mercaptoethanol (Sigma), 0.6 mM cysteine (Sigma), 0.5% insulin (Sigma), 10% (v/v) porcine follicular fluid, 10 IU pregnant mare serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan) and 1 mM dibutyryl cyclic AMP (Sigma) for the first 22 h of maturation at 38.5°C in 5% CO2 in air. The COCs were cultured for a further 20 h in the same medium without

hormonal and dibutyryl cyclic AMP supplementation. After cell culture, expanded COC cumulus cells were removed by gentle vortexing in PB1 medium [39] containing 0.1% hyaluronidase (Sigma). Oocytes were subsequently processed according to protocols for western blotting and immunostaining.

Immunocytochemistry of oocytes

Immunostaining of oocytes was performed as previously described with slight modifications [40]. COCs were denuded after 0 h, 14 h, 28 h, and 42 h in culture. After they were washed 3 times in PBS containing 0.1% PVA (PBS-PVA), denuded oocytes (DOs) were fixed with 4% paraformaldehyde (Sigma) in Dulbecco's PBS(-) containing 0.1% PVA at 4°C for 90 min. Then, DOs were placed in 0.5% Triton X-100 in PBS(-) containing 3% BSA(Sigma) at room temperature for 20 min, washed 3 times in PBS-PVA for 15 min each, and stored in PBS-PVA containing 1% BSA (Sigma) (PBS-PVA-BSA) at 4°C overnight or longer. Next, DOs were blocked with 10% Fetal Bovine Serum (FBS; Gemini bio-products, Calabras, CA) in PBS-PVA-BSA at room temperature for 45 min. Oocytes were incubated overnight at 4°C with rabbit anti-LC3 monoclonal antibody (Cell Signaling), which is the same antibody used in this study for western blotting, at a dilution of 1:200 in 10% FBS in PBS-PVA-BSA. In the control group, oocytes were incubated overnight at 4°C without antibodies. After 3

washes with PBS-PVA-BSA, oocytes were incubated with Alexa Fluor 488-labelled goat anti-rabbit antibody (Molecular Probes, Eugene, OR) at a dilution of 1:50 in PBS-PVA-BSA for 40 min at room temperature. After 3 washes with 0.1% Triton X-100 in PBS-PVA-BSA for 15 min each, the nuclei were labelled with 20 μg/ml propidium iodide (PI) (Sigma) for 60 min at room temperature. After washes with PBS-PVA-BSA, the oocytes were mounted on glass slides. Alexa Fluor 488 and PI generate green and red fluorescent signals, respectively. The samples were viewed using LSM700 confocal scanning laser microscope (Zeiss, Feldbach, Switzerland).

Results

I determined the immunolocalization of LC3 in porcine oocytes 0, 14, 28, and 42 h after culture. The fluorescence signal immunoreactived to LC3 protein is detected in every group although 42h group showed weak signaling intensity (Fig. 2A-D). Moreover, the dot- and ring-shape signals are detected on magnified picture (Fig. 2E).

2.3. Evaluation of autophagic activity over time in culture

Materials and methods

Western blot analysis of LC3-II

Protein extraction from oocytes was performed at 0, 14, 28, and 42 h. At each time point, 33 oocytes denuded by treatment of 0.1% hyaluronidase (Sigma) in PB1 medium were placed in 1× sodium dodecyl sulfate (SDS) sample buffer, 0.5 M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, and 20% glycerol. Lysates were cryopreserved at -80°C. The extraction was repeated 3 times, after which 99 oocyte lysates for electrophoresis were prepared by combining 3 samples of 33 oocytes. After heat denaturation, lysates were separated by 12% SDS-PAGE and transferred to Immobilon membranes (Millipore, Billerica, MA). After transfer, the membranes were blocked with 5% skimmed milk for 1 h at room temperature and washed several times with Tris-buffered saline containing 0.1% Tween20. The membrane was incubated with rabbit anti-LC3 monoclonal antibody (dilution 1:1000; Cell signaling, Danvers, MA) overnight at 4°C and then incubated with horseradish peroxidase-labeled anti-rabbit IgG (dilution 1:10000; Invitrogen, Carlsbad, CA). After several washes with TBS-T, the peroxidase activity was visualized using the ECL Plus western blotting detection system (GE Healthcare,

Piscataway, NJ). Signals were detected with an Image Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Results

I determined LC3-II protein levels in porcine oocytes in a time course of 0, 14, 28, and 42 h of culture in NCSU-23 medium. During the time course, LC3-II levels increased to maximum at 14 h and dropped to a minimum at 42 h (Fig. 3A). Using the LAS system (Fujifilm), average band density from 3 bands per group was determined to be 25.98%, 35.80%, 26.02%, and 12.21% at 0 h, 14 h, 28 h, and 42 h, respectively. We calculated the relative band density based on the numerical value of the density at 0 h (Fig. 3B). LC3-II densities were significantly different between 0 h and 14 h, 14 h and 28 h, and 28 h and 42 h (Fig. 3B).

2.4. Confirmation of autophagic new induction and degradation during oocyte maturation

Materials and methods

Inhibition of autophagosome induction

To inhibit autophagosome induction, wortmannin was added to the culture medium. A 10 mM stock solution of wortmannin in dimethyl sulfoxide (DMSO) was diluted in the culture medium to a final concentration of 50 μM. DMSO was added to the control culture, and all examined culture droplets contained 0.5% DMSO. After 14 h in culture, 99 oocytes from each control and treatment group were lysed for western blotting.

Inhibition of lysosomal degradation

To inhibit autophagosome degradation with lysosomal enzymes, E64d and papstatin A were added to the culture medium. A 5 mM E64d stock solution and a 2.6mM Pepstatin A stock solution were prepared in DMSO and 10% (v/v) acetic acid in DMSO, respectively. Then, they were diluted in the culture medium to final concentrations of 29.8 μM for E64d and 15.6 μM for Pepstatin A. DMSO containing 9.1% (v/v) acetic acid was added to control cultures, and all examined

culture droplets contained 0.6% DMSO containing 9.1% (v/v) acetic acid. After 42 h in culture, 99 oocytes from each control and treatment group were lysed for western blotting.

Results

To verify the occurrence of autophagic induction during in vitro maturation of porcine oocytes, we determined the LC3-II levels in the oocytes by western blotting 14 h after treatment with wortmannin, an inhibitor of autophagosome induction. We examined the differences in the LC3-II levels only at the 14 h time point because our previous time course data from western blotting indicated that the sample at 14 h contained the highest LC3-II amounts, making it easy to distinguish the differences between control and treatment groups. We found that compared to the control group, LC3-II levels in the treatment group are decreased by wortmannin treatment (Fig. 4A).

Using the same method as for wortmannin treatment, we treated oocyte cultures for 42 h with lysosomal protease inhibitors E64d and Pepstatin A to verify the occurrence of autophagic degradation during in vitro maturation of porcine oocytes. Compared to the LC3-II levels in the control group, the LC3-II levels in the treatment group were increased by E64d and Pepstatin A treatment (Fig. 4B).

Discussion

This chapter aimed to establish that autophagy occurs during porcine oocyte maturation, with the ultimate goal of understanding the autophagic process so that it can be reduced unintentional mistake for autophagy controlling in porcine oocyte maturation. We used the autophagic marker LC3-II to identify the presence of autophagosomes, and to demonstrate their induction and degradation in response to factor treatment.

It was first used western blotting to establish the presence of LC3-II and its quantitative changes over time in culture. The fact that LC3-II in oocytes cultured for 14 h is higher than that in oocytes cultured for 0 h can be seen as evidence for newly generated autophagosomes. Likewise, lower levels of LC3-II after 42 h than 0, 14, or 28 h after culture imply that autophagosomal degradation was occurring. However, it is not certain that autophagic activity was the highest after 14 h and lowest after 42 h of oocyte maturation in culture, because autophagy is such a dynamic process that it is difficult to evaluate autophagic activity by just measuring autophagosome amount on the basis of LC3-II levels. However, in spite of this kind of limitation of LC3-II measuring method for detection of autophagy, recent studies in autophagy field dictates that measuring LC3-II is the most persuasive method for determining autophagic activity. Moreover, most of autophagy studies have been using LC3-II detecting methods in measuring

autophagic activity [26]. Collectively, we are now certain that autophagosomes are present in porcine oocytes during in vitro maturation and that their amounts change over time in culture. This means that it is highly possible that autophagic activity changes over time in culture as autophagic activity reach the peak within 28 h after start culturing then gradually reduced. We next established the location of LC3 in porcine oocytes by immunolocalization. Although the LC3 antibody is immunoreactive to both LC3-I and LC3-II, we consider the signal intensity of total LC as representing that of LC3-II. Because, LC3-I levels showed very little variation in western blotting. Thus, the immunostaining results also support the presence of autophagosomes during the in vitro maturation of porcine oocytes. Moreover, dot- and ring-shapes in LC3 signal showed at magnified picture intensely support that LC3 signal is associated to autophagosome because these dot- and ring-shapes are known to represent autophagosomes [41, 42].

Next, we investigated whether autophagic induction occurs during in porcine oocyte in vitro culture, by treating 14 h cultures over the entire culture period with wortmannin, an inhibitor of autophagic induction. Wortmannin is widely used as an autophagic inhibitor based on its inhibitory effect on class III PI3K activity [33, 34] known to be a positive regulator of autophagic induction [35]. Our result shows that LC3-II levels were reduced by wortmannin treatment. It means that more newly generated autophagosomes exist in the control than in the

treatment group. Even though we could infer that autophagosome are newly generated, on the basis of the comparisons of LC3-II levels between 0 h and 14 h in the western blotting data, this result provides a direct evidence for autophagic induction in porcine oocytes cultured in vitro.

It was also demonstrated the occurrence of autophagic degradation, by treating 42 h cultures over the entire culture period with E64d and Pepstatin A, inhibitors of lysosomal proteases. During autophagic degradation, autolysosomes are formed by the fusion of autophagosomes and lysosomes immediately after the completion of autophagosome [36]. After this, the autolysosome is degraded by lysosomal proteases E64d and Pepstatin A, hinder autophagic degradation by inhibiting lysosomal proteases. The LC3-II protein is also degraded by lysosomal proteases during autophagic degradation [37]. Our observation that LC3-II levels were increased by E64d and Pepstatin A treatment means that more autophagosomes remained in the treatment group than in the control group. In other words, autophagosomes in the control group are more degraded than in the treatment group by lysosomal protease. Even though we could infer autophagic degradation by comparing western blotting data from the 14 h and 42 h time points, this result provides direct evidence for autophagic degradation in porcine oocytes cultured in vitro.

In this chapter, we provide evidence for the autophagic process during

porcine oocyte maturation in vitro by using the LC3-II marker to detect the presence of autophagosomes as well as their inception and termination. Moreover, our western blotting data of LC3-II showed that autophagic activity probably changes over the culture period. Therefore, the role of autophagy may be related to the in vitro maturation of porcine oocytes.

Referring to immunohistochemistry of LC3 in murine, LC3 was not detected in unfertilized oocytes [20, 43]. However, our immunohistochemistry result in porcine ovary surely showed LC3 signals. Our findings in porcine oocyte encourage us to investigate autophagy during porcine oocyte maturation.

Generally, autophagy is known to have a dual role in both cell survival [44] and cell death [45, 46]. Further investigation was required to determine whether the increase in autophagosomes after 14 h in porcine oocyte culture is related to the role of autophagy in cell survival or cell death. Therefore, in next chapter, we examined the reason of autophagic activation by testing which pathway autophagy pass through in vitro maturation.

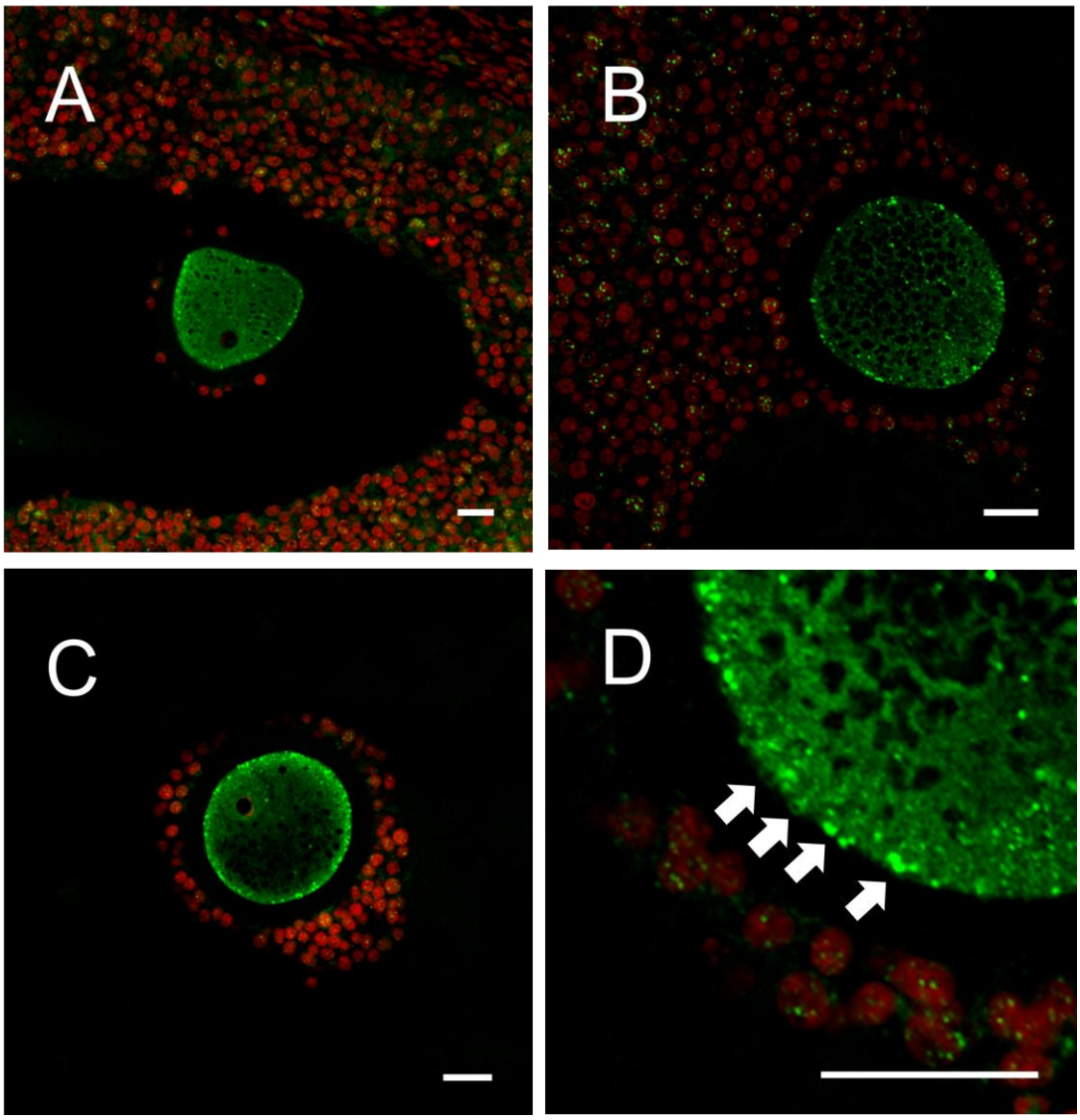

Figure 1. Microtubule-associated protein light chain 3 immunohisto-chemistry in the porcine ovary. Tissues were collected from porcine ovaries. Fixation was performed in 10% formaldehyde neutral buffer solution. The samples were then embedded in paraffin and cut to 5 μm. The cumulus cells and oocytes in follicles of sizes less than 1 mm (A), 1 mm (B), and over 2 mm (C) were photographed under a confocal scanning laser microscope. The picture of over 2 mm follicle (C) was obtained under magnification. Arrows indicate dot- or ring- shaped LC3 immunoreactive signals (D). The green and red indicate microtubule-associated protein light chain 3 (LC3) and the nucleus, respectively. Scale bar = 20 μm.

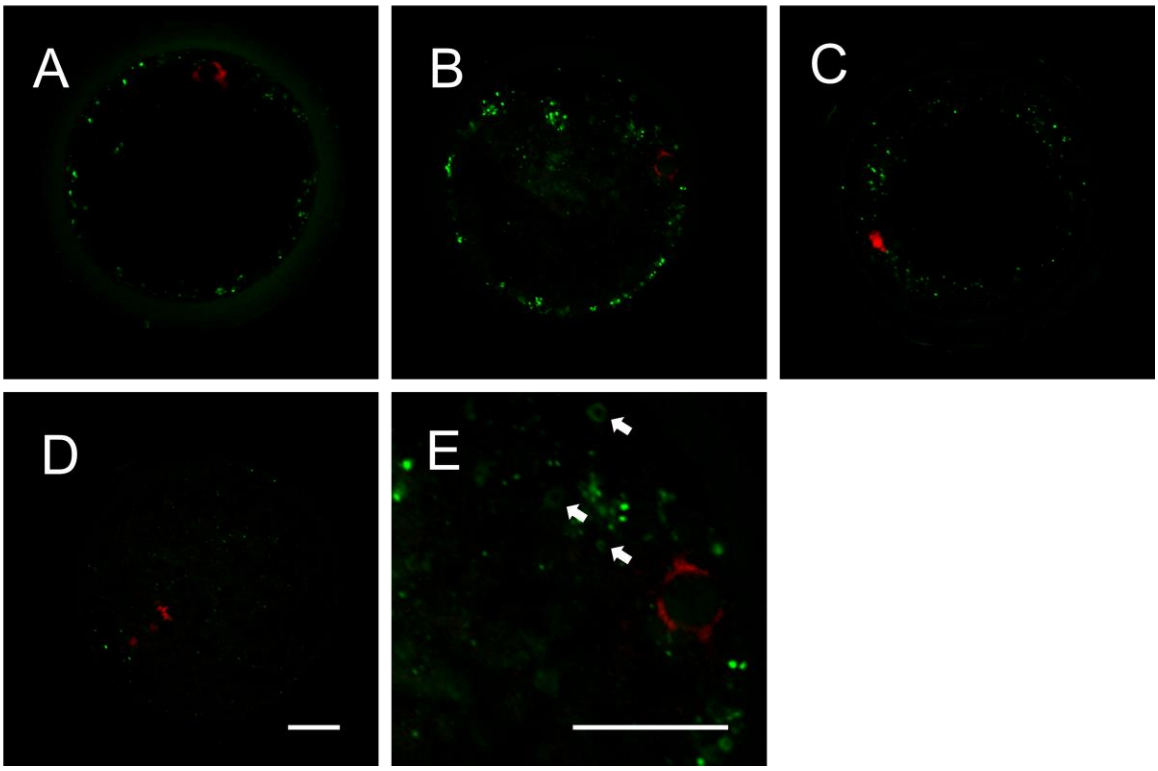

Figure 2. LC3 is localised in porcine oocytes during in vitro maturation. Immunostaining of LC3 was performed on oocytes cultured for 0 h (A), 14 h (B), 28 h (C), and 42 h (D). The picture of 14 h was performed to magnification. Arrows indicate dot- or ring-shape of LC3 immunoreactive signal (E). Oocytes expressing a representative signaling intensity for each time point, were selected for the picture. Green and red indicate LC3 and nucleus, respectively. Scale bar = 20 μm.

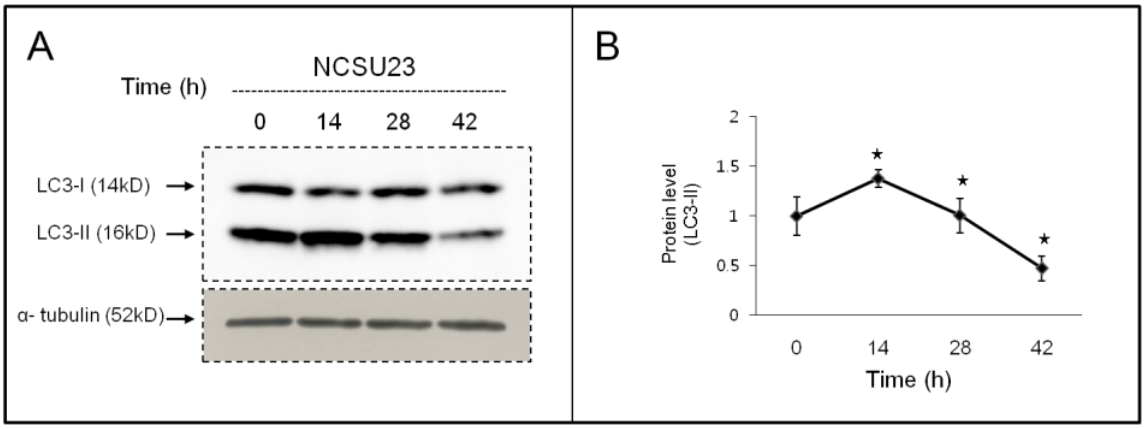

Figure 3. LC3-II levels change over time in culture. LC3-II levels were determined by western blotting (A). The protein density of LC3-II was calculated and the data are expressed based on the control (B). The lysate from each group of 33 oocytes was processed and cryo-preserved after 0 h, 14 h, 28 h, and 42 h in culture, and 99 oocytes were loaded in each lane. Data were the mean ± SD values. ★ $p < 0.05$ as compared with the previous time point. N = 3.

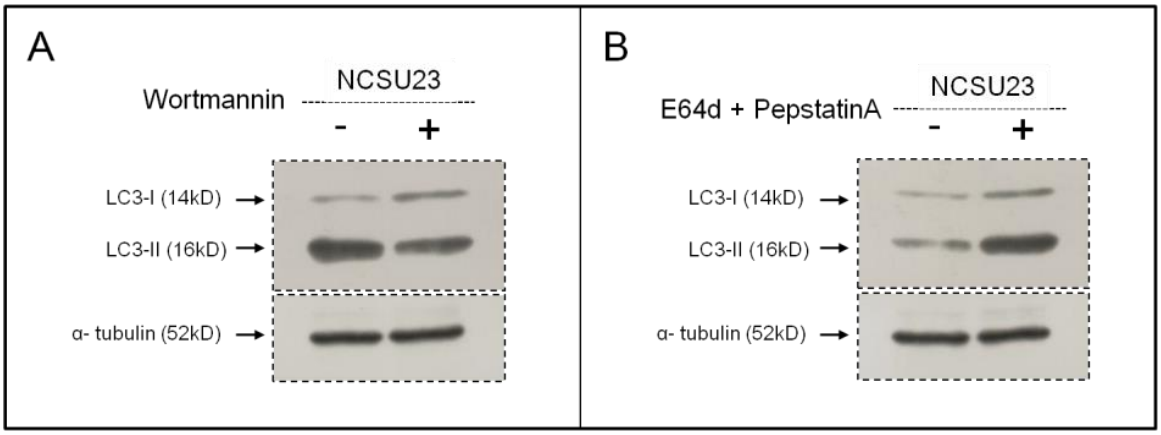

Figure 4. Autophagic inhibitors affected to LC3-II levels. After a 14 h treatment with wortmannin, LC3-II levels were determined by western blotting (A). After 42 h treatment with E64d and Pepstatin A, LC3-II levels were determined by western blotting (B). Lysate from 99 oocytes were loaded in each lane.

Chapter III

Autophagy pathway in porcine oocyte maturation

Introduction

Although we have known that autophagic activity changes in porcine oocyte during maturation in previous chapter, the reason for autophagy activation was still unclear. Therefore, in this chapter, we designed the experiment which could reveal which pathways in autophagy were used during porcine oocyte maturation.

However, before start examining autophagy pathway, we questioned whether culture medium may affect to our result for autophagic activation or nuclear progression may cause our previous result. To clarify this questions, we measured LC3-II by western blotting after culturing oocyte in TCM199 which is differ from NCSU-23 used in previous experiment. TCM199 medium contains no cAMP and do not need media change at 22 h. Then, we examined the effect of dbcAMP for autophagy. dbcAMP is well known reagent that mimics endogenous cAMP and inhibit nuclear progression in meiotic process in porcine oocyte [47]. We tested whether sustained GV stage by treatment of dbcAMP affect to autophagy.

About autophagy pathway, there are roughly two major pathways. One of them is mTORC1 pathway and another is AMPK pathway [48, 49].

mTORC1 is important for the regulation of protein translation, ribosome

biogenesis, and cell growth. At autophagic initiation process, mTORC1 is incorporated into the ULK1–Atg13–FIP200 complex and mTOR phosphorylates ULK1 and Atg13 [50].

Adenosine monophosphate-activated protein kinase (AMPK), which acts as a principal intracellular energy sensor, has been involved in the induction of autophagy [51]. AMPK is activated in various cellular and environmental stress conditions when AMP/ATP ratio is elevated and it generally acts by switching off ATP-requiring processes, while switching on ATP-generating catabolic pathways [52].

It has been reported that AMPK activates autophagic response presumably involves downregulation of the kinase activity of mammalian target of rapamycin (mTOR), which has been known as an important negative regulator of autophagy [53]. However, another research for autophagy demonstrated that AMPK promotes autophagy by directly activating Ulk1 [54]. Therefore, we examined which pathway autophagy in porcine oocyte use or whether autophagy uses both of pathways.

To investigate two pathways, we attempted to induce autophagy via each of two pathways. To know effect of mTOR, we treated mTOR inhibitor, rapamycin, for 28 h in culture, and LC3-II level was measured by western blotting. To know effect of AMPK, we treated AMPK activator, AICA-R, for 28 h in culture, then LC3-

II level was measured by western blotting. In this chapter, we demonstrate what pathway autophagy mainly use in porcine oocyte during maturation.

3.1. Effect of nuclear stage for autophagy

Materials and methods

Oocyte maturation in TCM199 media

Porcine ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory in a container within 2 h after extraction. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3–6 mm) with a 10 mL syringe attached to an 18-gauge needle. Compact cumulus-oocyte complexes (COCs) with uniform ooplasm were selected in phosphate buffered saline (PBS; Nissui Pharmaceutical, Ueno, Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St Louis, MO). After washing 3 times in 0.1% PBS-PVA, the COCs were cultured in TCM199 medium (Sigma) [38] supplemented with 0.1% of EGF (Sigma), 0.2% of FSH (Sigma) and 10% (v/v) porcine follicular fluid for the 42 h of maturation at 38.5°C in 5% CO2 in air. After cell culturing, expanded COC cumulus cells were removed by gentle vortexing in PB1 medium [39] containing 0.1% hyaluronidase (Sigma). The oocytes were subsequently processed according to protocols for western blotting.

Extended dbcAMP treatment in NCSU-23 medium

Porcine ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory in a container within 2 h after extraction. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3–6 mm) with a 10 mL syringe attached to an 18-gauge needle. Compact cumulus-oocyte complexes (COCs) with uniform ooplasm were selected in phosphate buffered saline (PBS; Nissui Pharmaceutical, Ueno, Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St Louis, MO). After washing 3 times in 0.1% PBS-PVA, the COCs were cultured in NCSU-23 medium [38] supplemented with 50 μM β-mercaptoethanol (Sigma), 0.6 mM cysteine (Sigma), 0.5% insulin (Sigma), 10% (v/v) porcine follicular fluid, 10 IU pregnant mare serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan) and 1 mM dibutyryl cyclic AMP (Sigma) for the first 22 h of maturation at 38.5°C in 5% CO2 in air. The COCs of control group were cultured for a further 20 h in the same medium without hormonal and dibutyryl cyclic AMP supplementation while the COCs of treatment group were cultured for a further 20 h in the same medium with dbcAMP. After cell culturing, expanded COC cumulus cells were removed by gentle vortexing in PB1 medium [39] containing 0.1% hyaluronidase (Sigma). The oocytes were subsequently processed according to protocols for western blotting

and orcein staining.

Orcein staining of porcine oocyte and observation of MII rate

Dunuded oocytes were washed 3 time in 0.1% of PBS-PVA. Subsequently, oocytes were mounted on slide glass and fixed in ethanol and acetic acid (3:1) fixative for 48 h. Oocytes were stained with solution containing 1% orcien (Merch) in 50% acetic acid for 2 h. Subsequently, oocytes were decolorized with the solution containing 20% of glycerol and acetic acid. Slides was sealed and detected with phase contrast microscope. Oocytes representing GV stage, from GVBD to metaphase I stage, from anaphase I to telophase I, and metaphase II stage was counted and sorted by direct observation via phase contrast microscope.

Results

In this experiment, we examined whether autophagy is affected by nucleus maturation. dbcAMP was used to block germinal vesicle break down. LC3-II of oocytes cultured in TCM199 media represented just same manner with LC3-II level of oocytes cultured in dbcAMP contained NCSU-23 media for 22 h (Fig.3A, 5A). In addition, LC3-II did not changed by prolonged treatment of

dbcAMP until 42 h in spite that oocyte maturation rate reduced significantly by prolonged treatment of dbcAMP (Fig. 5B, Table. 1). This result proves that autophagy is not affected by dbcAMP and nucleus maturation.

3.2. Examination of autophagy pathway

Materials and methods

Inhibition of mTOR by rapamycin

To inhibit mTOR, rapamycin was added to the culture medium. A 10 mM stock solution of wortmannin was prepared in dimethyl sulfoxide (DMSO) and diluted in the culture medium to final concentrations of 50 μM and 100 μM. DMSO was added to the control culture, and all examined culture droplets contained 0.5% DMSO. After 28 h in culture, 50 oocytes from each control and treatment group were lysed for western blotting. The extraction was repeated 4 times, after which 200 oocytes lysates for electrophoresis were prepared by combining 4 samples of 50 oocytes. Western blotting was conducted by following the same method explained above for western blotting procedure by using LC3 (Cell Signaling).

Activation of AMPK by AICA-R

To activate AMPK, AICA-R was added to the culture medium. A 10 mM stock solution of wortmannin was prepared in milli-Q and diluted in the culture medium to final concentrations of 500 μM and 1000 μM. Milli-Q was added to the control culture. After 28 h in culture, 50 oocytes from each control and treatment

group were lysed for western blotting. The extraction was repeated 2 times, after which 100 oocytes lysates for electrophoresis were prepared by combining 2 samples of 50 oocytes. Western blotting was conducted by following the same method explained above for western blotting procedure by using LC3 (Cell Signaling).

Results

There are two major pathways related to regulation of autophagy, which are PI3KC1-Akt-mTOR pathway and AMPK pathway [55, 56]. mTOR pathway plays inhibition role for autophagy and AMPK plays activate role for autophagy. In this experiment, we designed to induce autophagy by each mTOR inhibition and AMPK activation. We succeeded to induce autophagy by AICA-R which is AMPK activator while failed to induce autophagy by rapamycin which is mTOR inhibitor (Fig. 6). This result represents that autophagy pass through AMPK pathway rather than mTOR pathway.

Discussion

In this chapter, we examined the effect of another medium of TCM199 for autophagy. Two of distinct differences between NCSU-23 and TCM199 are the presence of dbcAMP and the kind of hormone. As our result, autophagic variation manner of porcine oocyte cultured in TCM199 showed just same as it in cultured in NCSU-23. This result means that autophagy affected by not media but something existed during oocyte maturation. Then, we suspected that it is possible that nuclear stage may affect to autophagy. To clarify whether autophagy is affected by nuclear stage, we inhibited nuclear progression in maturation by prolonged treatment of dbcAMP which is known to inhibitor of nuclear progression. In previous porcine researches, it was clearly established that dbcAMP keep GV stage [57]. Our result of nuclear stage observation also showed that GVBD is inhibited by prolonged treatment of dbcAMP. However, when we keep GV to 42 h, autophagic activity was not changed comparing to control group. This result means that autophagy was not affected by nuclear stage. By this result, the possibility that autophagy affect to nuclear progression was raised.

After then, we examined which pathway autophagy use between mTOR-ULK1 complex pathway and AMPK pathway [58]. We designed the experiment of autophagy inducing by mTOR inhibition or AMPK activation. As a result,

rapamycin which is inhibitor of mTOR could not induce autophagy while AICA-R which is activator of AMPK induced autophagy. This result suggests that autophagy in porcine oocyte during maturation use AMPK pathway rather than mTOR pathway or AMPK locate at up-stream of mTOR in porcine oocyte maturation. Generally, it has been known that mTOR pathway and AMPK pathway is not independent to each other [59]. Our result did not support the general idea which mTOR pathway and AMPK pathway is connected to each other. By recent research of autophagy, it has been reported that autophagy induced by AMPK-ULK1 activation independent with mTOR pathway in starvation circumstances [54]. Although artificially produced culture medium like as NCSU23 and TCM199 may supply oocytes less energy and nutrient than it of blood circulation system, a medium also contains energy and nutrient to cell growth. Therefore, we carefully infer that comparatively high concentration of hormones raises metabolic stress beyond to the capacity supplying energy in medium.

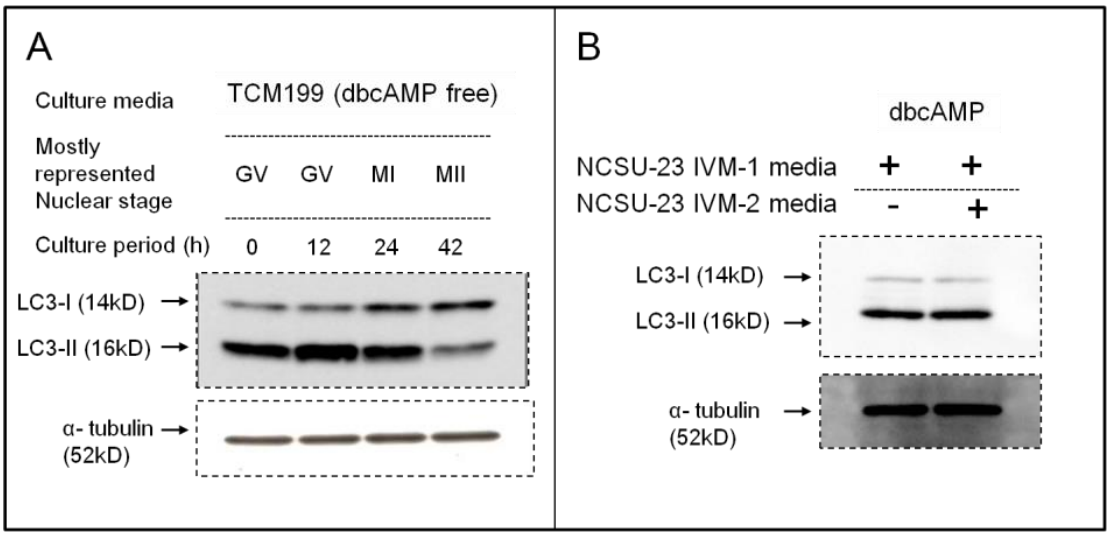

Figure 5. LC3-II levels by dbcAMP. LC3-II levels were determined by western blotting after 0 h, 12 h, 24 h, and 42 h culture in TCM199 dbcAMP free media (A). The protein density of LC3-II was determined by western blotting after 20 h prolonged dbcAMP treatment in culture (B). The lysate from each group of 99 oocytes were loaded in each lane.

Table 1. After prolonged treatment of dbcAMP, MII rate at 42h

dbcAMP concentration	No. of Oocytes examined	Total(%)	No.(%) of oocytes at GV	No.(%) of oocytes at GVBD~MI	No.(%) of oocytes at Ana~Tel	No.(%) of oocytes at MII stage
1mM(IVM-1) +0mM(IVM-2)	43	43(100)	9(20.93)	4(9.30)	1(2.32)	29(67.44)
1mM(IVM-1) +1mM(IVM-2)	43	43(100)	33(76.74)	2(4.65)	0(0)	8(18.60)

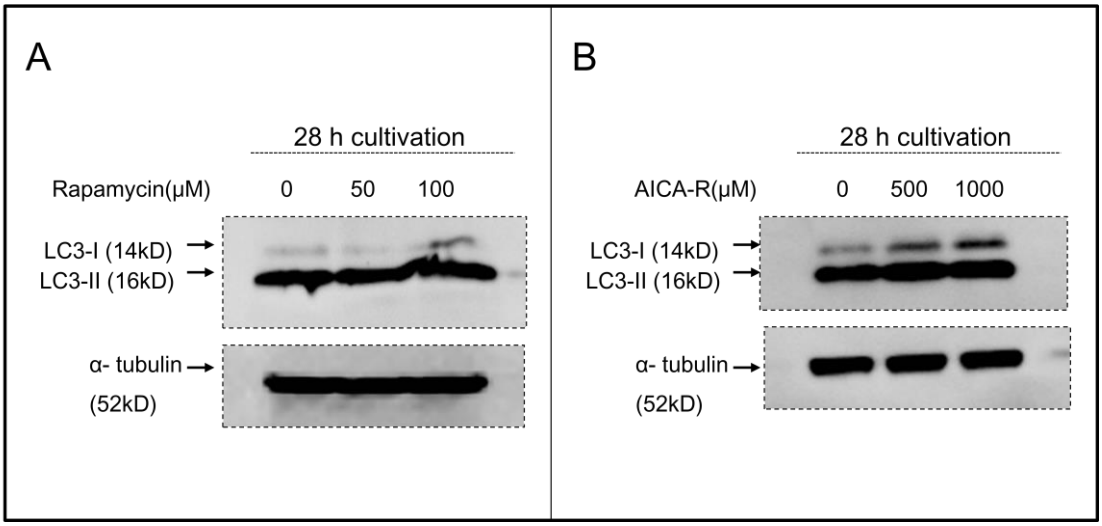

Figure 6. Measurement of LC3-II after treatment of rapamycin and AICA-R. LC3-II levels were determined with 28 h cultured 200 oocytes lysate in each lane. Each group treated rapamycin in concentration 0, 50, 100 μM (A). LC3-II levels were determined with 28 h cultured 100 oocytes lysate in each lane. Each group treated AICA-R in concentration with 0, 500, 1000 μM (B).

Chapter IV

Possible role of autophagy in porcine oocyte

Introduction

Our immunostaining results of a previous experiment showed ring- and dot- shaped LC3 signals mostly near the plasmic membrane in the oocyte samples (Fig. 1, 2). Referring to localization data, we hypothesized that autophagy is related to something located near the plasma membrane. Thus, we turned our attention to the relationship between autophagy and gap junctions. Mammalian follicular oocytes are surrounded by cumulus cells and they adhere to cumulus cells physically by gap junctions. Molecular exchanges and signal transportation between oocytes and cumulus cells is achieved through gap junction channels [27]. Gap junction channels are assembled from a ubiquitously expressed class of four-pass trans-membrane proteins termed connexins, with connexin43 (Cx43) being the most abundantly expressed connexin [28]. In autophagy research on somatic cells, it has been revealed that internalized gap junctions, including Cx43, are degraded by autophagy [29-31].

Therefore, in this chapter, we attempted to determine if internalized Cx43 is also degraded by autophagy in porcine oocytes. We examined the variation of intracellular Cx43 in porcine oocytes by performing western blotting with samples from 0 h, 14 h, 28 h, and 42 h after starting cultures.

Next, we examined the effect of autophagic dysfunction on a Cx43

population. The amount of intracellular Cx43 in porcine oocytes that were treated with 50 μM or 100 μM wortmannin for 28 h was measured by performing western blotting. Wortmannin is widely used as an autophagic inhibitor based on its inhibitory effect of class III PI3K activity, which is known to be a positive regulator of autophagic initiation [35].

4.1. Localization of Cx43 in porcine oocytes during in vitro

Materials and methods

Oocyte maturation

Porcine ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory in a container within 2 h of extraction. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3–6 mm) with a 10 mL syringe attached to an 18-gauge needle. Compact cumulus-oocyte complexes (COCs) with uniform ooplasm were selected in phosphate buffered saline (PBS; Nissui Pharmaceutical, Ueno, Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St Louis, MO). After washing 3 times in 0.1% PBS-PVA, the COCs were cultured in NCSU-23 medium [38] supplemented with 50 μM β-mercaptoethanol (Sigma), 0.6 mM cysteine (Sigma), 0.5% insulin (Sigma), 10% (v/v) porcine follicular fluid, 10 IU pregnant mare serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan) and 1 mM dibutyryl cyclic AMP (Sigma) for the first 22 h of maturation at 38.5°C in 5% CO2 in air. The COCs were cultured for a further 20 h in the same medium without hormonal and dibutyryl cyclic AMP supplementation. After cell culturing, expanded

COC cumulus cells were removed by gentle vortexing in PB1 medium [39] containing 0.1% hyaluronidase (Sigma). The oocytes were subsequently processed according to protocols for western blotting and immunostaining.

Immunohistochemistry of LC3 in porcine ovary

Porcine ovaries were fixed with 10% formaldehyde neutral buffer solution (Nacalai Tesque, Kyoto, Japan), embedded in paraffin wax and sectioned. Cross-sectioned samples (5 μM) of ovary tissue were placed on glass slides and dried at room temperature. The samples were then deparaffinized and incubated with HistoVT One (Nacalai Tesque) at 90°C for 30 min. After washing, Samples were treated with Blocking One (Nacalai Tesque) at 4°C for 1 h. Slide samples were incubated with rabbit anti-LC3 monoclonal antibody (dilution 1:200; Cell signaling, Danvers, MA) overnight at 4°C and then incubated with Alexa Fluor 488-labeled anti-rabbit secondary antibody (dilution 1:500; Invitrogen, Carlsbad, CA) and 20 μg/ml propidium iodide (PI) (Sigma Chemical, St Louis, MO) at 4°C for 2 h. After washing with PBS, the samples were viewed using a LSM700 confocal scanning laser microscope (Zeiss, Feldbach, Switzerland).

Results

We observed Cx43 by immunocytochemistry. The result of Immunocytochemistry for Cx43 showed that Cx43 signals localized both in the zona pellucida and near the plasma membrane of porcine oocytes during maturation (Fig. 7). Moreover, the magnified picture clearly showed that internalized Cx43 located near the plasma membrane (Fig. 7E). This result proves that monoclonal Cx43 primary antibody which we used in our experiment was profitable.

4.2. Quantitative analysis of Cx43 during oocyte maturation

Materials and methods

Time course analysis for western blot of Cx43

Protein extraction from oocytes was performed at 0 h, 14 h, 28 h, and 42 h. At each time point, 88 oocytes that had been denuded by 0.1% hyaluronidase (Sigma) in PB1 medium were placed in 1× sodium dodecyl sulfate (SDS) sample buffer, 0.5 M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, and 20% glycerol. Lysates were cryopreserved at -80°C. After heat denaturation, lysates were separated by 4%–15% gradient SDS-PAGE (Bio-Rad, Hercules, CA) and transferred to Immobilon membranes (Millipore, Billerica, MA). After the transfer, the membranes were blocked with Blocking One (Nacalai Tesque) for 1 h at room temperature and washed several times with Tris-buffered saline containing 0.1% Tween20 (TBS-T). The membranes were incubated with rabbit anti-Cx43 monoclonal antibody (dilution 1:1000; Cell signaling, Danvers, MA) overnight at 4°C and then incubated with horseradish peroxidase-labeled anti-rabbit IgG (dilution 1:10000; Invitrogen, Carlsbad, CA). After several washes with TBS-T, the peroxidase activity was visualized by using an ECL Plus western blotting detection system (GE Healthcare, Piscataway, NJ). Signals were detected with an Image

Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Statistical Analysis

Western blotting experiment for Cx43 was repeated at least three times. Data were analyzed by using STATVIEW (Version 5.0, SAS Institute Inc., Cary, NC, USA). Comparison between two groups was made with Student's t-test. For all statistical analyses, a P value less than 0.05 was considered significant.

Results

Cx43 protein levels in porcine oocytes were determined by western blotting. Lysate of denuded oocytes was prepared at a time course of 0, 14, 28, and 42 h for cultures in NCSU-23 medium. Lysate of each 99 oocytes were loaded in a lane. During 42 h of the time course, Cx43 levels increased to a maximum at 14 h and gradually dropped to a minimum at 42 h (Fig. 8A). By using the LAS system (Fujifilm), the band density of Cx43 and α-tubulin was converted to numerical values for each group of 0 h, 14 h, 28 h, and 42 h. Then, the Cx43/α-tubulin ratio was statistically calculated and compared to the other groups based on the Cx43/α-tubulin ratio at 0 h (Fig. 8B). The Cx43/α-tubulin ratio was significantly different between 0 h and 14 h, 14 h and 28 h, and 28 h and 42 h (Fig. 8B).

4.3. Effect of autophagy for Cx43

Materials and methods

Effect of Inhibiting autophagic initiation on Cx43

To inhibit the generation of autophagosomes, wortmannin was added to the culture medium. A 10 mM stock solution of wortmannin was prepared in dimethyl sulfoxide (DMSO) and diluted in the culture medium to final concentrations of 50 μM and 100 μM. DMSO was added to the control culture, and all examined culture droplets contained 0.5% DMSO. After 28 h in culture, 50 oocytes from each control and treatment group were lysed for western blotting. The extraction was repeated 3 times, after which 150 oocytes lysates for electrophoresis were prepared by combining 3 samples of 50 oocytes. Western blotting was conducted by following the same method explained above for western blotting procedure by using both LC3 (Cell Signaling) and Cx43 (Cell Signaling) primary antibodies.

Results

Cx43 and LC3-II protein levels were examined in the oocytes by western blotting after each sample was taken at 28 h of treatment with 50 μM and 100 μM wortmannin during in vitro maturation in an NUSU-23 culture system. The level of Cx43 was increased by wortmannin treatment when LC3-II was decreased by wortmannin treatment (Fig. 9A). By using the LAS system (Fujifilm), the band densities of Cx43, LC3-II and α-tubulin were converted to numerical value at each group. Then, the Cx43/α-tubulin ratio and the LC3-II/α-tubulin ratio were calculated and compared to the other groups based on the control group (Fig. 9B). Data were analyzed by using STATVIEW (Version 5.0, SAS Institute Inc.). Comparison between two groups was made with Student's t-test. Each of Cx43/α-tubulin ratio and LC3-II/α-tubulin ratio was significantly different between 0 μM and 50 μM as they showed a P value less than 0.05. However, Each of Cx43/α-tubulin ratio and LC3-II/α-tubulin ratio between 50 μM and 100 μM did not show significant differences as they showed a P value more than 0.05. Cx43/α-tubulin ratio between 0 μM and 100 μM did not also show significant differences as they showed a P value more than 0.05 while LC3-II/α-tubulin ratio was significantly different between 0 μM and 100 μM as they showed a P value less than 0.05.

Discussion

In the previous chapter, we examined the reason for autophagy activation. In this chapter, we examined what kind of functions the activated autophagy have during porcine oocyte maturation.

We hypothesized that autophagy in porcine oocytes is related to gap junctions because our results with respect to the localization of autophagosomes showed that autophagosomes exist mostly near the plasma membrane of oocytes (Fig. 1, 2). In other cells, it has been already shown that Cx43, the main component of gap junction, was degraded by autophagy [30]. We postulated that oocytes whose sizes are bigger than any other cells are supposed to have more gap junctions and that their autophagic activity may be related with gap junctions during oocyte maturation. To confirm this, we conducted Cx43 observation during porcine oocyte maturation.

We observed Cx43 by immunocytochemistry and western blotting. The result of Immunocytochemistry for Cx43 showed that Cx43 signals localized both in the zona pellucida and near the plasma membrane of porcine oocytes during maturation (Fig. 7). Moreover, the magnified picture clearly showed that internalized Cx43 located near the plasma membrane (Fig. 7E). This result proves that monoclonal Cx43 primary antibody which we used in our experiment was

profitable. Through a quantitative analysis of Cx43 during oocyte maturation by western blotting, the overall Cx43 population was found to have changed during the culturing time. Four time points were examined during maturation with 14 h intervals as we took samples at 0, 14, 28, and 42 h. In our NCSU-23 porcine oocyte culture system, 14, 28, and 42 h oocytes mostly represent GV stage, MI stage, and MII stage oocytes, respectively. For western blotting results, the level of Cx43 was changed during the time course as it showed a maximum at 14 h and minimum at 42 h (Fig. 8). It has been reported that Cx43 dynamics of porcine COCs are affected by gonadotropin and they contribute to earlier oocyte maturation [60]. In present study, although we did not examine the reason for variation in Cx43 level in porcine oocytes, we verified for the first time that the variation of Cx43 in porcine oocytes during maturation. However, our results for the time course analysis of Cx43 did not correspond to those of an earlier study by another group. A group showed that Cx43 of porcine COCs exhibits lower levels around 14 h rather than at 0 h or 28 h [61], possibly because of the difference between oocytes and cumulus cells in terms of Cx43 expression. Apart from Cx43 in COCs, quantitative analysis of Cx43 in porcine oocytes showed that Cx43 expression was the highest at 14 h and the lowest at 42 h (Fig. 8). The important point to note is that the results of this quantitative analysis of Cx43 coincide with the results of quantitative analysis of LC3-II in previous chapter, in which LC3-II

was the highest at 14 h and the lowest at 42 h (Fig. 3). These results for LC3-II and Cx43 monitoring support our idea that there is a relationship between autophagy and gap junctions.

Next, to confirm that autophagy really degrades gap junction proteins, we next inhibited the generation of autophagosomes by using wortmannin, which is an inhibitor of autophagic initiation, during maturation period and then subsequently measured the level of Cx43 by performing western blotting. We used wortmannin at final concentrations of 50 and 100 μM for in culturing and sampled the oocytes after 28 h. We chose 28 h as the sampling point, which was the mid-point between 14 (peak of autophagic activity) and 42 h (achievement of MII stage), in order to maintain the link between inhibition of autophagy and reaching the MII stage at 42 h. The amount of Cx43 was increased by autophagic inhibition when LC3-II amount was decreased (Fig. 9A). Moreover, when we intentionally introduced saturation at high concentration of wortmannin (100 μM), both Cx43 and LC3-II showed slight variations compared to 50 μM group (Fig. 9B). However, we noticed that Cx43 of 0 μM and 100 μM also represent no significant differences although Cx43 was more detected in 100 μM rather than 0 μM when we observed with unaided eyes. We presume that Cx43 proliferation was slightly disturbed by too high concentration of 100 μM wortmannin. By these results, we concluded that larger population of Cx43 was remained because of autophagic dysfunction.

Although wortmannin not only inhibit PI3K Class III but also temporarily inhibit PI3K Class I which is engaged with autophagy inhibition, our result of LC3-II reduction by wortmannin means that wortmannin surely act as autophagy inhibitor in porcine oocyte. Therefore, it is proved that autophagy in porcine oocyte was inhibited by wortmannin and inhibited autophagy by wortmannin caused Cx43 accumulation in oocyte. Conclusively, this result provides direct evidence that autophagy degrades Cx43, the main gap junction protein, in porcine oocytes as well as in other cells.

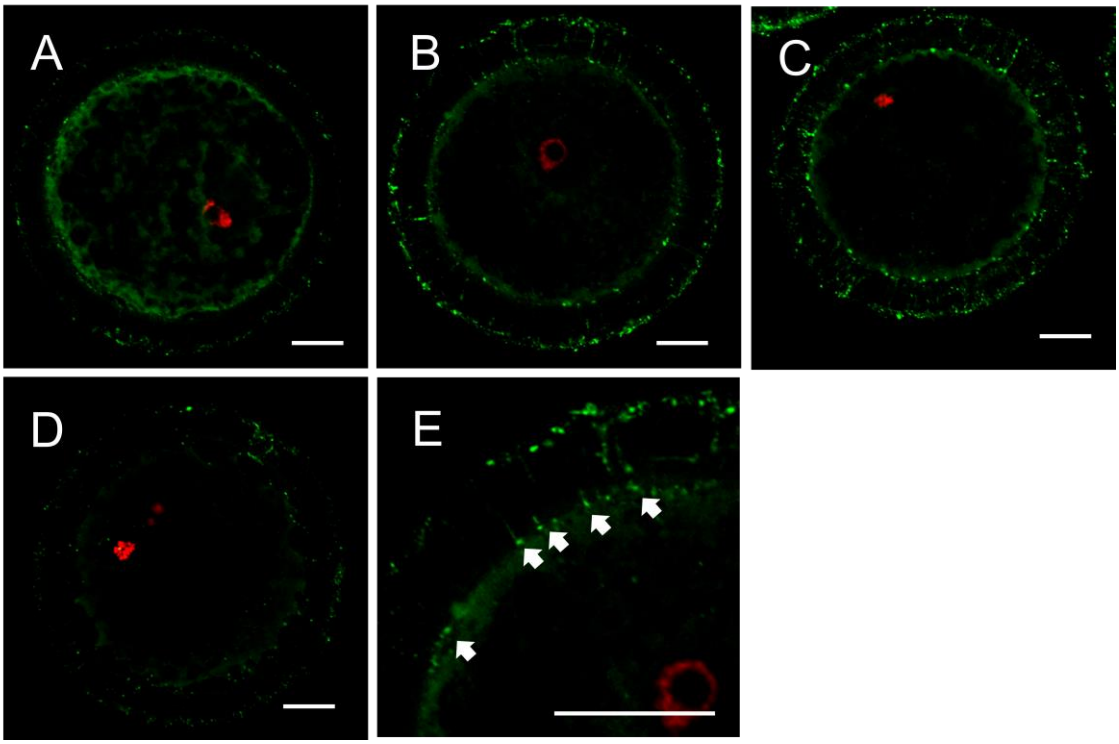

Figure 7. Cx43 is localized in porcine oocytes during maturation. Immuno-cytochemistry of connexin 43 (Cx43) in oocytes cultured for 0 h (A), 14 h (B), 28 h (C), and 42 h (D) was examined. The picture of 14 h (B) was obtained under magnification. Arrows indicate internalized Cx43 signals (E). Oocytes, with a nucleus on their equatorial planes and expressing a representative signaling intensity for each time point were selected and their pictures were obtained. The green and the red indicate Cx43 and the nucleus, respectively. Scale bar = 20 μm.

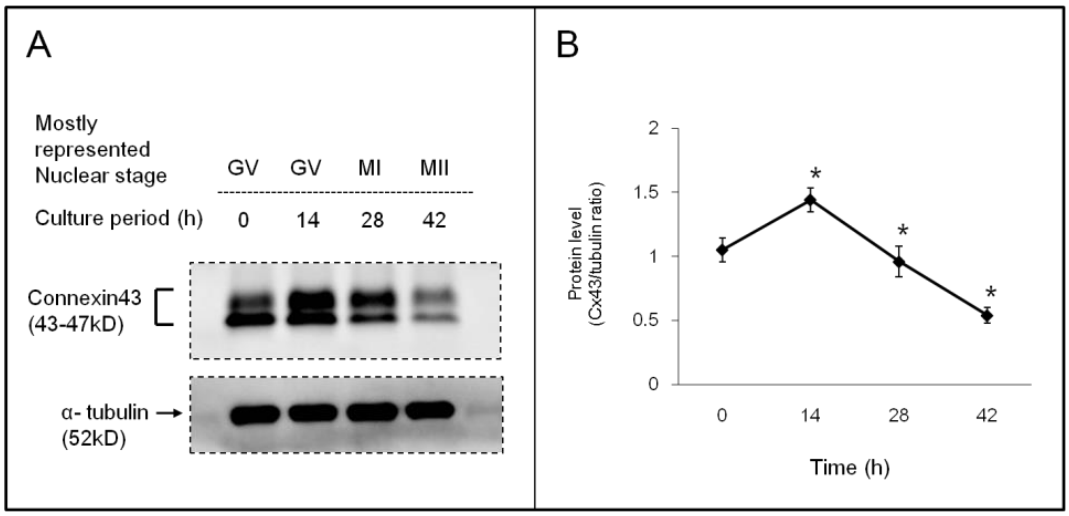

Figure 8. Connexin 43 levels change over time during culturing. Connexin 43 (Cx43) levels were determined by performing western blotting during maturation (A). The Cx43/α-tubulin ratio was calculated and the data are expressed relative to the value at 0 h (B). The lysate from each group of 88 oocytes was processed and cryo-preserved after 0 h, 14 h, 28 h, and 42 h in culture, and 88 oocytes were loaded into each lane. The data were analyzed by using Student's t-test (*P < 0.05; n = 3). The data are shown as the mean ± SD. *P < 0.05 as compared with each previous time point.

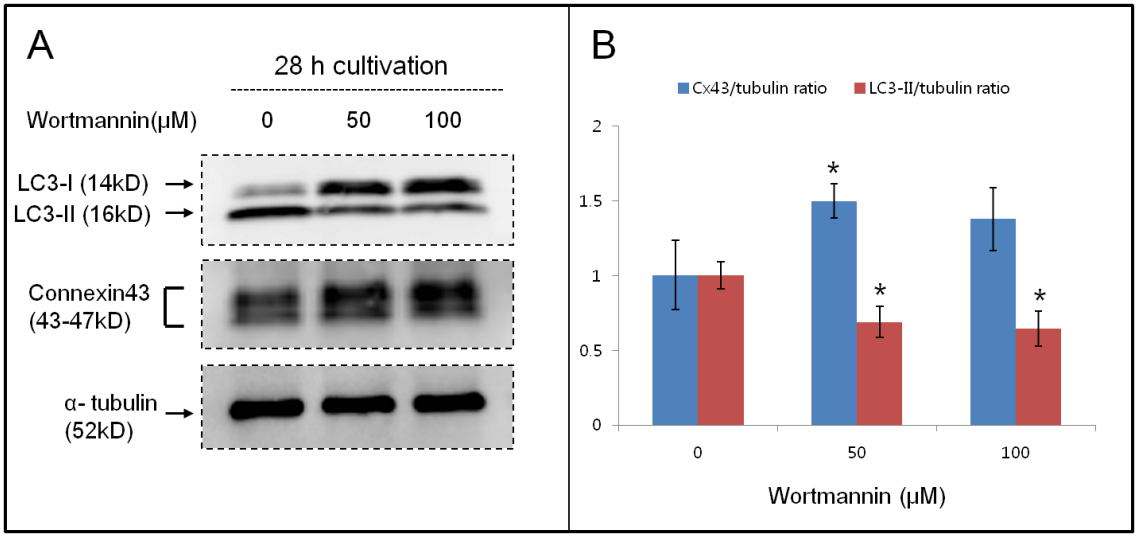

Figure 9. Change in connexin 43 population due to autophagic inhibition. The initiation of autophagy was inhibited by 50 μM and 100 μM wortmannin during maturation. The levels of connexin 43 (Cx43) and microtubule-associated protein light chain 3 (LC3) amount were determined by western blotting (A). The Cx43/α-tubulin ratio and LC3-II/α-tubulin ratio were calculated and the data are expressed based on the value of control group (B). The lysate from each group of 50 oocytes was processed and cryo-preserved after 28 h in culture. All processes for preparing lysate were repeated 3 times. 150 oocytes which each repeat of 3 times is unified were loaded into each lane. The red colored bar in the graph represents LC3-II/α-tubulin ratio and the blue colored bar in the graph represent Cx43/α-tubulin ratio (B). The data were analyzed by using Student's t-test (*$P < 0.05$; $n = 3$). The data are shown as the mean ± SD. *$P < 0.05$ as compared with each control group.

Chapter V

Effect of autophagy for oocyte maturation

Introduction

So far, we verified autophagy, examined the reason of autophagy activation and investigated its possible function in porcine oocyte. In this chapter, we examined how much autophagy affect to oocyte maturation. We inhibited autophagic initiation process and degradation process among five steps of autophagic process by wortmannin and E64d/PepstatinA mixture, respectively. Then, MII rate was measured. Wortmannin, which plays a role in blocking the induction of autophagosomes, has been widely used as an autophagy inhibitor based on its inhibitory effect on class III phosphatidylinositol 3-kinase (PI3K) activity [33, 34] which is known to be a positive regulator of autophagic induction [35]. The lysosomal protease inhibitors, E64d and PepstatinA, hamper autophagic degradation by inhibiting lysosomal proteases. According to the research by other group, it has been reported that 3-methylamine (3-MA) inhibited meiotic progression of porcine oocytes. Because it has been known that 3-MA, which is a PI3K inhibitor, have less stable effect for inhibiting autophagy than wortmannin, wortmannin was used for inhibiting autophagy in this study. By this experiment, we intended to evaluate the potentiality that autophagy could be regulator of oocyte maturation.

5.1. Relationship with autophagy and oocyte maturation

Materials and methods

Inhibition of autophagosome induction

To inhibit autophagosome induction, wortmannin was added to NCSU23 culture medium. A 10 mM stock solution of wortmannin was prepared in dimethyl sulfoxide (DMSO) and diluted in the culture medium to a final concentration of 50 μM. DMSO was added to the control culture, and all examined culture droplets contained 0.5% DMSO. After 42 h in culture, oocytes from each control and treatment group were fixed in ethanol and acetic acid solution (3:1) for orcein staining.

Inhibition of lysosomal degradation

To inhibit autophagosome degradation with lysosomal enzymes, E64d and papstatin A were added to NCSU23 culture medium. A 5 mM E64d stock solution and a 2.6mM Pepstatin A stock solution were prepared in DMSO and 10% (v/v) acetic acid in DMSO, respectively. Then, they were diluted in the culture medium to final concentrations of 29.8 μM for E64d and 15.6 μM for Pepstatin A. DMSO containing 9.1% (v/v) acetic acid was added to control cultures, and all examined

culture droplets contained 0.6% DMSO containing 9.1% (v/v) acetic acid. After 42 h in culture, oocytes from each control and treatment group were fixed in ethanol and acetic acid solution (3:1) for orcein staining.

Inhibition of autophagosome induction and degradation simultaneously

To inhibit autophagic initiation and degradation, Wortmannin and E64d/pepstatin A mixture were added to NCSU23 culture medium with final concentration of 50 μM, 29.8 μM and 15.6 μM, respectively. After 42 h in culture, oocytes from each control and treatment group were fixed in ethanol and acetic acid solution (3:1) for orcein staining.

Orcein staining of porcine oocyte and observation of MII rate

Dunuded oocytes were washed 3 time in 0.1% of PBS-PVA. Subsequently, oocytes were mounted on slide glass and fixed in ethanol and acetic acid (3:1) fixative for 48 h. Oocytes were stained with solution containing 1% orcien (Merch) in 50% acetic acid for 2 h. Subsequently, oocytes were decolorized with the solution containing 20% of glycerol and acetic acid. Slides was sealed and detected with phase contrast microscope. Oocytes representing GV stage, from GVBD to metaphase I stage, from anaphase I to telophase I, and metaphase II stage was counted and sorted by direct observation via phase contrast

microscope.

Results

To know that autophagy participate in oocyte maturation, maturation rate of porcine oocytes was measured after induction of autophagy dysfunction. We disturbed autophagosome generation by wortmannin treatment and autophagosome degradation by E64d and Pepstatin A mixture. Inhibition of autophagosome generation reduced maturation rate (Table. 2, 4) while inhibition of autophagosome degradation did not affect to maturation rate (Table. 3).

Discussion

There was a previous research suggesting that wortmannin inhibited oocyte maturation in porcine oocyte [61]. And also, other group used 3-methylamine which is similar reagent with wortmannin and showed the evidence that 3-methylamine inhibit meiotic resumption [42]. In our experiment, the result showed that wortmannin treatment caused reduction of MII rate. The difference between other group and us is that we showed not only reduction of maturation rate in wortmannin treatment but also reduction of autophagic activity. In spite that PI3K class III is essential materials that trigger autophagy, the thing that wortmannin inhibit autophagy by inhibiting PI3K class III has been ignored in research of oocyte maturation until now. Regarding to treatment of E64d and Pepstatin A mixture, MII rate did not represent significant differences. One of possible reason is that E64d and Pepstatin A inhibit only a few kinds of cathepsins (cathepsin B, L, D and E) which are lysosomal protease. There still exist any other cathepsins such as cathepsin A, C, F, G, H and etc. and it is thought that other cathepsins might compensate for autophagy degration. Our result indicate that autophagy probably participate in oocyte maturation.

Table 2. After treatment of wortmannin, MII rate at 42h

Concentration of Mixture (uM)	No. of Oocytes examined	total	No.(%) of oocytes at GV	No.(%) of oocytes at GVBD~MI	No.(%) of oocytes at Ana~Tel	No.(%) of oocytes at MII stage
0	97	91 (93.8)	13 (13.4)	6 (6.2)	1 (1.0)	73 (75.3)
50	106	94 (88.7)	22 (20.8)	31 (29.0)	0 (0)	41 (38.7)

Table 3. After treatment of E64d and pepstatin A mixture, MII rate at 42h

Concentration of Mixture (uM)	No. of Oocytes examined	total	No.(%) of oocytes at GV	No.(%) of oocytes at GVBD~MI	No.(%) of oocytes at Ana~Tel	No.(%) of oocytes at MII stage
0	101	98 (97.0)	11 (10.9)	6 (5.9)	0 (0)	81 (80.2)
30+16	102	96 (94.1)	10 (9.8)	7 (6.9)	2 (2.0)	77 (75.5)

Table 4. After treatment of wortmannin plus E64d and pepstatin A mixture, MII rate at 42h

Concentration of Mixture (uM)	No. of Oocytes examined	total	No.(%) of oocytes at GV	No.(%) of oocytes at GVBD~MI	No.(%) of oocytes at Ana~Tel	No.(%) of oocytes at MII stage
0	53	53 (100)	8 (15.1)	7 (13.2)	2 (3.8)	36 (68.0)
40+30+16	53	53 (100)	17 (32.1)	12 (22.6)	3 (5.7)	21 (39.6)

Chapter VI

General Discussion and Summary

In this study, we investigated autophagy in porcine oocytes using LC3 detection method. In mice, an LC3 signal was detected in unfertilized oocytes at the MII stage although the LC3 signal intensity was lower than that in fertilized oocytes [5]. In this study, we revealed for the first time that autophagosomes exist in porcine oocytes. As shown in chapter I, autophagic activity was changed during culture. Autophagy activity was the highest at 14 h and lowest at 42 h. In other words, autophagic activity reached at peak at least until 28 h after starting of culture of porcine oocytes and then it gradually decreased. We attempted to find the reason for this variation in autophagic activity during porcine oocyte maturation because it is possibile that autophagy contributed to oocyte maturation rather than in cell death. Actually, it is known that autophagy plays two kinds of role in other cells: cell survival role and cell death role [62, 63]. Thus, we tried to determine which role autophagy performs between the two during porcine oocyte maturation by investigating the reason and the function in autophagic activity. In chapter II, we discovered that autophagy is not affected by nucleus stage. And, we knew that one of important factor which affect to autophagy in porcine oocyte maturation is AMPK which is energy sensor of cellular system. Subsequently, In chapter III, we hypothesized that autophagy in porcine oocytes is related to gap junctions because our results of the localization of autophagosomes show that autophagosomes exist mostly near the plasma membrane of oocytes. Besides, it

has been already reported that Cx43, main component of gap junction, is degraded by autophagy in other cells. We postulated that oocytes whose sizes are bigger than any other cells are supposed to have more gap junctions and that their autophagic activity may reflect relationship between autophagy and gap junctions during oocyte maturation. To investigate a relationship between autophagy and gap junction, we monitored not only LC3 for autophagy but also Cx43 for gap junctions during porcine oocyte maturation. We monitored Cx43 by immunocytochemistry and western blotting. The result of Immunocytochemistry for Cx43 showed that Cx43 signals localized both in the zona pellucida and near the plasma membrane of porcine oocytes during maturation. We showed, for the first time, internalized Cx43 of porcine oocyte by immunocytochemistry. Through a quantitative analysis of Cx43 during oocyte maturation by western blotting, the overall Cx43 population was found to have changed during the culturing time. We examined four points during maturation with 14 h intervals as we took samples at 0 h, 14 h, 28 h, and 42 h. In our NCSU23 porcine oocyte culture system, 14 h, 28 h, and 42 h oocytes mostly represent GV stage, MI stage, and MII stage oocytes, respectively. For western blotting results, the level of Cx43 was changed during the time course as it showed a maximum at 14 h and minimum at 42 h. Referring to other research, it has been reported that Cx43 dynamics of porcine COCs are affected by gonadotropin and they contribute to earlier oocyte maturation [60]. In

our study, although we did not examine the reason for variation in Cx43 level in porcine oocytes, we verified for the first time that the variation of Cx43 in porcine oocytes during maturation. However, our results for the time course analysis of Cx43 did not correspond to those of an earlier study by another group. Another group showed that Cx43 of porcine COCs exhibits lower levels around 14 h rather than at 0 h or 28 h [61], possibly because of the difference between oocytes and cumulus cells in terms of Cx43 expression. Apart from Cx43 in COCs, our quantitative analysis of Cx43 in porcine oocytes showed that Cx43 expression was the highest at 14 h and the lowest at 42 h. The important point to note is that the results of this quantitative analysis of Cx43 coincide with the results of quantitative analysis of LC3-II, in which LC3-II was the highest at 14 h and the lowest at 42 h. These results for LC3-II and Cx43 monitoring support our idea that there is a relationship between autophagy and gap junctions. At least, it is denied that autophagy do not degrade Cx43 by these results. Thus, these results could be not a direct evidence but an indirect evidence that autophagy degrade Cx43 in porcine oocytes during maturation. It could be possible that, only in healthy oocytes, an elevation in the gap junction population may need autophagy activation to supply more energy for more metabolic activity which caused by hormonal stimulation.

To confirm that autophagy really degrades gap junction proteins, we next inhibited the generation of autophagosomes by using wortmannin, which is an

inhibitor of autophagic initiation, during maturation period and then subsequently measured the level of Cx43 by performing western blotting. The amount of Cx43 was increased by autophagic inhibition when LC3-II amount was decreased. Moreover, when we intentionally introduced saturation at high concentration of wortmannin (100 μM), both Cx43 and LC3-II showed slight variations compared to 50 μM group. Therefore, we concluded that a large concentration of Cx43 was present because of autophagic dysfunction. This result provides direct evidence that autophagy degrades Cx43, the main gap junction protein, in porcine oocytes as well as in other cells.

There is another PI3K inhibitor, 3-methylamine, which is known to PI3K class III inhibitor. It has been reported that 3-methylamine inhibited porcine meiotic progression [64]. In our experiment, although we did not use 3-methylamine to inhibit autophagy because 3-methylamine has been known to have an opposite effect to autophagic inhibition particularly in a circumstance of nutrient deficiency [33], the fact that PI3K classIII inhibition inhibited meiotic progression also support our idea that autophagy may participate in meiotic resumption. Another study that used wortmannin during porcine COC maturation suggested that PI3K regulate the Cx43 during porcine meiotic resumption [61]. Our discovery that autophagy degrade Cx43 in porcine oocyte can be the bridge when revealing the relationship between PI3K and Cx43.

Through this study, we noticed that autophagic activity and Cx43 change in porcine oocytes during maturation with similar manner. We suggest that activation of autophagy induced by AMPK activation and activated autophagy take a part in degrading gap junction. We also suggest that autophagy affect to oocyte maturation.

Referring previous research of others and our result, we infer that autophagy may replenish energy to oocytes, when oocytes demand more energy due to increment of metabolic activity caused by gonadotropin stimulation, by degradation and recycling of the long lived protein including gap junction (Fig. 10).

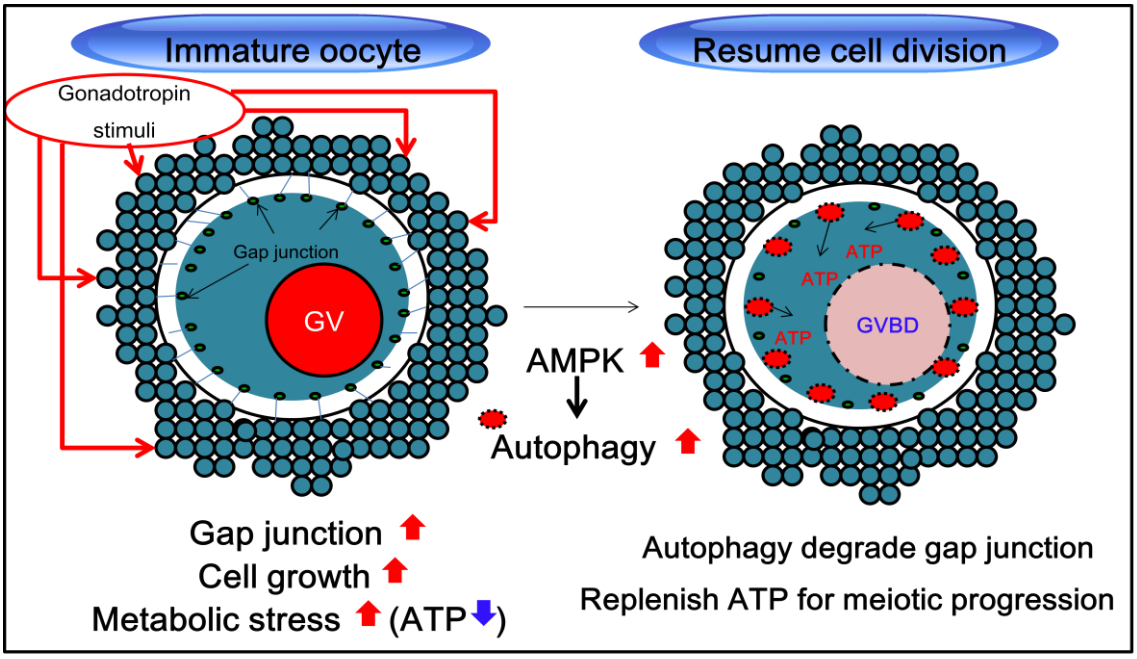

Figure 10. Proposed model of the autophagy degrade Cx43. Gonadotropin stimulate gap junction expression and cell growth. By progressing cell growth, metabolic stress is increased and ATP is depleted gradually. An elevation of AMP/ATP ratio activates AMPK. Consequently, autophagy which is induced by AMPK degrade gap junction protein and other long-lived protein. Autophagy produce energy and amino acid which is required for meiotic progression.

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