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Autophagy and ubiquitin-mediated proteolysis may not be involved in the degradation of spermatozoon mitochondria in mouse and porcine early embryos

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Summary

The mitochondrial genome is maternally inherited in animals, despite the fact that paternal mitochondria enter oocytes during fertilization. Autophagy and ubiquitin-mediated degradation are responsible for the elimination of paternal mitochondria in *Caenorhabditis elegans*; however, the involvement of these two processes in the degradation of paternal mitochondria in mammals is not well understood. We investigated the localization patterns of light chain 3 (LC3) and ubiquitin in mouse and porcine embryos during preimplantation development. We found that LC3 and ubiquitin localized to the spermatozoon midpiece at 3 h post-fertilization, and that both proteins were colocalized with paternal mitochondria and removed upon fertilization during the 4-cell stage in mouse and the zygote stage in porcine embryos. Sporadic paternal mitochondria were present beyond the morula stage in the mouse, and paternal mitochondria were restricted to one blastomere of 4-cell embryos. An autophagy inhibitor, 3-methyladenine (3-MA), did not affect the distribution of paternal mitochondria compared with the positive control, while an autophagy inducer, rapamycin, accelerated the removal of paternal mitochondria compared with the control. After the intracytoplasmic injection of intact spermatozoon into mouse oocytes, LC3 and ubiquitin localized to the spermatozoon midpiece, but remnants of undegraded paternal mitochondria were retained until the blastocyst stage. Our results show that paternal mitochondria colocalize with autophagy receptors and ubiquitin and are removed after *in vitro* fertilization, but some remnants of sperm mitochondrial sheath may persist up to morula stage after intracytoplasmic spermatozoon injection (ICSI).

Keywords: Autophagy, Mitochondria, Ubiquitin-mediated proteolysis

Introduction

Mitochondria are essential cell organelles that govern energy production and various signalling pathways, and they have their own DNA (Zhou *et al.*, 2011). While oocytes and spermatozoa have their own mitochondrial DNA (mtDNA), it is widely believed that mtDNA is maternally inherited, despite the fact that paternal mitochondria enter the ooplasm during fertilization in most species (Ankel-Simons & Cummins, 1996; Al Rawi *et al.*, 2011). One possible reason is that paternal mtDNA is diluted with an excess copy number of oocyte mtDNA. Each oocyte contains approximately 10^5 to 10^8 copies of mtDNA, exceeding that of spermatozoon by a factor of at least 10^3 (Birky, 2001; Jansen & de Boer, 1998). Another possible reason is that paternal mitochondria are selectively degraded and eliminated from the cytoplasm of the embryo after fertilization. Spermatozoon-derived mitochondria packed into the midpiece disappear

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during early embryogenesis (Aitken, 1995), typically by the 8-cell stage in the mouse (Cummins *et al.*, 1997). Digestion of paternal mtDNA in embryos is also observed (Kaneda *et al.*, 1995; Nishimura *et al.*, 2006). Although several studies have supported the notion that spermatozoon mitochondria are actively destroyed by the egg (Szollosi, 1965; Hiraoka & Hirao, 1988; Shalgi *et al.*, 1994; Sutovsky *et al.*, 1999, 2000, 2003), the precise mechanism is still unknown.

Autophagy, a conserved process of cell degradation, selectively removes mitochondria from mammalian and yeast cells (Wang & Klionsky, 2011). Autophagy commences with the formation of a specific membrane structure, the phagophore, which expands to engulf part of the cytosol. The phagophore, which contains the cytosol and its organelles, subsequently fuses with the lysosome for degradation (Yorimitsu & Klionsky, 2005). Two recently published papers have provided additional evidence for the involvement of autophagy, and p62 in particular, in sperm mitochondrion degradation in *Drosophila* and mammals (Politi *et al.*, 2014; Song *et al.*, 2014). Ubiquitination of proteins has recently been proposed to trigger selective autophagy (Kraft *et al.*, 2010), and autophagy receptors such as p62 and NBR1 can bind to ubiquitin and light chain 3 (LC3, an autophagy marker) (Kraft *et al.*, 2010), which is suggestive of interplay between the ubiquitin–proteasome system and autophagy in the removal of mitochondria. However, the mechanism of mitophagy is still elusive.

Degradation of paternal mitochondria in *Caenorhabditis elegans* occurs by fertilization-induced autophagy (Al Rawi *et al.*, 2011; Sato & Sato, 2011; Zhou *et al.*, 2011). Immediately after fertilization, spermatozoon-derived components trigger autophagy in the vicinity of spermatozoon mitochondria in *C. elegans.* Al Rawi *et al.* (2011) also demonstrated that mouse fertilization induces recruitment of LC3 to the spermatozoon midpiece, indicating that autophagy of paternal mitochondria may be conserved in mammals. Recently, by using two transgenic mouse strains, one bearing green fluorescent protein (GFP)-labelled autophagosomes and the other bearing red fluorescent protein (RFP)-labelled mitochondria, Luo & Sun (2013) suggested that autophagy is not responsible for the degradation of spermatozoon mitochondria and mtDNA in the mouse embryo after fertilization. While their results provide important insights on the elimination of paternal mitochondria in mouse embryos, the mechanism of autophagy remains unclear. For example, it is not known whether Atg5/Atg7 and LC3 (Nishida *et al.*, 2009) are involved in paternal mitochondrial removal.

To understand better the elimination of spermatozoon mitochondria in mammals, we traced parental mitochondria, and determined the localization of autophagy and ubiquitination markers in mitochondria in mouse and porcine embryos following fertilization. To investigate the involvement of autophagy in paternal mitochondria clearance, we used a chemical modulator of autophagy, 3-methyladenine (3-MA), which inhibits autophagy by blocking class III phosphoinositide 3-kinases (Blommaart *et al.*, 1995). This compound has been widely used as a pharmacological tool in autophagy studies (Sheng *et al.*, 2013). For example, it inhibits autophagy in tobacco culture cells under conditions of sucrose deprivation (Takatsuka *et al.*, 2004). Rapamycin, which inhibits mammalian target of rapamycin (mTORC1) and induces autophagy in mammalian cells, *Saccharomyces cerevisiae* and *Drosophila melanogaster*, was also used in this study (Blommaart *et al.*, 1995; Noda and Ohsumi, 1998; Kamada *et al.*, 2000; Rusten *et al.*, 2004). We also studied the elimination of spermatozoon mitochondria by autophagy and ubiquitin-mediated degradation following intracytoplasmic spermatozoon injection (ICSI).

Materials and methods

Ethics statement

Animals were cared for and used in accordance with guidelines set forth by the Animal Research Institute Committee of Chungbuk National University, Korea. Mice were housed in a temperature-controlled room with appropriate dark−light cycles, fed a regular diet, and maintained under the care of the Laboratory Animal Unit, Chungbuk National University, Korea. Mice were killed by cervical dislocation. This study was approved by the Animal Research Institute Committee, Chungbuk National University (approval no. CB-R28).

Antibodies and chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise. Rabbit polyclonal anti-LC3B (cat. no. 2775) and rabbit polyclonal anti-ubiquitin (cat. no. ab19247) antibodies were purchased from Cell Signaling (Danver, MA, USA) and Abcam (Cambridge, UK). Alexa Fluor® 488 and 568 goat anti-rabbit antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

Mitochondrial staining

To localize spermatozoon mitochondria, mice and porcine spermatozoa were incubated with 200 nM MitoTracker® Red CMXRos dye (Life Technologies, Carlsbad, CA, USA) for 30 min at 37°C in fertilization medium, before injection or *in vitro* fertilization (IVF).

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Mouse oocyte collection, IVF, ICSI, and embryo culture

Female mice were superovulated by 6 IU pregnant mare serum gonadotropin (PMSG), followed by 6 IU human chorionic gonadotropin (hCG) at 46–48 h later. Mice were euthanized 14–18 h thereafter. The oviducts were removed and dissected free from surrounding tissues using fine needles. For IVF, 5–10 cumulus–oocyte complexes (COCs) were transferred into each well of a 4-well plate. Each well contained 500 μl of human tubal fluid (HTF) medium. With a 20 μl micropipette, a spermatozoon suspension was transferred into each well containing oocytes (final spermatozoon concentration was approximately 10^6/ml). The oocytes and spermatozoon were incubated for 4 h in a humidified atmosphere of 5% CO_2 in air at 37°C.

For ICSI, a drop (~5 μl) of a dense spermatozoon mass from the cauda epididymis of a mouse was placed at the bottom of a 1.5 ml tube containing 200 μl of TL-HEPES medium for 30 min at 37°C to allow spermatozoa to swim up. ICSI was carried out with a piezo-driven unit using a previously described method (Tesarik *et al.*, 2002; Zheng *et al.*, 2012), except that our experiment was performed at room temperature in HEPES-CZB medium containing 5 μg/ml cytochalasin B (Sigma). Whole spermatozoon were injected into oocytes.

After IVF and ICSI, the embryos were washed several times and cultured in K-modified simplex optimized medium in a humidified atmosphere of 5% CO_2 in air at 37°C. Zygotes were sampled at 3, 6, 9, 12, and 15 h post-fertilization, and 2-cell embryos to blastocysts were sampled at 40, 53 and 64 h post-fertilization. Oocytes were collected at different culture times and used for immunostaining or microinjection. Zygotes or embryos at different developmental stages were fixed and stained with MitoTracker® Red (MTR), Hoechst 33342 (Invitrogen, 10 mg/ml), and an anti-LC3B or anti-ubiquitin antibody.

Porcine oocyte collection, IVF and embryo culture

Ovaries from prepubertal gilts were transported to the laboratory in saline (0.9% NaCl (w/v)) containing 100 μg/ml kanamycin sulphate at 37°C. They were then washed once with 0.04% cetrimide solution (w/v) and twice with saline. COCs were collected from follicles (3–6 mm in diameter) and washed twice in 35 mm Petri dishes containing Dulbecco's phosphate-buffered saline (DPBS) supplemented with 4 mg/ml bovine serum albumin (BSA). Groups of 50 COCs were cultured in 500 μl of maturation medium for 20–22 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C. After incubation, COCs were washed twice, transferred to fresh hormone-free maturation medium, and cultured for an additional 20–22 h.

COCs cultured for 44 h in maturation medium were stripped of the cumulus cells by gentle aspiration with a glass pipette. Oocytes were washed three times with TALP medium, and groups of 30–35 oocytes were transferred into each well of a 4-well multidish containing 250 μl of IVF medium previously equilibrated at 38.5°C. The spermatozoon suspension was added to the wells to obtain final concentration of 3000 cells/oocyte. At 2 h post-insemination (hpi), oocytes were washed twice with fresh PZM-5 medium by gentle pipetting with a glass pipette and incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

After 2 h (in the case of IVF), putative zygotes were washed three times with PZM-5 medium previously equilibrated overnight, transferred into microdrops at a rate of 5 μl of PZM-5 media/embryo, covered with mineral oil (embryo-tested) in a Petri dish, and incubated at 38.5°C for 7 days. Zygotes were sampled at 3, 6, 9, 12, and 15 h post-fertilization. Zygotes at different developmental stages were fixed and stained with Hoechst 33342 stain (Invitrogen) and an anti-LC3B or anti-ubiquitin antibody.

3-MA or rapamycin functional analysis

Embryos produced from IVF or ICSI were collected and washed three times with M16 medium and then cultured in the same medium containing 2 mM 3-MA (Sigma) or 50 ng/ml rapamycin (Sigma). Embryos in the control group were incubated with an equivalent volume of dimethylsulfoxide (DMSO). After IVF or ICSI, embryos were treated with compounds until the blastocyst stage, and embryos at each stage were stained with the LC-3B antibody. Each experiment was repeated at least three times. Images of treated embryos were captured with a phase-contrast microscope (Olympus America Inc., Center Valley, PA, USA).

Confocal microscopy

The protocol was similar to the one described previously (Jin *et al.*, 2012; Sun *et al.*, 2011, 2012). Oocytes were fixed in 4% paraformaldehyde (w/v) in PBS for 30 min at room temperature and then transferred to membrane permeabilization solution (0.5% Triton™ X-100 (v/v)) for 20 min. After 1 h in blocking buffer (1% BSA (w/v) in PBS), oocytes were incubated overnight at 4°C or for 4 h at room temperature with a 200-fold dilution of rabbit anti-LC3B (2775) or a 50-fold dilution of rabbit anti-ubiquitin antibody. After three washes with washing buffer (0.1% Tween® 20 (v/v) and 0.01% Triton X™-100 (v/v) in PBS), the oocytes were labelled with a 100-fold

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dilution of Alexa Fluor® goat-anti-rabbit 488 IgG or 568 IgG for 1 h at room temperature. The oocytes were stained with Hoechst 33342 for 10 min, followed by three washes in washing buffer. The oocytes were mounted onto glass slides, coverslipped, and examined with a confocal laser-scanning microscope (Zeiss LSM 710 META, Weimar, Germany). At least 30 oocytes were examined from each group.

Statistical analysis

At least three replicates were performed for each experiment. Statistical analyses were conducted using analysis of variance. Differences between treated groups were evaluated with Duncan's multiple comparison test. Data were expressed as means ± standard error of the mean (SEM), and P-values < 0.05 were considered significant.

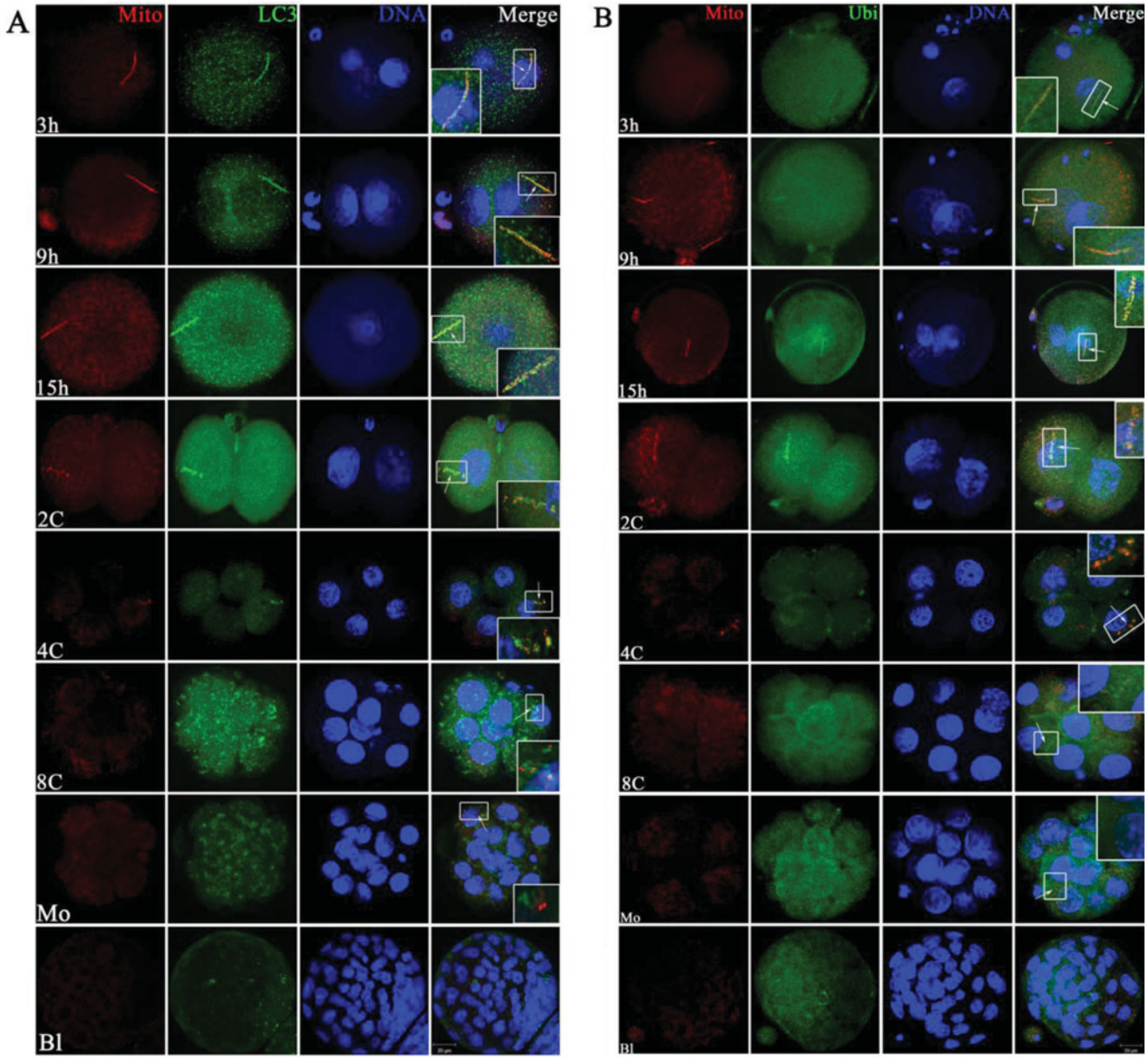

Figure 1 LC3 and ubiquitin localized to the spermatozoon midpiece in the mouse embryo from 3 h after fertilization until the 16-cell stage. (*A*) The localization of LC3 to paternal mitochondria decreased in 2-cell stage embryos and then disappeared by the 4-cell stage. Paternal mitochondria were absent from 8-cell stage embryos. (*B*) Ubiquitin localized to the spermatozoon midpiece from 3 h after fertilization until the 8-cell stage as well as to paternal mitochondria. The localization of ubiquitin to maternal mitochondria decreased in 2-cell stage embryos and then disappeared by the 4-cell stage. (*C*) The tails of mouse and porcine spermatozoa were negative for LC3 and ubiquitin staining. Bl, blastocyst; Mo, morula; Ubi, ubiquitin. MTR, red; LC3, green; and DNA, blue.

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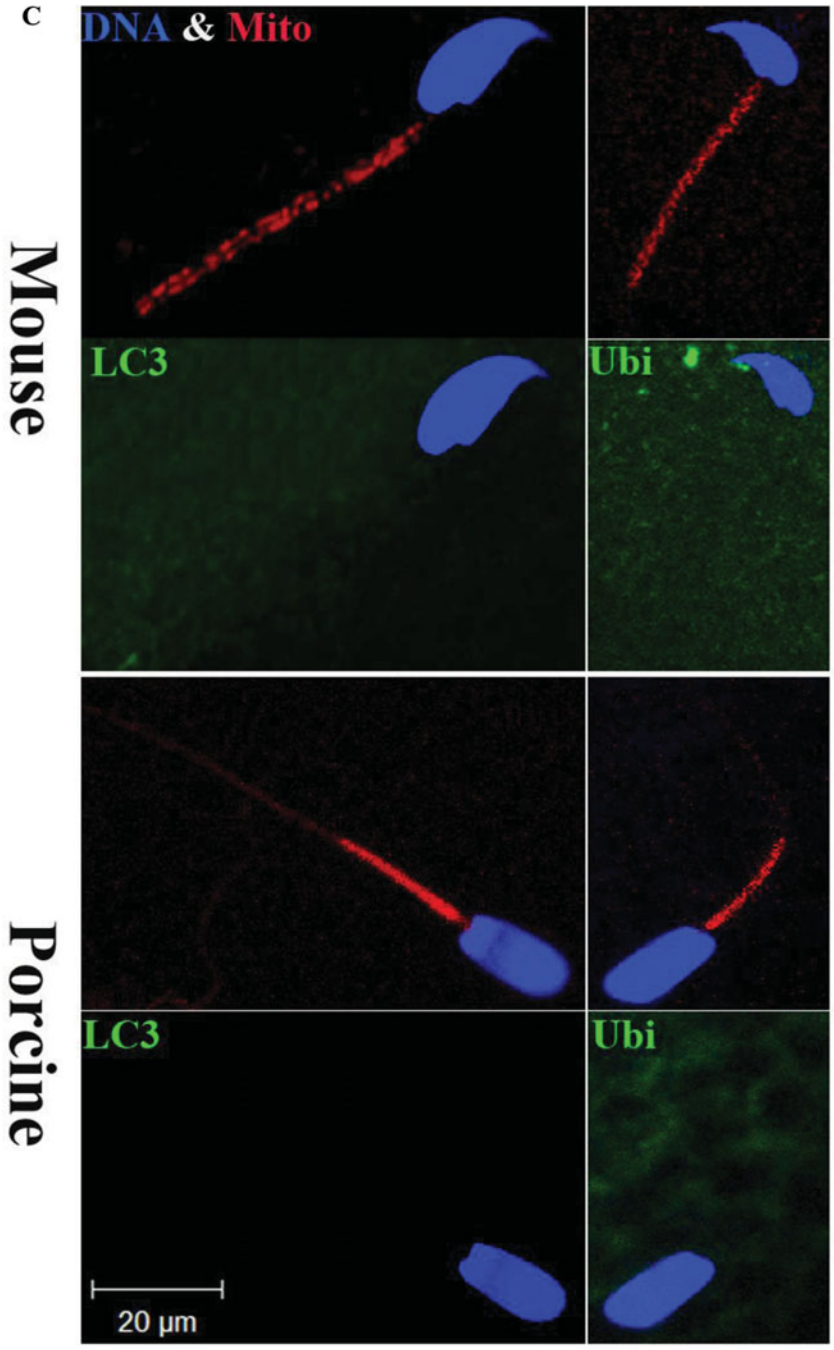

Figure 1 Continued.

Results

To monitor the distribution and ubiquitination of spermatozoon mitochondria after fertilization and embryogenesis, we prelabelled spermatozoon with a vital fluorescent probe, MTR, to track paternal mitochondria, followed by immunostaining with an anti-LC3B (Fig. 1*A*) or anti-ubiquitin antibody (Fig. 1*B*), or both LC3B and ubiquitin antibodies (Fig. 1*C*). In fertilized mouse oocytes before pronuclear fusion (3–15 h), MTR-positive paternal mitochondria were detected (Fig. 1*A*, *B*). The autophagy marker, LC3, was localized to mitochondria (Fig. 1*A*). In dividing embryos ($n = 67$), the staining of paternal mitochondria decreased gradually during development from the 2-cell to 8-cell stage (Fig. 1*A*). Likewise, ubiquitin was localized to paternal mitochondria from the early pronuclear stage until the 4-cell stage (Fig. 1*B*). In embryos beyond the morula stage, the staining of paternal mitochondria, LC3, and ubiquitin

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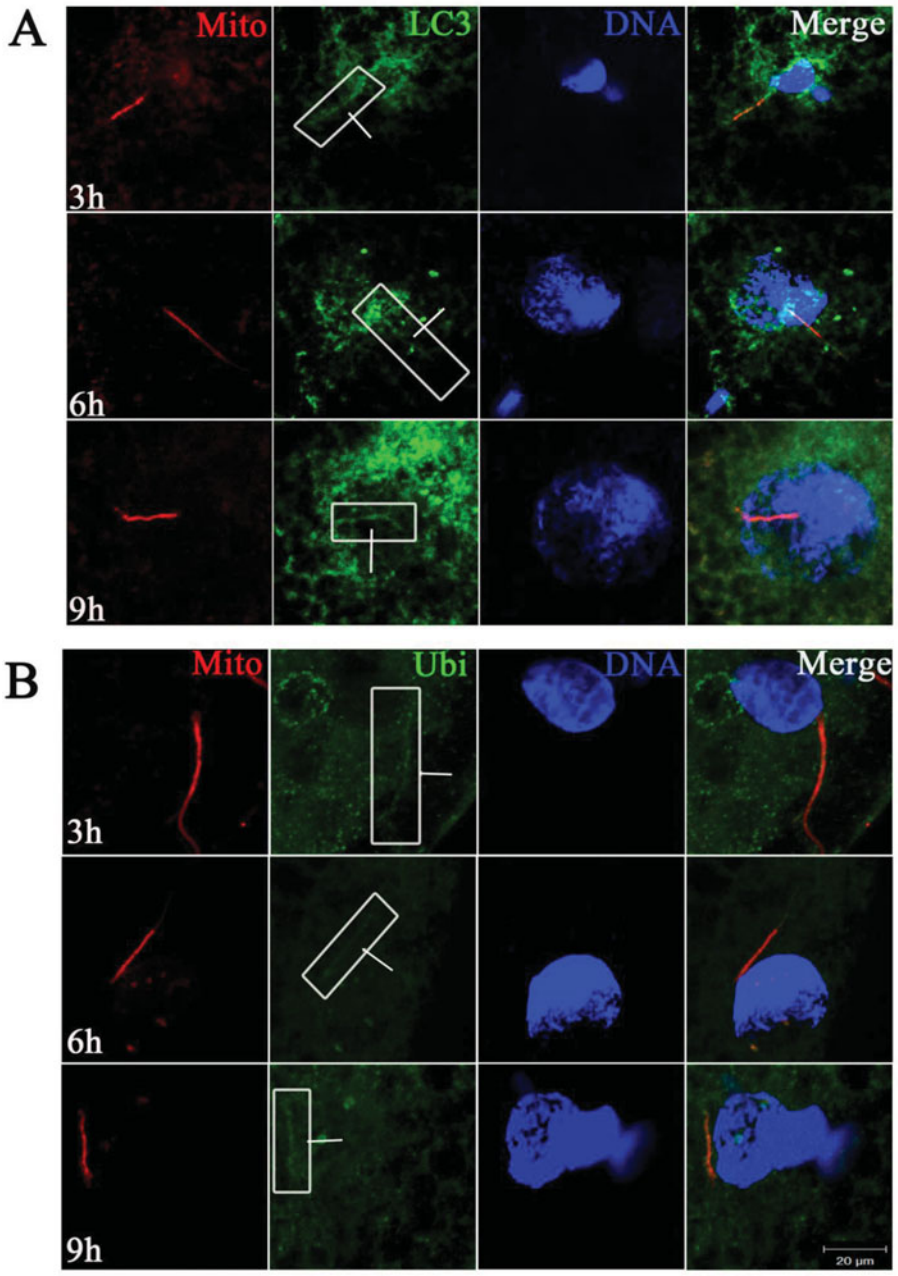

Figure 2 LC3 and ubiquitin localized to the spermatozoon midpiece in porcine embryos from 3–12 h after fertilization. (*A*) The localization of LC3 to paternal mitochondria decreased at 6 h and then disappeared by 12 h. (*B*) The localization of ubiquitin to paternal mitochondria decreased at 6 h and then disappeared by 12 h. MTR, red; LC3, green; and DNA, blue. Ubi, ubiquitin.

was examined in 40 morula stage embryos, with only three embryos staining positive for a paternal mitochondria signal (7.5%) (Fig. 1*A*, *B*). In agreement with results from the mouse, LC3 was localized to paternal mitochondria in porcine zygotes at the pronuclear stage of development (Fig. 2*A*). Specifically, LC3 was localized to paternal mitochondria at the early pronuclear stage (3 h) until the last 15 h culture in porcine embryos (Fig. 2*A*). Likewise, ubiquitin was localized to paternal mitochondria at the early pronuclear stage (Fig. 2*B*). In later stages of spermatozoon mitophagy in porcine embryos, the localization of LC3 and ubiquitin was similar to that in mouse embryos. However, the degradation of paternal mitochondria was faster in porcine embryos than in mouse embryos.

To investigate the effects of autophagy activation or inhibition on the clearance of paternal mitochondria,

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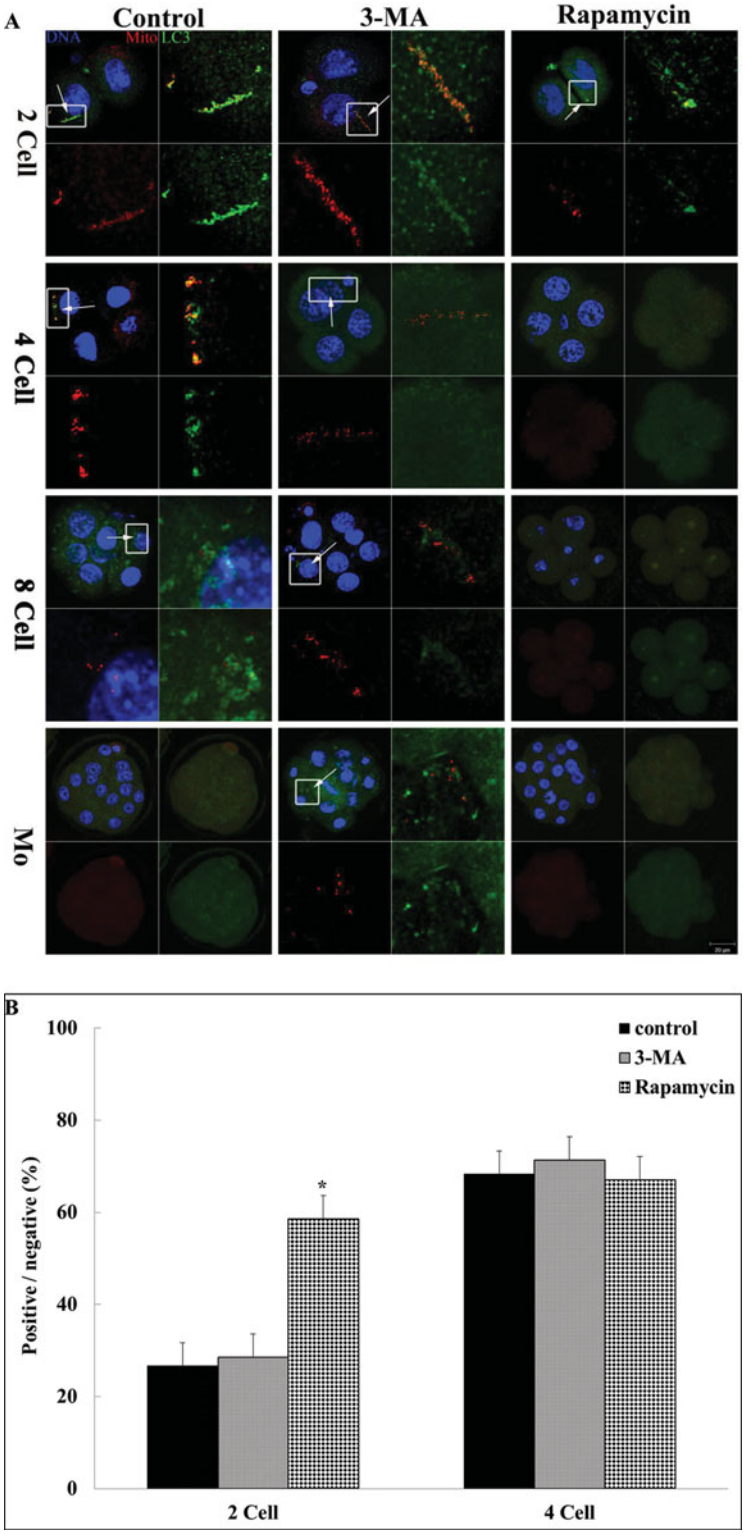

Figure 3 *In vivo* fertilized zygotes at 3 h post-fertilization (15 h after hCG injection) were collected and then cultured in HTF medium in the presence or absence of 3-MA or rapamycin. (*A*) LC3 localized to paternal mitochondria in embryos treated with 3-MA or rapamycin. LC3 signs correspond to the absence and presence of paternal mitochondria in 2-cell or 4-cell stage embryos. MTR, red; LC3, green; and DNA, blue. (*B*) The percentage of 2-cell or 4-cell stage embryos with paternal mitochondria. Mo, morula.

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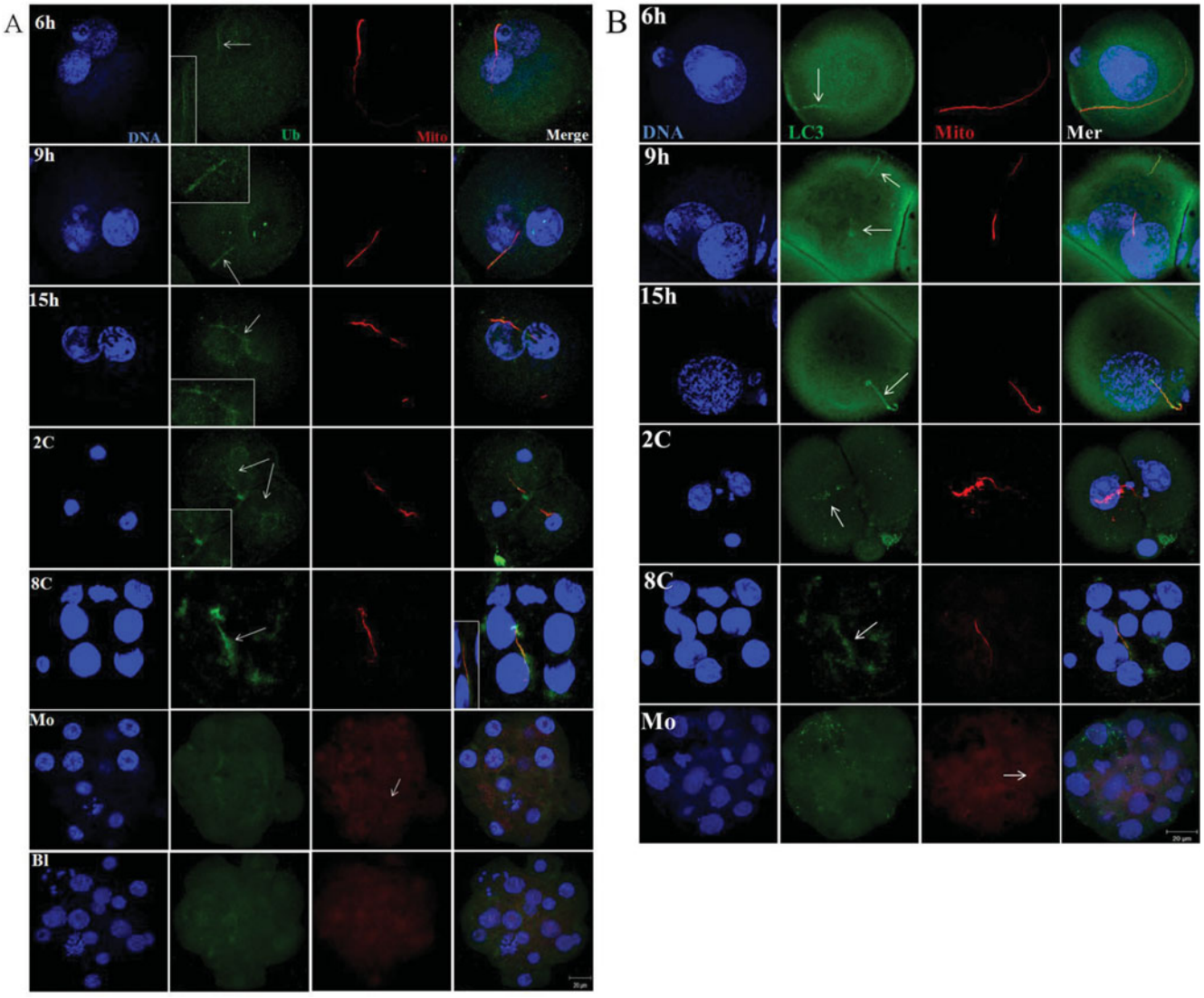

Figure 4 Degradation of paternal mitochondria after ICSI was delayed. (*A*) At 6 h after ICSI, ubiquitin localized to the spermatozoon midpiece in injected zygotes in the mouse. Ubiquitin also localized to paternal mitochondria. Paternal mitochondria were present in embryos from the zygote stage until the morula stage. (*B*) LC3 immunoreactivity and paternal mitochondria were present in single-cell stage to 8-cell stage embryos after injection, and LC3 localized to paternal mitochondria. Paternal mitochondria were absent from spermatozoon tails in morula stage embryos, but not LC3 immunoreactivity. Bl, blastocyst; Mo, morula. MTR, red; LC3, green; and DNA, blue.

we exposed single-cell stage embryos to rapamycin, an autophagy activator, or 3-methyladenine (3-MA), an autophagy inhibitor. As shown in Fig. 3*A*, LC3 localized to paternal mitochondria in embryos treated with 3-MA or rapamycin. Although rapamycin decreased the staining of paternal mitochondria in 2-cell stage embryos, there was no difference after 3-MA treatment (Fig. 3*B*). Both compounds did not affect the staining of paternal mitochondria in 4-cell stage embryos (Fig. 3*B*).

When MTR-labelled whole spermatozoon were used for ICSI, autophagosomes colocalized with paternal mitochondria in early pronuclear stage zygotes (Fig. 4*A*). Paternal mitochondria were detectable in at least, Furthermore, LC3 did not localize entirely to mitochondria after the 8-cell stage (Fig. 4*A*). It also localized to spermatozoon tails, indicating that spermatozoon mitochondria in mouse embryos after ICSI were not degraded by autophagy. Ubiquitin also localized to spermatozoon tails (Fig. 4*B*). Degradation of paternal mitochondria was delayed until the morula stage in 46.1% (18/39) of embryos after ICSI compared with 7.5% of embryos after IVF (Fig. 4*A*, *B*). In addition, single-cell stage embryos were treated with 3-MA or rapamycin. After ICSI, mitochondria remained in 68.6% (24/35) of morula stage embryos

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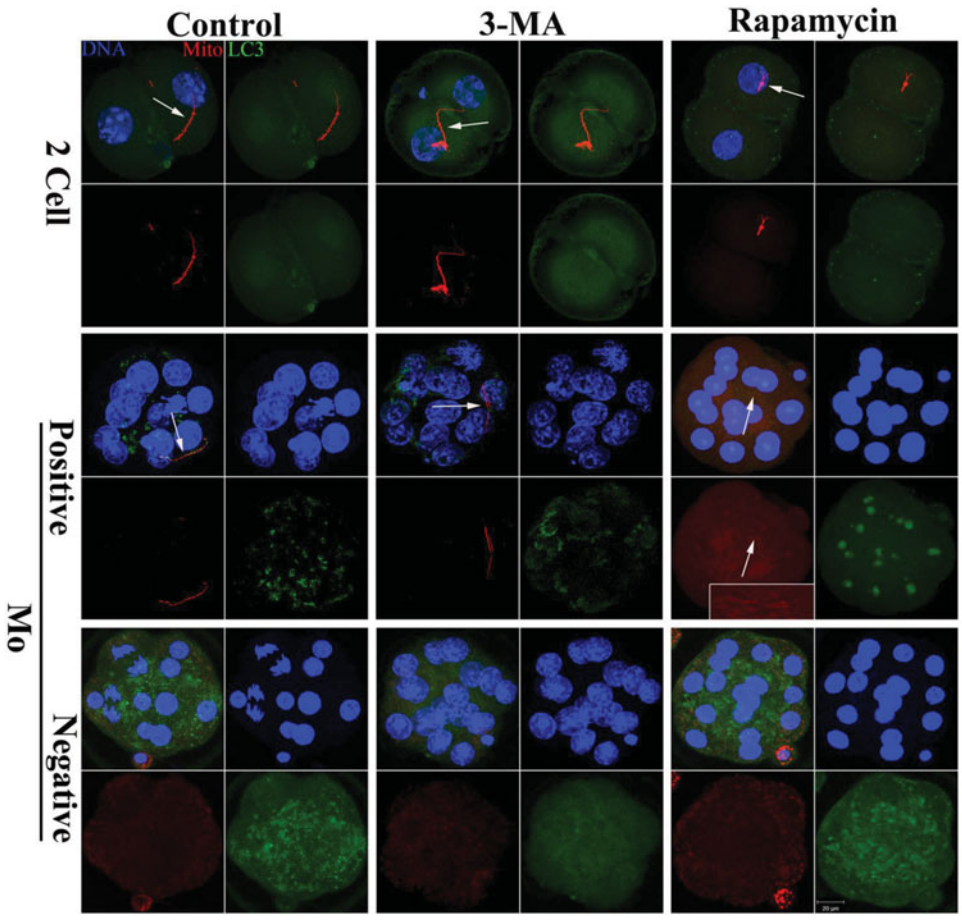

Figure 5 After ICSI, oocytes were cultured in HTF medium in the presence or absence of 3-MA or rapamycin after stage. (*A*) LC3 localized to paternal mitochondria in embryos treated with 3-MA or rapamycin. The localization of LC3 corresponds to the absence or presence of spermatozoon tails in morula stage embryos. MTR, red; LC3, green; and DNA, blue. Mo, morula.

treated with 3-MA compared with 47.7% (21/44) of embryos treated with rapamycin (Fig. 5). $P<0.01$

Discussion

A previous report has shown ubiquitin-mediated proteolysis to mediate the removal of paternal mitochondria in fertilized mammalian embryos (Sutovsky *et al.*, 1999, 2000, 2003). Conversely, autophagy is the primary mechanism for the removal of paternal mitochondria in *C. elegans* embryos (Al Rawi *et al.*, 2011; Sato & Sato, 2011; Zhou *et al.*, 2011). Lysosomes also participate in the degradation of paternal mitochondria (Sutovsky *et al.*, 2000). We showed that the autophagy marker LC3 localized to paternal mitochondria in both *in vivo* and *in vitro* pronuclear and early cleavage embryos. In addition, we observed the localization of ubiquitin to paternal mitochondria, as previously reported (Sutovsky *et al.*, 1999, 2000, 2003). Our results from 3-MA and rapamycin experiments indicate that autophagy is not the sole mechanism of mitochondria removal in mammalian cells.

Recently, Luo *et al.* (2013) have demonstrated the fate of paternal-derived mitochondria in mouse zygotes using two transgenic mouse strains that expressed either GFP-LC3 or RFP-P62 (Luo *et al.*, 2013; Sato & Sato, 2013). In their study, they show that GFP-LC3 colocalizes to single-cell stage embryos, but it is dissociated at the four-cell stage, which is in agreement with our results in mouse and porcine embryos generated by IVF. Although rapamycin accelerated paternal mitochondria removal in early stage embryos *in vitro*, the role of autophagy in the elimination of paternal mitochondria under physiological conditions is not clear. In somatic cells, paternal mitochondria removal may occur via Atg5–Atg5-independent autophagy (Nishida *et al.*, 2009).

Previous studies show that paternal mitochondrial DNA is absent from embryos produced by ICSI (Houshmand *et al.*, 1997; Torroni *et al.*, 1998), suggesting that paternal mitochondria clearance occurs

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even in the absence of normal fertilization. In our study, the colocalization of autophagy and proteasome markers on paternal mitochondria was observed in ICSI embryos and blastocyst stage embryos. The delay in the clearance of paternal mitochondria in ICSI or treatments 3-MA group indicates that a spermatozoon-derived factor(s) and/or fertilization-specific component(s) may be involved in this process. The precise mechanism of paternal mitochondria clearance is open to investigation.

In conclusion, our results show that autophagy and proteasome markers colocalize to paternal mitochondria in pronuclear and early cleavage embryos. Furthermore, paternal mitochondria are degraded prior to the morula stage in naturally fertilized murine and porcine embryos but not in mouse ICSI embryos where they can persist up to the morula stage.

Acknowledgements

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