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## Effects of Maternal Aging on Expression of Sirtuin Genes in Ovulated Oocyte and Cumulus Cells

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**Abstract:** Sirtuins, a family of NAD<sup>+</sup>-dependent protein deacetylases, regulate important physiological events including aging and cell metabolism, mainly by protecting cells/tissues from oxidative damage. Ovarian aging decreases the quality of oocytes through induction of mitochondrial dysfunction and increases in DNA strand breaks by accumulation of reactive oxygen species. However, involvement of sirtuins in regulating oocyte quality with aging has not been determined. Here, we found the expression of sirtuin genes (*Sirt1-7*) in mouse ovaries and isolated oocytes and cumulus cells in a cell-specific manner. Based on real-time RT-PCR, all seven sirtuin genes were detected in the ovary with *Sirt2* transcript levels showing the highest abundance. Oocyte expressed high levels of *Sirt6*, whereas the expressions of *Sirt1*, *Sirt2*, *Sirt4*, and *Sirt6* were high in cumulus cells. When comparing samples from young and aged mice, oocyte levels of *Sirt1-7* mRNA were not different. However, *Sirt2* and *Sirt6* transcript levels were decreased in cumulus cells of aged mice. Our findings suggest a possible association of *Sirt2* and *Sirt6* transcript levels in cumulus cells with impaired oocyte quality in aged mice. Further understanding the roles of these sirtuins in cumulus cell and oocyte could provide a better strategy to minimize aging-related decline in oocyte quality.

**Key words:** Sirtuin, Aging, Ovary, Cumulus cells, Oocyte

### Introduction

Sirtuins, a family of NAD<sup>+</sup>-dependent protein deacetylases, are highly conserved proteins expressed in

multiple tissues [1]. Seven sirtuin members have been identified in mammals (SIRT1-7) and they show distinct subcellular localizations: SIRT1, 6 and 7, are found in nucleus, SIRT2 are in the cytoplasm, and SIRT3, 4, and 5 are in the mitochondria [2]. These sirtuin genes are implicated in many physiological events, including aging, cell metabolism, apoptosis, and cell cycle regulation [3]. Sirtuins protect cells/tissues from oxidative damage by regulating reactive oxygen species (ROS) production and/or scavenging ROS [4, 5]. Sirtuins suppress apoptosis in response to cellular DNA damage by regulating of ATP production in the mitochondria [6, 7].

Ovarian aging leads a remarkable decline in the ovarian follicle pool and oocyte reserve, accompanied by increases in low-quality oocytes incapable of early embryonic development [8]. Structural degradation and chromosome damage that occurs during long periods of arrest at diakinesis of prophase I stage in oocytes likely contribute to chromosome abnormalities and aneuploidy. Also, accumulation of ROS with aging is associated with decreases in oocyte quality [9]. Oxidative stress deteriorates the quality of oocytes by inducing of mitochondrial dysfunction leading to low ATP content as well as increases in DNA strand breaks [10–12].

A recent study demonstrated that treatment with sirtuin inhibitors or *Sirt3* knock down increased intracellular ROS levels in cultured mouse preimplantation embryos resulting in decreases in blastocyst formation, implantation failure and retarded fetal growth during *in vitro* fertilization and embryo transfer. Although these results indicate that SIRT3 plays a protective role in preimplantation embryos against stress conditions during *in vitro* fertilization and culture [13], physiological functions of sirtuins in oocytes remain to be elucidated. Here, we investigated the expression of sirtuin genes in the mouse ovary, and

**Table 1.** List of primers for real-time RT-PCR

Gene	Forward primer	Reverse primer
Sirtuin1	CCTTGGAGACTGCGATGTTA	GTGTTGGTGGCAACTCTGAT
Sirtuin2	GCAGTGTCAGAGCGTGGTAA	CTAGTGGTGCCTTGCTGATG
Sirtuin3	CTGACTTCGCTTTGGCAGAT	GTCCACCAGCCTTCCACAC
Sirtuin4	GCTTGCCTGAAGCTGGATT	GATCTTGAGCAGCGGAACTC
Sirtuin5	AGCCAGAGACTCAAGACGCCA	AGGGCGAGCTCTCTGTCCACC
Sirtuin6	TCGGGCCTGTAGAGGGGAGC	CGGCGCTTAGTGGCAAGGGG
Sirtuin7	GGCACTTGTTGTCTACACG	GTGATGCTCATGTGGGTGAG
Histone-H2A	ACGAGGAGCTCAACAAGCTG	TATGGTGGCTCTCCGTCTTC

ovulated cumulus cells and oocytes. We also compared the expression levels of sirtuins between young and aged mice. All *Sirt1-7* transcripts were detected in ovaries with *Sirt2* being the predominant one. Although the levels of *Sirt1-7* mRNA in oocytes were not different between young and aged mice, *Sirt2* and *Sirt6* transcript levels were decreased in cumulus cells of aged mice.

## Materials and Methods

### Animals

To obtain cumulus-oocyte complexes (COCs) for gene expression analyses, female ICR mice at 7 or 44 weeks of age (CLEA Japan, Tokyo, Japan) were treated with a single i.p. injection of 5 IU of pregnant mare serum gonadotropin (PMSG) (Calbiochem, Cambridge, MA) followed 48 h later with 10 IU of human chorionic gonadotropin (hCG) (ASKA Pharmaceutical, Tokyo, Japan) administered i.p. At 14–16 h after hCG treatment, ovulated COCs were obtained from oviductal ampullas. Ovaries were also obtained from ICR mice at 7 or 44 weeks of age at 48 h after PMSG treatment. For *in vitro* fertilization (IVF), ovulated COCs were obtained from ICR mice at 7–12 or 44–46 weeks of age as described above due to limited number of animals available for this study. The care and use of animals was approved by the Animal Research Committee, St. Marianna University School of Medicine.

### IVF and preimplantation embryo culture

Sperm from ICR male mice (10–12 weeks of age) were collected into human tubal fluid (HTF) media (NKsystem, Osaka, Japan) and incubated for 60–90 min at 37°C in a 5% CO<sub>2</sub> and 95% air to facilitate capacitation reaction. ICR mice at 7–12 or 44–46 weeks of age received one i.p. injection of 5 IU PMSG followed, at 48 h later, with another injection of 10 IU hCG. At 15 h later, COCs were collected from the oviductal ampulla. COCs were then placed in 100 µl HTF with sperm ( $3 \times 10^6$ /ml). Fertilization was carried out for 5–6 h and inseminated oocytes

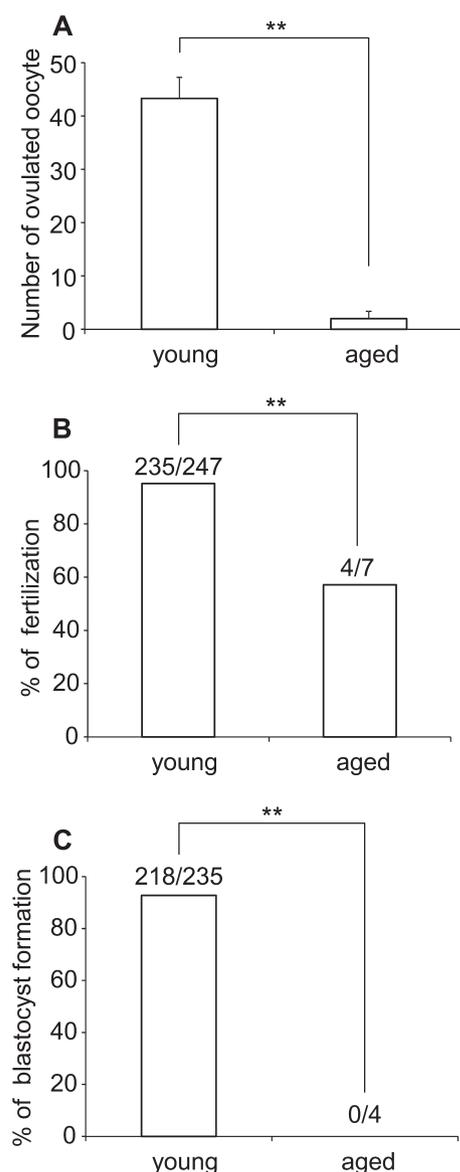
were removed into 30 µl droplets of KSOM media (Millipore, MA, USA) under mineral oil (IrvineScientific, Santana, USA) and incubated at 37°C. Two-cell embryos were scored 24 h later and all oocytes not inseminated were removed from the droplet. Some embryos were allowed to develop to the blastocyst stage as previously described [14].

### Isolation of cumulus cells and oocytes

For quantitative real-time RT-PCR, oocytes and cumulus cells were isolated from ovulated COCs by separating denuded oocytes from cumulus cells by mechanical pipetting at 1–2 min after 300 µg/ml hyaluronidase treatment.

### Quantitative real-time RT-PCR

Quantitative real-time RT-PCR of transcript levels in ovarian cells and whole ovaries was performed using a SmartCycler (Takara, Tokyo, Japan) as described [14, 15]. Total RNA was extracted using an RNeasy Micro Kit (QIAGEN Sciences, Valencia, CA), and cDNA was synthesized using a Sensicript RT Kit (QIAGEN) according to the manufacturer's protocol. Real-time PCR was performed using SYBR premix Ex Taq (Takara) as follows: 15 min at 95°C and then 45 cycles of 15 sec at 95°C and 60 sec at 60°C. The primers used are shown in Table 1. To determine the absolute copy number of target transcripts, cloned plasmid cDNAs for individual gene were used to generate a calibration curve. Purified plasmid cDNA templates were measured, and copy numbers were calculated based on absorbance at 260 nm. A calibration curve was created by plotting the threshold cycle against the known copy number for each plasmid template diluted in log steps from 10<sup>5</sup> to 10<sup>1</sup> copies. Each run included standards of diluted plasmids to generate a calibration curve, a negative control without a template, and samples with unknown mRNA concentrations. Data were normalized based on histone H2a transcript levels.



**Fig. 1.** Effects of aging on ovulation (A), fertilization (B), and embryo development (C). Ovulated COCs were obtained from either young (7–12 weeks of age;  $P = 7$ ) or aged (44–46 weeks of age;  $P = 4$ ) ICR mice treated with PMSG followed 48 h later with hCG (mean  $\pm$ SEM). *In vitro* fertilization was performed using ovulated COCs and fertilized oocytes were cultured for 5 days. The ability of oocyte for fertilization was judged by development of 2-cell stage embryo, whereas developmental competence of fertilized oocytes was evaluated by blastocyst formation at the end culture (mean  $\pm$ SEM). \*\*,  $P < 0.01$  vs. young

#### Statistical analysis

Chi-square test was performed to compare the proportion of fertilization and blastocyst formation. Other differ-

ences were evaluated using the Mann–Whitney U-test. Data are mean  $\pm$ SEM.

## Results

#### *Impaired oocyte quality in aged mice*

To assess the oocyte quality in aged mice (44–46 weeks of age) used for current experiments, we performed IVF and embryo cultures. As shown in Fig. 1A, the numbers of ovulated COCs in young animals were more than 20-fold higher than those of aged mice. We found 38% decreases in proportions of embryos at 2-cell-stage in aged mice as compared with those in young mice, indicating decreased fertilization potential in oocytes derived from aged mice (Fig. 1B). Although more than 90% of fertilized embryos from young animals developed to blastocysts, no embryos were reached the blastocyst stage in aged mice (Fig. 1C).

#### *Expression of Sirtuin genes in mouse ovaries and ovulated cumulus cells and oocytes*

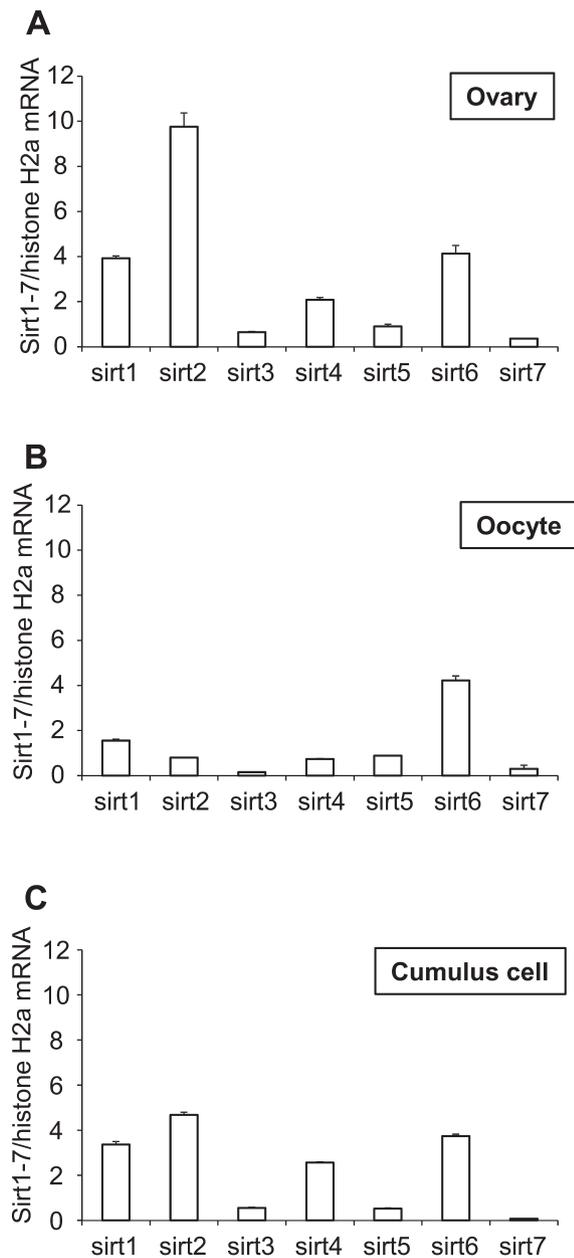
Using young animals, we detected all sirtuin genes (*Sirt1-7*) in ovaries before ovulation by quantitative real-time RT-PCR. The levels of *Sirt2* transcripts were highest among *Sirt1-7* genes, whereas the expression of *Sirt3* and *Sirt7* mRNA was minimum (Fig. 2A). We also determined the expression levels of sirtuin genes in isolated oocytes and cumulus cells. In oocytes, *Sirt6* showed highest expression levels, whereas the levels of *Sirt3* and *Sirt7* transcripts were low similar to ovaries (Fig. 2B). In cumulus cells, the expressions of *Sirt1*, *Sirt2*, *Sirt4*, and *Sirt6* were high, although the levels of *Sirt3*, *Sirt5*, and *Sirt7* transcripts were low (Fig. 2C).

#### *Decreases in Sirt2 and Sirt6 transcript levels in ovulated cumulus cells of aged mice*

To evaluate an association of the expression levels of sirtuins with impaired oocyte quality in aged mice, we compared their levels in ovulated oocytes and cumulus cells between young and aged mice. The levels of *Sirt2* and *Sirt6* mRNA in cumulus cells of aged mice were decreased more than 45% as compared with those in young animals (Fig. 3A), whereas *Sirt4* transcript levels were increased in aged mice. In oocytes, however, there was no difference in *Sirt1-7* transcript levels between young and aged mice (Fig. 3B).

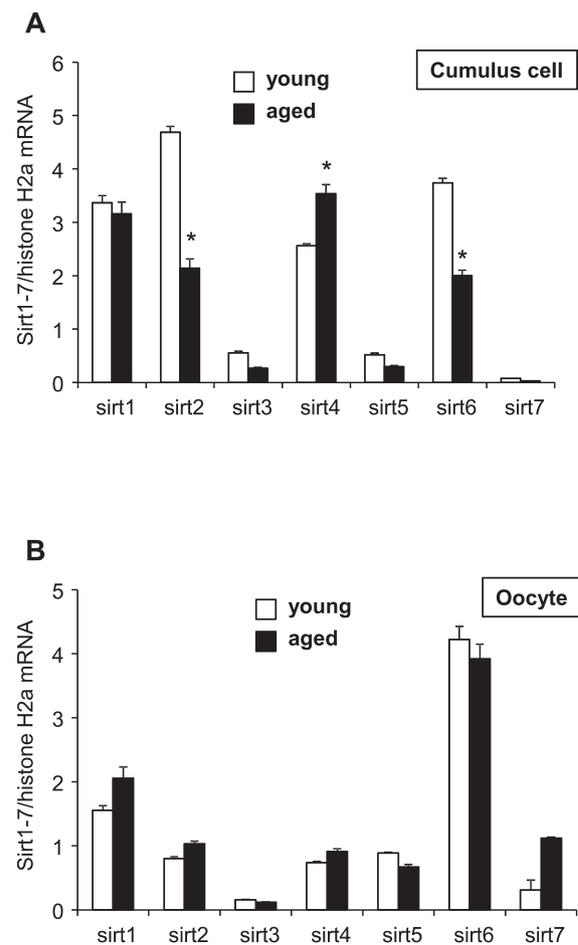
## Discussion

This study shows the presence of *Sirt1-7* genes in mouse ovaries as well as ovarian cells, oocyte and cu-



**Fig. 2.** Expression of sirtuin genes (*Sirt1-7*) in ovaries (A) and ovulated oocytes (B) and cumulus cells (C). Ovaries were dissected from ICR mice at 7 weeks of age, whereas oocytes and cumulus cells were isolated from cumulus-oocyte complexes ovulated from those animals. Transcript levels of *Sirt1-7* genes were quantified by real-time RT-PCR. Levels of all mRNAs were normalized based on those for histone H2a in the same sample (mean  $\pm$ SEM,  $P = 5$ ).

mulus cells. Among sirtuin genes, *Sirt2* and *Sirt6* expression was decreased in aged cumulus cells, suggesting its association with impaired oocyte qualities in aged



**Fig. 3.** Effect of aging on transcript levels of sirtuin genes (*Sirt1-7*) in ovulated oocytes (A) and cumulus cells (B). Oocytes and cumulus cells were isolated from cumulus-oocyte complexes ovulated from either young (7 weeks of age) or aged (44 weeks of age) ICR mice. Transcript levels of *Sirt1-7* genes were quantified by real-time RT-PCR. Levels of all mRNAs were normalized based on those for histone H2a in the same sample (mean  $\pm$ SEM,  $P = 5$ ). \*,  $P < 0.05$  vs. young.

animals. Unexpectedly, the levels of *Sirt1-7* mRNA in ovulated oocyte were not changed in aged mice, despite their low fertilization and embryo development potentials.

Age-related cellular disorders, including damages to mitochondria, DNA and telomeres are driven by ROS and have been shown to occur in diverse tissues [16–18]. Previous studies demonstrated that sirtuins eliminated these cytotoxic effects of ROS by regulating its production and/or disposal [4, 5]. In addition to our findings demonstrating a decline of *Sirt2* and *Sirt6* expressions in cumulus cells of aged mice, age-associated decreases

in *Sirt1* expression were detected in mouse hypothalamus [19], mouse and human vascular endothelial cells [20], rat left ventricular tissue [21] and hippocampus [22] as well as human anterior lens capsule [23]. Thus, these data suggest a potential role of sirtuins in protecting cells from oxidative damage associated with aging.

Cumulus cells communicate with oocytes via gap junctions and through transport of essential molecules and nutrients to support oocyte growth during folliculogenesis. Indeed, cumulus cells protected oocytes against oxidative stress-induced apoptosis by transporting glutathione into oocytes [24]. Apoptosis of cumulus cells increased in aged mice [25, 26] leading to decreases in oocyte quality [27]. DNA fragmentation in cumulus cells also increased with aging [26] and results in poor oocyte quality [28]. Furthermore, age-dependent increased apoptotic potential in oocytes was mediated by cumulus cells, but not by changes within the oocyte itself. These findings are consistent with our results showing impaired oocyte quality with aging associated with low expression levels of *Sirt2* and *Sirt6* expressions in cumulus cells, but not in oocytes. We also found increases in *Sirt4* transcript levels in aged cumulus cell. This change may further regulate cumulus cell functions. Although global gene expression changes in human cumulus cells obtained from ovulated COCs in IVF program were intensively studied, no apparent differences in *Sirt1-7* gene expression between good and poor quality COCs [29–31]. This discrepancy may be derived from the different experimental design that did not focused on the age-related impaired oocyte quality.

Majority of mice with *Sirt1* deletion died before weaning, and some survivors exhibited infertility both in male and female [32]. However, no mice for *Sirt6* null animals were available to examine their reproductive status, because the animals died shortly after birth [6]. Furthermore, no data on fertility changes were reported for *Sirt2* null mice, although they developed normally [33]. *Sirt3*, *Sirt4* and *Sirt5* deficient mice were fertile, whereas no precise data on fertility was available on *Sirt7* knockout mice [34]. Therefore, the importance of *Sirt2* and *Sirt6* expressions in cumulus cells could not be derived from these gene deletion studies.

Here, we demonstrated as association of decreases in *Sirt2* and *Sirt6* transcript levels in cumulus cells of ovulated COCs with impaired oocyte quality in aged mice. Future studies in *Sirt2* and *Sirt6* double knockdown oocyte or oocyte-specific double knockout mouse could reveal the contribution of these sirtuins to oocyte quality. Although the ability of several compounds including resveratrol to upregulate sirtuins expression is controversial [35, 36], identification of reliable sirtuin-activating

compounds and their clinical application in aged women could lead to novel approaches to recover impaired oocyte quality by aging.

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