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ORIGINAL RESEARCH

Impact of endoplasmic reticulum stress on oocyte aging mechanisms

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ABSTRACT: Endoplasmic reticulum (ER) stress is associated with several aging-related diseases; however, the mechanism underlying age-related deterioration of oocyte quality is unclear. Here, we used post-ovulatory, *in vivo* aged mouse oocytes as a model. Super-ovulated oocytes harvested from the oviduct at 14 h and 20 h post-hCG injection were designated as 'fresh' and 'aged', respectively. Embryo development following IVF was compared between fresh, aged and ER stress-induced oocytes. Expression of the ER stress marker GRP78 was examined at each stage. To evaluate the effect of salubrinal, an ER stress suppressor, on embryo development following IVF, expression levels of GRP78 and phospho-eukaryotic initiation factor 2 alpha were compared between aged and salubrinal-treated aged oocytes. Embryo transfer of salubrinal-treated aged oocytes was performed to examine the safety of salubrinal. Similar to aged oocytes, ER stress-induced oocytes showed lower fertilization rates and poor embryo development. Following IVF, expression of GRP78 decreased with embryo development. GRP78 expression was significantly higher in aged oocytes than in fresh oocytes. Salubrinal lowered GRP78 levels and improved embryo development. No adverse effect of salubrinal treatment was found on the birth weight of pups or on organogenesis in mice. The limitation of this study was that protein kinase-like ER kinase was the only ER stress pathway examined; the role of IRE1 and ATF6 pathways was not considered. Nevertheless, salubrinal can significantly improve embryo development in *in vivo* aged oocytes undergoing ER stress. Hence, regulation of ER stress might represent a promising therapeutic strategy to overcome poor oocyte quality.

Key words: oocyte aging / mouse oocyte / endoplasmic reticulum / ER stress / GRP78 / PERK / eIF2α / salubrinal

Introduction

Aging is widely known to reduce female fecundity and worsen reproductive outcomes. Low pregnancy rates and high miscarriage rates result in fewer live births. The age-associated decline in female fertility is largely attributed to a decrease in oocyte quality caused by ovarian aging (American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee of the American Society for Reproductive Medicine, 2014; Heffner, 2004). Oocyte quality is a key determinant of embryo development (Miao *et al.*, 2009). Although the mechanisms underlying the decline in oocyte quality remain unknown, oocyte quality is thought to be responsible for fertilization and subsequent embryo development. Oxidative stress damages various cellular components, including mitochondria, lipids, proteins, enzymes and DNA. In particular, mitochondria are a key target of oxidative stress as they affect cell survival and death. As well as with other age-related disorders, mitochondrial dysfunction may play a central role in the qualitative deterioration of aged oocytes.

In vivo, post-ovulatory aging of oocytes that are not fertilized immediately after ovulation is known to significantly affect the development of mammalian oocytes (Tarin *et al.*, 2000), frequently resulting in a lower fertilization rate (Smith and Lodge, 1987; Takahashi *et al.*, 2003), chromosomal anomalies (Saito *et al.*, 1993; Mailhes *et al.*, 1998) and poor embryo development (Marston and Chang, 1964; Tarin *et al.*, 1999). We have previously studied the mechanisms of defective embryo development using *in vivo* aged oocytes (Igarashi *et al.*, 1997; Takahashi *et al.*, 2003; Igarashi *et al.*, 2005; Igarashi *et al.*, 2015) and found that *in vivo* aged mouse oocytes represents a good model for studying low-quality oocytes (Igarashi *et al.*, 1997). Using post-ovulatory, *in vivo* aged mouse oocytes, we demonstrated that transplantation of mitochondria from somatic cells did not improve the low fertilization and embryo development rate of aged mouse oocytes in spite of a significantly improved mitochondrial function (Igarashi *et al.*, 2016). These results suggest that other factors could be involved in oocyte aging and that endoplasmic reticulum (ER) stress could be a possible candidate.

ER stress is essential for maintaining cellular function through coordinated upregulation of several signaling pathways such as the unfolded protein response (UPR). The UPR plays a fundamental role in maintaining cellular homeostasis under normal physiological conditions. However, prolonged or above-threshold and unresolved ER stress may lead to apoptosis. Malfunctioning of the ER stress response has been implicated in several age-related diseases, such as neurodegenerative disorders (Katayama *et al.*, 1999), diabetes mellitus (Ozcan *et al.*, 2004), arteriosclerosis (Kedi *et al.*, 2009) and cancer (Lee, 2007). Similarly, ER stress in oocytes and preimplanting embryos may be involved in oocyte aging and affect embryo development. In addition, alterations of calcium homeostasis are closely related to ER stress (Luciani *et al.*, 2009; Fu *et al.*, 2011), since *in vivo* aged mouse oocytes have been shown to exhibit dysregulated intracellular calcium due to dysfunctional ER (Igarashi *et al.*, 1997). Such evidence suggests the possibility that ER stress is relevant to oocyte aging.

The present study investigated the involvement of ER stress in mechanisms of oocyte aging, using an *in vivo* aged mouse oocyte model. We also examined whether regulation of ER stress could improve defective embryo development in these *in vivo* aged oocytes.

Materials and methods

Ethical approval for animal studies

This study was performed with permission from the Committee of Animal Experimentation at the Yamagata University Faculty of Medicine (approval number, 30056).

Reagents

Human tubal fluid (HTF) medium was used for oocyte maintenance and IVF. K-supplemented simplex optimized medium (KSOM) was used for embryo culture. HTF and KSOM media were used following equilibration with 5% CO_2 in air at 37°C. HEPES-HTF medium was used at the time of handling mouse oocytes in contact with air. Unless otherwise stated, media contained 10% Serum Substitute Supplement (Irvive Scientific, USA). Salubrinal, a specific inhibitor of the eukaryotic initiation factor 2 alpha (eIF2α) phosphatase, was purchased from Sigma Aldrich (SML0951, USA). Reagents for culture media preparation were of tissue-grade quality. The antibody against glucose-regulated protein 78 (GRP78) was purchased from Abcam (ab21685, UK), whereas antibodies against eIF2α (#9722S) and phospho-eIF2α (#9721S, Ser51) were purchased from Cell Signaling Technology (USA). Secondary anti-mouse or anti-rabbit antibodies were purchased from GE Healthcare (UK). Other reagents were obtained from Sigma Aldrich (USA) unless otherwise indicated.

Oocyte preparation

Oocyte preparation was performed as described previously (Igarashi *et al.*, 1997). In brief, B6C3F1 hybrid female mice (4–6 weeks of age) were super-ovulated by intraperitoneal injection of 10 IU equine chorionic gonadotropin (CG) (Asuka, Japan), followed by intraperitoneal injection 48 h later with 10 IU hCG (Asuka). Unfertilized oocytes were harvested from the oviduct at 14 or 20 h post-hCG treatment, using a fine needle to disperse droplets of HEPES-HTF medium containing 300 IU/ml hyaluronidase. Then, cumulus cells were removed by repeated aspiration with a narrow-bore pipette. Cumulus-free oocytes were washed five times in HEPES-HTF medium and incubated in HTF medium at 37°C with 5% CO_2 in air. Oocytes harvested from the oviduct at 14 h and 20 h post-hCG treatment were designated as 'fresh oocytes' and 'aged oocytes', respectively.

IVF and embryo culture

The IVF procedure and assessment of embryonic development were performed as described previously (Takahashi *et al.*, 2009). In brief, denuded oocytes in 1000 µl HTF medium were placed in a 35-mm plastic dish, covered with mineral oil, and cultured in 5% CO_2 in air at 37°C until sperm insemination. Sperm were collected from the cauda epididymis of male ICR mice (10 weeks of age) and preincubated in 300 µl HTF medium for 90 min to allow capacitation. Then, the sperm were added to 1000 µl HTF containing denuded oocytes at a final concentration of $1–2 \times 10^5$ spermatozoa/ml. The mixture was incubated for 4 h in HTF medium at 37°C in 5% CO_2 in air to allow for fertilization. The fertilized embryos were washed three times in HEPES-HTF to remove the remaining spermatozoa, and then cultured in KSOM for 5 days in 5% CO_2 in air at 37°C. The fertilized oocytes were assessed as two-cell embryos at 24 h after insemination. Embryo development at each stage was assessed visually every 24 h after insemination.

Treatment with ER stress inducer and inhibitor

To induce ER stress, denuded oocytes were treated with thapsigargin or tunicamycin (both Sigma Aldrich, USA). Thapsigargin is a high-affinity specific inhibitor of Ca^{2+}-ATPase on the sarcoplasmic reticulum and ER, while tunicamycin inhibits N-linked glycosylation. Both compounds are commonly used to induce ER stress experimentally in various cell types (Foufelle and Fromenty, 2016). Denuded oocytes were incubated in HTF medium with thapsigargin (5 µM, 2 h) or tunicamycin (5.0 µg/ml, 4 h). By targeting dephosphorylation, salubrinal inhibits global protein translation and has been described as a specific inhibitor of ER stress-induced apoptosis in various cell types (Foufelle and Fromenty, 2016). To examine the inhibitory effect of salubrinal on ER stress, denuded oocytes were incubated in HTF medium with salubrinal (0, 1, 5 or 10 µM) and dimethyl sulfoxide (10, 9, 5 or 0 µM, respectively) for 1 h before IVF.

Western blotting

Western blotting was performed to examine GRP78, eIF2α and phospho-eIF2α (Ser51) expression in oocytes and at each stage of embryo development. Fifty denuded oocytes or embryos were washed three times in phosphate-buffered saline (PBS), resuspended in 15 µl PROPREP (iNtRON Biotechnology, Korea), and incubated for 20 min at −20°C to extract proteins. After centrifugation for 5 min at 13 000 RPM and 4°C, samples were mixed with 1% (w/v) sodium dodecyl sulfate and boiled for 10 min. Samples were then applied onto 10% (w/v) polyacrylamide gels for electrophoresis (500 V, 25 mA) and transferred (100 V, 100 mA) to polyvinylidene fluoride membranes. Membranes were washed with Tris-buffered saline with 0.5% (v/v) Tween-20 (TBS-T) three times and blocked with 5% (w/v) skimmed

milk in TBS-T for 60 min. Membranes were then incubated with primary anti-GRP78 (1:4000), anti-eIF2α (1:500), ant-phospho-eIF2α (1:500) or anti-ß-actin (1:500) in 5% skimmed milk in TBS-T overnight at 4°C. Thereafter, membranes were washed three times in 5% skimmed milk with TBS-T and incubated with anti-rabbit or anti-mouse secondary antibodies (1:5000) in 5% skimmed milk with TBS-T for 60 min at room temperature. After three more washes with TBS-T, membranes were exposed using 20% (v/v) Immunostar (Wako, Japan).

Analysis of blastomere death by fluorescence microscopy

To identify the non-viable cells, we used propidium iodide (PI; Thermo Fisher Scientific) and Hoechst 33342 (Thermo Fisher Scientific, Japan) staining as described previously (Farin *et al.*, 2001; Safian *et al.*, 2016). Blastomere death was evaluated by double staining with PI-Hoechst using a fluorescence microscope (DMI3000B; Leica, Germany). Blastocysts were washed three times with PBS and fixed in 4% (v/v) paraformaldehyde for 1 h at room temperature. After three more washes with PBS, blastocysts were incubated for 15 min in 1000 µl HEPES-HTF with 5 µl Hoechst 33342 and 10 µl PI. Blastocysts were washed three times with HEPES-HTF and observed under a fluorescence microscope. The rate of dead blastomeres was calculated as the ratio of PI-positive blastomeres to Hoechst 33342-positive blastomeres.

Transfer of blastocysts derived from aged oocytes and salubrinal-treated aged oocytes

We prepared pseudo-pregnant ICR female mice (10 weeks of age) by mating them with castrated ICR male mice. Defining the mating day as P0, blastocysts derived from the aged oocytes and the salubrinal-treated aged oocytes were transferred on P3. Then, pregnant mice were sacrificed on P19 and pups were obtained from the uterus. Birth weight and number of live pups were counted.

Statistical analysis

Except for the embryo transfer experiment, all procedures were performed interdependently at least three times. Fertilization rate and embryo development following IVF were compared using the χ^2 test. The significance of differences between groups was judged by one-way ANOVA followed by *post hoc* analysis with Tukey's test using GraphPad PRISM Version 5 (GraphPad Software, USA). All the data are expressed as mean ± standard error. Significant differences were defined as $P < 0.05$.

Results

Post-ovulatory aging and ER stress lower the fertilization rate and impair embryo development

Aged oocytes showed significantly lower rates of fertilization and embryo development than fresh oocytes (Fig. 1A). ER stress-induced oocytes treated with thapsigargin showed similar fertilization and embryo development patterns as aged oocytes (Fig. 1B). Interestingly, oocytes in which ER stress was induced by tunicamycin presented a completely different embryo development pattern compared to aged oocytes or thapsigargin-treated oocytes (Fig. 1C), showing deteriorating embryo development intensely from the morula stage onward.

ER stress is critical for cell survival, but excessive or prolonged ER stress can lead to apoptosis. We hypothesized that defective embryos development observed in aged and ER stress-induced oocytes was caused by an apoptotic process and was responsible for deterioration of blastomeres. The rate of dead blastomeres, defined by the ratio of PI-positive to Hoechst 33342-positive blastomeres, was significantly higher in blastocysts derived from aged and ER stress-induced oocytes compared with those from fresh oocytes (Fig. 1D and E).

GRP78 expression increases in aged oocytes

GRP78 is an established and reliable ER stress-related marker. Therefore, western blotting analysis was carried out to examine the level of GRP78 during embryo development. GRP78 expression was highest in MII oocytes and one-cell embryos, and then decreased with the embryo development after IVF (Fig. 2A). We hypothesized that the excessive ER stress in aged oocytes was closely related to poor embryo development, and thus we compared GRP78 levels between aged and fresh oocytes. Using thapsigargin-treated oocytes as a positive control, we found that GRP78 expression was significantly higher in aged oocytes than in fresh ones (Fig. 2B).

Salubrinal treatment improves embryo development of aged oocytes

We next investigated the effects of salubrinal, on embryo development. As fresh oocytes showed satisfactory embryo development (Fig. 1), we used aged oocytes as controls to evaluate the potential effects of salubrinal on improving embryo development. Aged oocytes were pretreated with 1, 5 and 10 µM salubrinal for 1 h at the MII stage before IVF. We chose this stage as it showed the highest GRP78 expression and thus the ER stress inhibitor was expected to have maximum effect. Salubrinal significantly increased post-IVF embryo development at all the concentrations used as compared to non-treated samples (Fig. 3A). Specifically, development to the four-cell stage and morula stage, blastocyst formation and the number of hatching/hatched blastocysts were significantly improved following 5 µM salubrinal treatment (Fig. 3A). The rate of dead blastomeres in blastocysts derived from 5 µM salubrinal-treated aged oocytes was significantly lower than in blastocysts from non-treated aged oocytes (Fig. 3B and C).

Salubrinal treatment alters the expression of GRP78, eIF2α and phospho-eIF2α in aged oocytes

Even though the expression of GRP78 was significantly increased in aged oocytes as compared with fresh ones, 5 µM salubrinal treatment significantly decreased the GRP78 level in aged oocytes, and this trend continued at least 4 h after treatment (Fig. 4A and B). Expression of eIF2α did not change significantly between fresh, aged and 5 µM

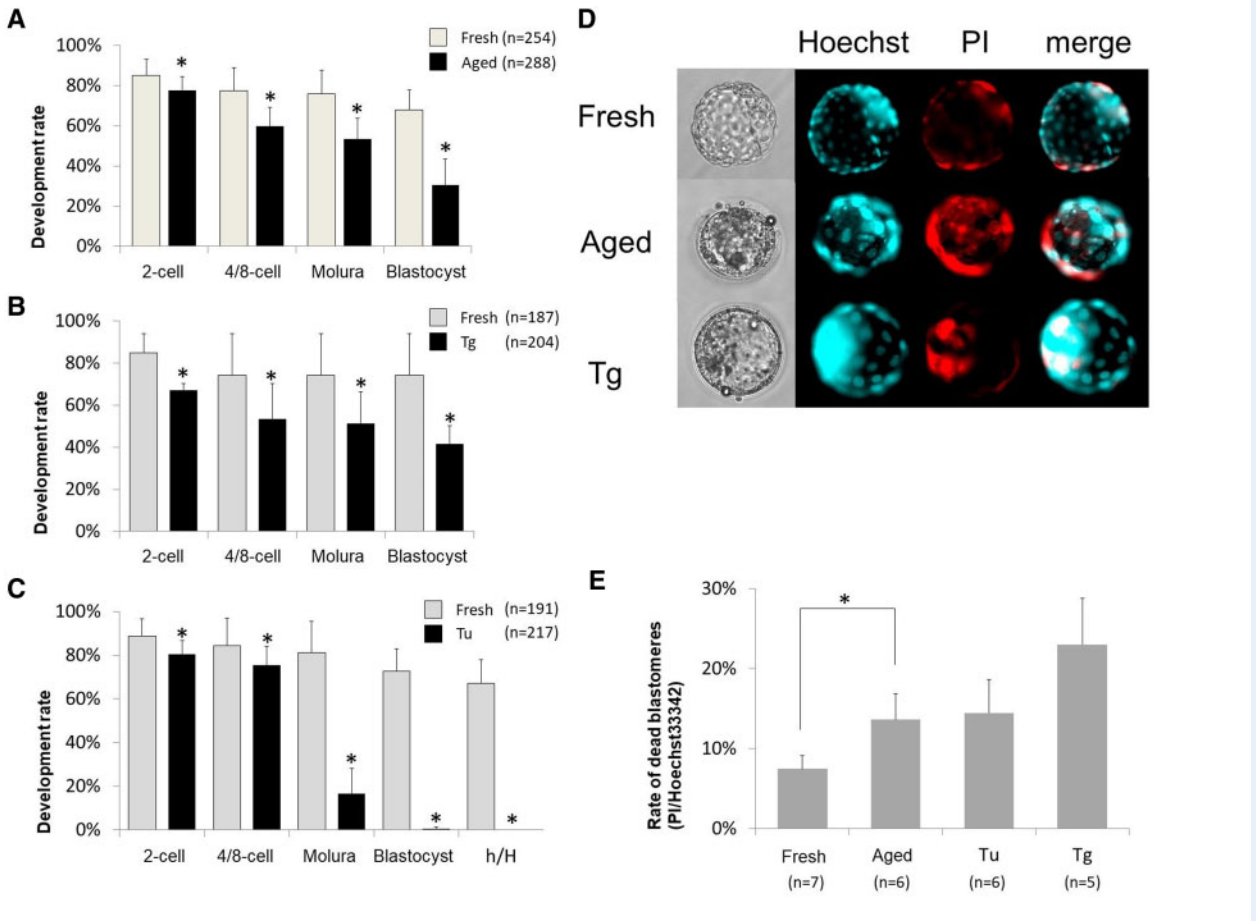


Figure 1 Embryo development and rate of dead cells. (**A**) Fertilization and embryo development in fresh and aged oocytes. (**B**) Fertilization and embryo development in oocytes, in which Endoplasmic reticulum (ER) stress was induced by 5 μM thapsigargin. (**C**) Fertilization and embryo development in oocytes, in which ER stress was induced by 5 μg/ml tunicamycin. (**D**) PI/Hoechst33342 immunofluorescence staining showing dead blastomeres in each blastocyst. (**E**) Rates of dead blastomeres in fresh oocytes, aged oocytes and oocytes treated with either thapsigargin or tunicamycin. Bars with different letters represent significant differences ($P < 0.05$). Embryo development data represents the mean ± standard error of at least five determinations performed independently. Immunofluorescence staining data represents the mean ± standard error of five determinations performed independently. *$P < 0.05$; h, hatching embryo; H, hatched embryo; Tg, thapsigargin; Tu, tunicamycin.

salubrinal-treated aged oocytes (Fig. 4A and C). Even though phospho-eIF2α expression was decreased significantly in aged and 5 μM salubrinal-treated oocytes, a 4 h incubation after salubrinal treatment significantly increased the level of phospho-eIF2α (Fig. 4A and D).

Salubrinal treatment is safe for delivered pups

As salubrinal is known to suppress protein synthesis through phospho-eIF2α and this might result in embryonic lethality or teratogenesis, we examined the safety of salubrinal to delivered pups. To validate the safety of salubrinal treatment, we transferred embryos derived from salubrinal-treated aged oocytes to mice. Sixteen blastocysts were transferred in each mouse (four blastocysts per horn of each pseudo-pregnant female mouse). Salubrinal treatment had no effect on pup weight and no congenital malformation was observed (Fig. 5). Although no significant effect on the rate of live births ($P = 0.20$) was seen, the implantation rate was significantly higher in the salubrinal-treated group than in the aged group.

Discussion

In the present study, we show that ER stress was higher in *in vivo* aged mouse oocytes than in the fresh oocytes. Moreover, ER stress decreased with embryo development. Importantly, ER stress regulation by pretreatment of MII oocytes with salubrinal suppressed the protein kinase-like ER kinase (PERK) pathway involved in ER stress and significantly improved the embryo development in post-ovulatory aged mouse oocytes. These data suggest that an increase in ER stress in post-ovulatory aged oocytes, especially in MII oocytes, might contribute to oocyte aging.

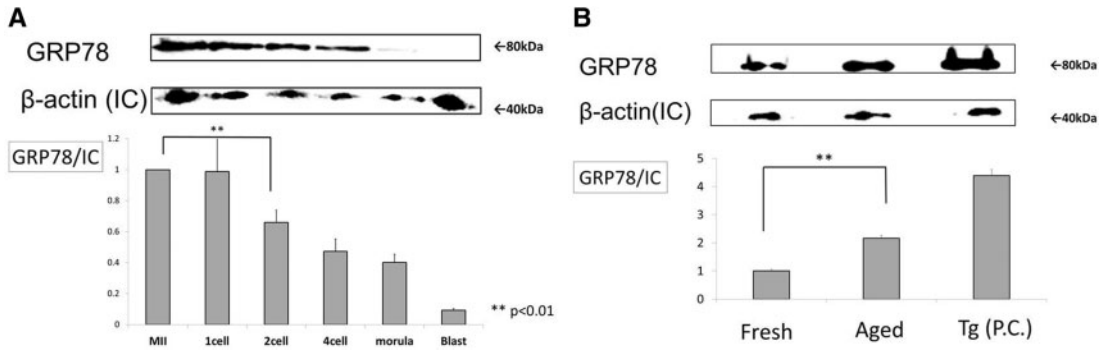


Figure 2 GRP78 expression. (**A**) Changes in GRP78 expression during embryo development after IVF of fresh oocytes. GRP78 bands normalized to β-actin are shown in the lower panel, with the GRP78 band density in MII oocytes set to 1.0. (**B**) GRP78 expression in fresh, aged and ER stress-induced oocytes (5 μM thapsigargin-treated oocytes were used as positive control). Lysates equivalent to 50 oocytes were loaded onto each lane and β-actin was used as internal control. Each value represents the mean ± standard error of three determinations performed independently. *$P < 0.05$, **$P < 0.01$; Blast, blastocyst; Tg, thapsigargin; IC, internal control; PC, positive control. Full blot images are in Supplementary information.

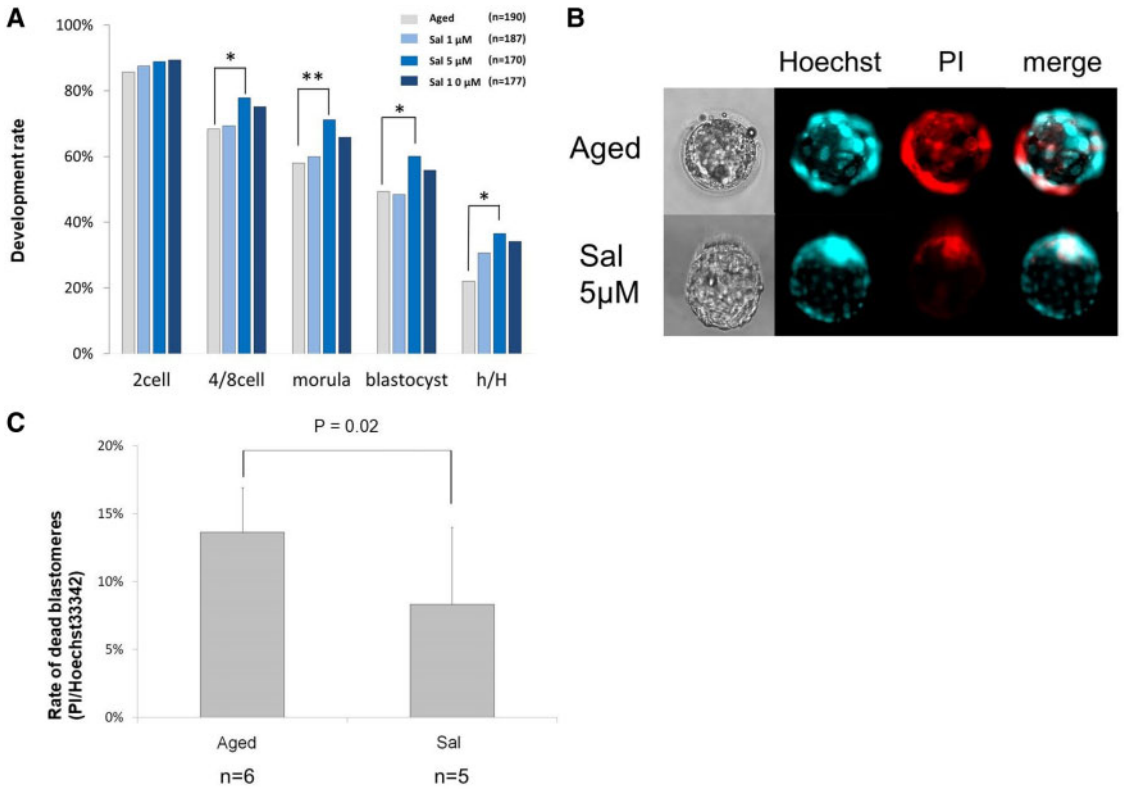


Figure 3 Embryo development and rate of dead blastomeres in salubrinal-treated and untreated aged oocytes. (**A**) Effect of 1, 5 and 10 μM salubrinal treatment before IVF on embryo development of aged oocytes. (**B**) PI/Hoechst33342 immunofluorescence staining showing dead blastomeres in blastocysts. (**C**) Rate of dead blastomeres in aged oocytes treated with 5 μM salubrinal and untreated oocytes. Embryo development data represents the mean ± standard error of seven determinations performed independently. Immunofluorescence staining data represents the mean ± standard error of five determinations performed independently. *$P < 0.05$, **$P < 0.01$; h, hatching embryo; H, hatched embryo; Sal, salubrinal.

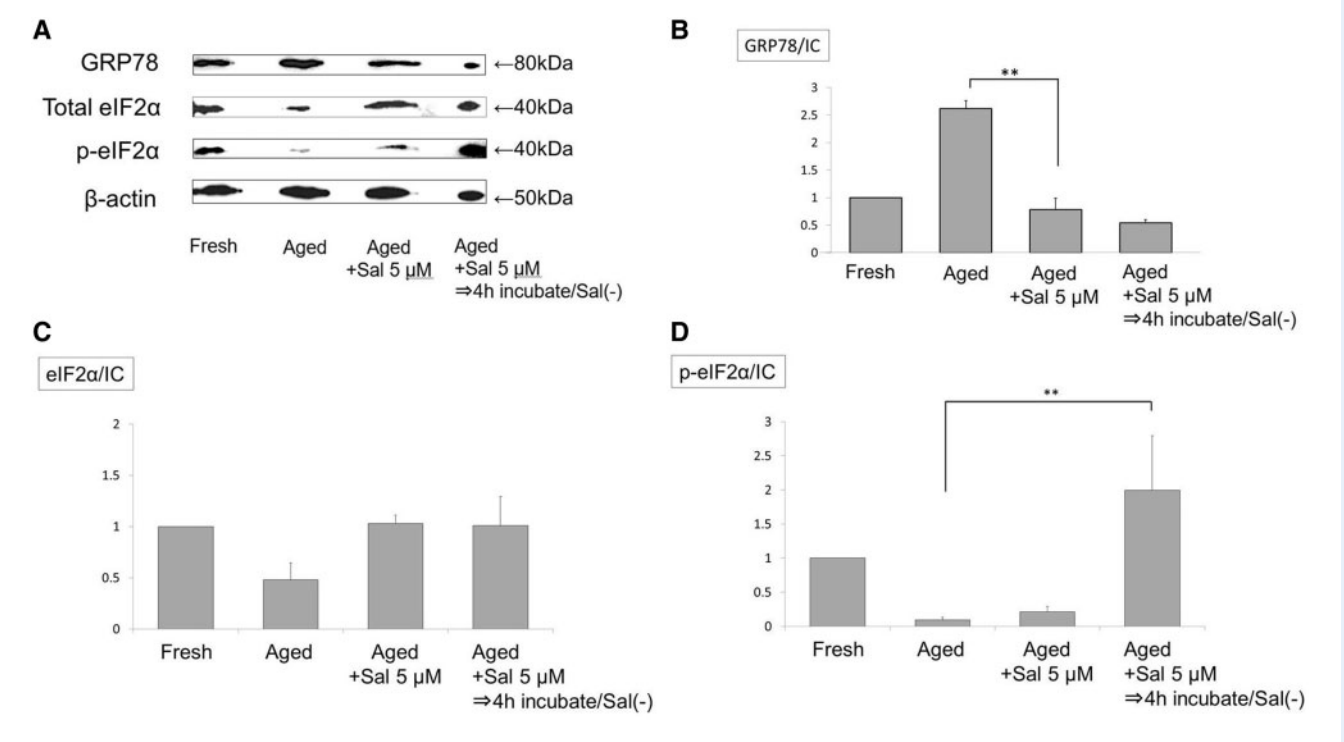
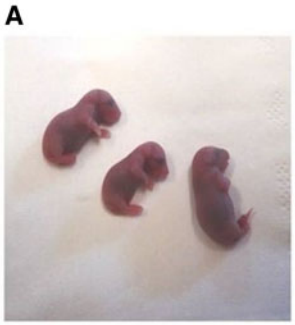
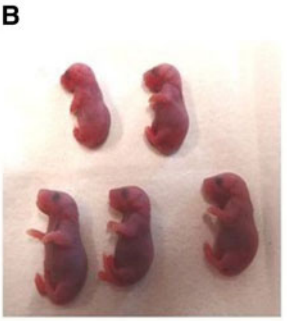


Figure 4 Effect of salubrinal on the expression of ER stress marker. (**A**) Expression of three markers of ER stress (GRP78, eIF2α, phospho-eIF2α) in fresh, aged and 5 μM salubrinal-treated aged oocytes and in salubrinal-treated aged oocytes allowed to recover for 4 h. Full blot images are in Supplementary information. (**B**) GRP78 expression. (**C**) eIF2α expression. (**D**) Phospho-eIF2α expression. Lysates equivalent to 50 oocytes were loaded onto each lane, and β-actin was used as internal control. Each value represents the mean ± standard error of three determinations performed independently. IC, internal control; Sal, salubrinal.

In the present study, aged oocytes displayed poorer embryo development, a higher rate of dead blastomeres in blastocysts and higher expression of GRP78 in MII oocytes compared to fresh oocytes. The same features were observed in MII oocytes subjected to pharmacologically induced ER stress. Thapsigargin, a high-affinity specific inhibitor of Ca^{2+}-ATPase on the sarcoplasmic reticulum and ER, seemed to reduce the store of ER immediately, whereas tunicamycin, an inhibitor of N-linked glycosylation, appeared to cause ER stress gradually by accumulation of unfolded protein. These differences could be responsible for the dissimilar patterns of embryo development shown in Fig. 1B and C. Previous reports showed that ER stress could be induced in one-cell mouse embryos by tunicamycin and sorbitol (Zhang *et al.*, 2012a,b) or in two-cell mouse embryos by tunicamycin alone (Basar *et al.*, 2014); in both the cases, a significantly lower blastocyst formation rate was observed. Accordingly, ER stress induced at the MII or earlier embryo stage is closely related to subsequent embryo development. Therefore, expression of GRP78 was highest in MII oocytes and one-cell embryos from fresh oocytes and decreased thereafter. These data suggest that oocytes just after ovulation were exposed to excessive or prolonged ER stress. The excessive ER stress observed in aged oocytes could be responsible for defective embryo development. Although it is not well understood how ER stress affects mammalian oocytes and embryos, inadequate ER stress may also have a negative impact on embryo development (Latham, 2015, Michalak and Gye, 2015, Latham, 2016). Importantly, GRP78 is a central regulator of the ER stress-induced UPR and appropriate GRP78 expression is essential for embryonic cell growth in preimplantation embryos (Luo *et al.*, 2006). In the case of *in vitro* maturation, the expression of GRP78 in cumulus-oocyte complexes at 44 h of maturation culture was significantly increased compared to that at 22 h (Park *et al.*, 2018). Therefore, the regulation of ER stress in oocytes and preimplantation embryos is thought to be important for normal embryo development. Because our previous report demonstrated that mitochondrial transfer to rejuvenate aged oocytes was insufficient to improve embryo development (Igarashi *et al.*, 2016), our results in this study suggest that simultaneous targeting of mitochondrial dysfunction and ER stress might be beneficial to rejuvenate aged oocytes.

We demonstrate that ER stress regulation by pretreatment of MII oocytes with salubrinal suppressed the PERK pathway of ER stress and significantly improved the embryo development in post-ovulatory aged mouse oocytes. Accumulation of unfolded or misfolded proteins in the ER lumen activates the UPR signaling pathways to maintain ER homeostasis. ER homeostasis is essential for the intracellular calcium homeostasis and the synthesis of various proteins and lipids, explaining why the disruption of ER homeostasis causes apoptosis. GRP78 synthesis is stimulated by a variety of environmental and physiological stress conditions, and GRP78 maintains the homeostasis by releasing and activating three stress sensors, activating transcription factor 6 (ATF-6), inositol-





C

	Maternal mouse	Transferred embryos	Pregnancy	Live births	Birth weight (g)
Control group	Mouse 1	8	1	1	1.30
	Mouse 2	8	3	3	1.33 ± 0.24
	Total	16	4 (25%)	4 (25%)	1.24 ± 0.19
Sal treated group	Mouse 3	8	6	5	1.19 ± 0.16
	Mouse 4	8	4	2	1.38 ± 0.22
	Total	16	10 (63%)*	7 (44%)	1.33 ± 0.20

Figure 5 Pups delivered following embryo transfer of salubrinal-treated or untreated aged blastocysts. (**A**, **B**) Pups originating by the transfer of blastocysts derived from aged (A) or salubrinal-treated (B) aged oocytes into pseudo-pregnant female mice. (**C**) Implantation rate, delivery rate and pups' body weight at birth. *$P < 0.05$; Sal: salubrinal.

requiring enzyme 1 (IRE1) and PERK (Lee, 2005). The ATF6 signaling pathway mainly induces the expression of GRP78 by inducing transcriptional activation of ER stress-response elements 1 and 2. The IRE1 signaling pathway mainly causes ER-associated protein degradation, which reduces the amount of unfolded proteins. PERK leads the phosphorylation of eIF2α. Phosphorylated eIF2α binds eIF2B to disturb the exchange of GDP to GTP in cells, and therefore it decreases the translation of new proteins in the ER (Lin *et al.*, 2019). There is some evidence suggesting that targeting modulators or regulators of ER stress may improve ER stress-related diseases (Liu *et al.*, 2012; Hetz *et al.*, 2013; Sapra *et al.*, 2014; Li *et al.*, 2015). The effect of tauroursodeoxy-cholic acid (TUDCA) and salubrinal on ER stress relief has been studied extensively in preimplantation embryos (Wu *et al.*, 2012; Zhang *et al.*, 2012a,b; Basar *et al.*, 2014; Wu *et al.*, 2015). In spite of the documented positive effect of TUDCA on the development of preimplantation embryos under stress conditions (Zhang *et al.*, 2012a,b), here, we chose to focus on salubrinal as an ER stress inhibitor, because of its well-known characteristics and pharmacokinetics. Salubrinal can inhibit the dephosphorylation of eIF2α and keep it in a phosphorylated state, resulting in the suppression of protein translation and a decrease in protein synthesis required to maintain ER homeostasis (Fullwood *et al.*, 2012; Boyce *et al.*, 2005). In the *in vitro* mouse cumulus-oocyte complex maturation process, addition of salubrinal to culture medium was shown to revert the thapsigargin-induced deterioration of mitochondrial activity in oocytes and embryo developmental competence (Wu *et al.*, 2012). Female ALMS1 mutant mice exhibit defects in ovulation and decreased oocyte quality following the onset of obesity. Obesity is an important factor that elicits ER stress-induced subfertility. Treatment of this obesity and subfertility in female mice (obese Blobby mice) with salubrinal once a day for four days has been shown to improve ovulation and mitochondrial activity (Wu *et al.*, 2015). In addition, oocytes from obese Blobby mice have exhibited impaired embryo development that could be improved by treatment with salubrinal. These reports strongly suggest that salubrinal effectively counteracts the ER stress-related deterioration of oocyte quality and defective embryo development. Here, we demonstrate that ER stress contributed to the aging mechanism in oocytes, and salubrinal restored embryo development and apoptosis in post-ovulatory aged mouse oocytes. Importantly, in our experimental conditions, only 1 h of MII oocytes pretreatment with salubrinal was sufficient to suppress ER stress and improve oocyte quality and embryo development. This finding suggests that short-term exposure and minimal use of salubrinal can contain costs and decrease embryo toxicity in clinical applications.

No clinical data about embryonic lethality or teratogenesis of salubrinal has existed. To validate the effect of salubrinal treatment on the embryo and fetus, we transferred salubrinal-treated embryos. Salubrinal-treated embryos showed normal implantation ability. Moreover, pups derived from salubrinal-treated embryos were healthy and showed normal growth and no congenital malformations. These results indicate that trophoectoderm differentiation and placental development were not affected by salubrinal treatment in our experimental conditions. Additionally, the short exposure of oocytes to salubrinal does not seem to exert any adverse influence on the intrauterine embryo development or organogenesis. Our data indicate a significantly higher implantation rate in the salubrinal-treated group

than in the aged group. Even though the live birth rate did not change significantly between these two groups, a protocol that optimizes salubrinal treatment might be expected to improve the result of *in vivo* experiments.

The limitation of the present study is that we assessed only the PERK pathway of the UPR as a possible aging mechanism in oocytes, without investigating the role of other pathways. We used salubrinal as a specific inhibitor of the PERK pathway, as no adequate inhibitors of the IRE1 or ATF6 pathways exist. In this study, we considered enough to examine whether salubrinal contributed to the improvement of embryo development by proceeding with at least one of UPRs.

In conclusion, in the present study, ER stress was strongly associated with aging of post-ovulatory aged mouse oocytes. Salubrinal, an inhibitor of the ER stress-related PERK pathway, significantly improved the embryo development of aged mouse oocytes through the suppression of apoptosis in blastomeres. Based on our findings, we propose that targeting ER stress regulation is a new therapeutic strategy to improve oocyte quality.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

I.S. and H.I. contributed to study conception and design, acquisition, analysis and interpretation of data, and to drafting the manuscript. J.K., K.M., K.T. and M.N. contributed to acquisition and analysis of data. S.N. contributed to drafting the manuscript and provided critical discussions. All authors approved the final manuscript.

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Conflict of interest

The authors have no conflict of interest to disclose.

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