Age-associated changes in bovine oocytes and granulosa cell complexes collected from early antral follicles

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Abstract

Purpose To assess the age-associated changes in oocytes and granulosa cells derived from early antral follicles (EAFs).

Method Gene expression analysis of granulosa cells of the EAFs using a genome analyzer (Illumina) and in vitro culture of oocyte-granulosa cell complexes (OGCs) of EAFs (400–700 μm in diameter) collected from ovaries of aged (>120 months) and young (<50 months) cows.

Results Gene expression profiles in granulosa cells of EAFs of aged cows, which included changes in genes that encode chaperone proteins and antioxidants. In vivo development of EAFs, as determined by oocyte diameter of EAFs and AFs (3–6 mm in diameter), appeared to be impaired in aged cows and the OGCs of aged cows contained low GSH compared to younger counterparts. When the OGCs were cultured in a medium containing low estradiol (E_2 , 0.1 $\mu\text{g}/\text{mL}$), the ratio of antrum formation was higher for OGCs from aged animals than that from young animals, while higher abnormal fertilization rate and lower total cell number of the blastocysts were observed in the OGCs of aged cows compared with those of young cows. On the contrary, when the OGCs were cultured in a medium containing 10 $\mu\text{g}/\text{mL}$ E_2 , the ratio of antrum formation and fertilization outcome

was comparable between the two age groups, whereas the total cell number of the blastocysts was still low in the aged group.

Conclusion Aging affects the gene expression profiles of the granulosa cells, and impairs in vitro developmental ability of OGCs collected from EAFs.

Keywords Cows · Early antral follicle · Gene expression · Granulosa cells · Aging

Introduction

Age-associated sub fertility has been commonly observed in mammalian females, with the quality of oocytes gradually declines over the age of 35. Cows retain their reproductive ability for much longer than rodents, and show similar oocyte selection process and age-associated endocrinal events as that in humans [1–3]. Japan has a traceability system that helps researchers obtain information about the breed and day of birth of cows at any of the slaughter houses.

A decrease in the ability of oocytes to fertilize and develop to the blastocyst stage has been observed with increasing age in cows as well as human with reproductive capacity [4–11]. In addition to the low quality of oocytes, ovarian reserve also declines with age in humans [12] and the reduced antral follicle counts reflect the sub fertility in reproductive aging women as well as cows [13, 14]. Thus, it is important to leverage early developmental-stage follicles as an oocyte source in aged females. Although no reports in cows, the effects of maternal aging on developmental ability of premature oocytes has been reported in other species. Xu et al. [15] demonstrated that the growth rate of pre antral follicles derived from aged rhesus monkeys was slower than that of pre antral follicles derived from younger monkeys, when tested under in vitro conditions. In addition, in vitro culture of oocytes derived from early antral follicles (EAFs) of aged mice (63–67 weeks old) exhibited lower rates of pseudo-antrum formation

Capsule N Itami conducted culture experiment and wrote this paper and R Kawahara-Miki conducted gene expression analysis and wrote this paper. Their contributions are equal.

N Itami conducted culture experiments and R Kawahara-Miki conducted gene expression analysis and both authors contributed equally to this study.

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and early developmental ability than those from their younger counterparts [16]. However, the mechanism by which maternal aging influences the developmental ability of oocytes originating from EAFs remains to be elucidated.

Oocyte growth is supported by surrounding granulosa cells [17], and a deterioration in the characteristics of these granulosa cells derived from full-grown antral follicles have been observed in aged women and cows [18–21]. Result from real time RT-PCR experiments have revealed that the expression of some genes including those that encode for antioxidants in mouse ovary was affected by aging [22]. However, researchers have not reported age-associated changes in the characteristics of granulosa cells derived from EAFs in any animals. Therefore, elucidation of the comprehensive gene expression profile of the granulosa cells surrounding immature oocytes as well as the developmental ability of oocytes from EAFs may enhance our understanding of age-associated events influencing oogenesis in animals that retain their reproductive ability for long periods.

In the present study, we conducted comprehensive gene expression analysis of granulosa cells derived from EAFs of young and aged cows using next generation sequencing technology (NGS). In addition, we compared the character of oocyte-granulosa cell complexes (OGCs) of EAFs between these cows by examining the diameter of oocytes, number of granulosa cells, and GSH content in the OGCs. Furthermore, we cultured OGCs derived from EAFs of young and aged cows using two types of media containing low and high concentration of estradiol; low estradiol concentration was based on the corresponding levels in follicular fluid [23]. High concentration of estradiol was based on those reported in previous reports, which yielded high antrum OGCs formation in vitro [24], because antrum formation is a primary marker of follicular development in vitro [25, 26]. We also compared the developmental ability of the oocytes grown under in vitro conditions.

Materials and methods

Drugs, media, and collection of ovaries

All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Ovaries of Japanese Black Cows (*Bos taurus*) were collected from a slaughterhouse, maintained at 30 °C in phosphate-buffered saline (PBS) containing antibiotics, 10 mM sucrose, and 10 mM glucose, and transported to the laboratory within 4 h. The medium used for the in vitro culture of OGCs collected from EAFs was based on a previous report [24] with a slight modification; our medium contained 0.1 or 10 µg/mL 17β-estradiol. TCM-199 medium supplemented with 10 % FCS (570H, ICN; Costa Mesa, CA, USA) and antibiotics was used

for in vitro maturation (IVM medium). The medium used for in vitro culture (IVC medium) of the embryos was based on synthetic oviductal fluid (SOF) [27] and contained 5 % FCS, 1.5 mM glucose, and amino acids.

Collection and in vitro culture of OGCs

To avoid the using of ovaries without adequate follicle development and ovulation, we collected ovaries with a functional luteum and morphologically healthy large dominant (>8 mm) follicles. Ovaries were washed with 70 % ethanol and the ovarian surface was sliced. EAFs that were 400–700 µm in diameter were collected under a stereomicroscope using an 18G needle connected to a syringe and moved into TCM-199 medium containing 3 mg/mL bovine serum albumin (BSA, Nacalai, Kyoto, Japan). After opening the EAFs, OGCs were dissected from the EAFs with fine forceps and were washed in culture medium, transferred individually to 200 µL of culture medium in a well (96-well plate, Becton Dickinson; NJ, USA), and cultured for 16 days. Half of the medium was replaced with fresh medium and the rate of antrum formation was examined for each donor every 4 days (4, 8, 12, and 16 days of culture).

Definition of aged and young cows

In previous reports, oocytes from cows aged more than 120 months old had some abnormalities compared with those from young cows aged 25–50 months [9, 10, 28]. In the present study, cows aged more than 120 months were defined as aged cows and cows aged 20–35 months were defined as young cows. OGCs collected from aged and young cows were pooled and randomly selected for each experiment. The age of donor cows used for experiments is expressed as mean ± SE in Table 1.

In vitro maturation, fertilization, and culture of oocytes

After the initial culture of oocytes in vitro, OGCs with antrum cavities were further cultured for 23 h to facilitate IVM. In vitro fertilization and in vitro culture were conducted as described previously [11, 28]. After fertilization, OGCs were washed and cultured in IVC medium containing 5 % fetal calf serum (FCS), and then incubated at 38.5 °C in an atmosphere of 5 % CO₂ in air (5–10 OGCs/50 µL IVC medium). Forty-eight hours after fertilization, oocytes were denuded from surrounding cumulus cells and the cleavage rate (embryo development over 5-cell stage) was assessed. The cleaved embryos were then cultured for 5 days (1 embryo/2 µL IVC medium) to assess blastulation rate and total number of blastocysts. Cultures from day 2 to day 7 were performed at 38.5 °C in an atmosphere of 5 % CO₂, 5 % O₂, and 90 % N₂ with maximum humidity. To assess nuclear

Table 1 Number and age of donor cows and number of OGCs used for each experiment

Exp. medium ^a	Number	Young cows		No. cows	Aged cows	
		Age Ave $\pm$ SE	No. of OGCs		Age Ave $\pm$ SE	No. of OGCs
1	11	28.3 $\pm$ 0.7	110	11	164 $\pm$ 6.1	110
2	10	28.8 $\pm$ 0.7	91	10	147.8 $\pm$ 7.5	92
3	10	29.1 $\pm$ 1.4	100	10	149.1 $\pm$ 5.7	100
4 Low E ₂	34	29.8 $\pm$ 1.0	358	43	153.7 $\pm$ 4.0	341
High E ₂	39	28.6 $\pm$ 0.7	395	49	160.4 $\pm$ 4.5	400

^a In experiment 4, OGCs were cultured in medium containing 0.1 or 10 μ g/ml estradiol

maturation, fertilization, and total cell number of the blastocysts, denuded oocytes or embryos were fixed in 4 % para-formaldehyde and mounted on glass slides with an anti fade reagent containing DAPI (Pro-long gold anti fade reagent with DAPI; Invitrogen, OR, USA), and observed under a fluorescence digital microscope (BZ-8000, Keyence, Tokyo, Japan).

Gene expression analysis

Total RNA of granulosa cells from young and aged cows were extracted using the RNAqueous Kit (Life Technologies Corp., Carlsbad, CA, USA) following the manufacturer's protocol. The RNA quality and quantity were assessed on a 2100 Bio analyzer by using the RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA, USA). Libraries were prepared using a TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA), and used to generate clusters on the Illumina Cluster Station. Four lanes per group were sequenced on the Genome Analyzer II (Illumina) as 50-bp reads, and the image analysis and base calling were performed with CASAVA ver.1.7.0 (Illumina) following the manufacturer's instructions. High-quality sequences (those passing the default quality-filtering parameters in Illumina GA pipeline GERALD stage) were retained and aligned to the bovine genome sequence (bosTau4) and the exon–exon splice junction database downloaded from the UCSC sequence and annotation database [<http://hgdownload.cse.ucsc.edu/downloads.html#cow>]. Both raw and normalized (reads per kilo base of exon per million reads of mapped reads, [RPKM]) [29] read counts were obtained using CASAVA and Genome Studio ver. 2010.2 (Illumina). In genes showing normalized gene counts greater than one, the fold-change of the normalized counts between the young and aged cows was calculated.

The datasets of differentially expressed genes were interpreted in the context of their biological processes and functions and by their networks and pathways using Ingenuity Pathways Analysis (IPA; Ingenuity Systems Inc., Redwood City, CA, USA). A detailed description of the method for performing IPA can be found at www.ingenuity.com. The

Fisher's exact test was used in the analysis of gene set enrichment in functional categories.

Experimental design

Experiment 1

Granulosa cells were harvested from OGCs of EAFs. A comprehensive gene expression analysis of granulosa cells was conducted and results were compared between the two age groups. Total number of OGCs collected and age in months (average $\pm$ SE) of the donors are listed in Table 1. Ten OGCs were collected from EAFs (400–700 μ m in diameter) of each donor cow, and granulosa cells were collected from OGCs using a Pasteur pipette. The granulosa cells were washed in PBS containing polyvinyl alcohol, and used for RNA extraction.

Experiment 2

The number of granulosa cells comprising OGCs and the diameter of oocytes collected from OGCs derived from the EAFs were examined. Ten OGCs were collected from each EAF of the donor cows (donor information is shown in Table 1). Oocytes were removed from OGCs and their diameters were measured. The diameter of oocytes was examined under a digital microscope (Keyence, Tokyo, Japan). A total of 91 and 92 oocytes were successfully collected from OGCs of aged and young cows, respectively. After oocyte collection, the granulosa cells were transferred to micro tubes and separated by rigorous pi petting after 0.25 % trypsin EDTA treatment. Total cell number was counted using hemo cytometer to obtain an average granulosa cell number per OGC of each donor cow. In addition, 10 full-grown oocytes were collected from each antral follicle (3–6 mm in diameter) of the same ovaries. The diameter of the full-grown oocytes were also measured and compared between the two age groups.

Experiment 3

GSH content of OGCs collected from EAFs was examined. Ten OGCs were collected from each donor cow (Table 1) and the average GSH content per OGC of each donor cow was obtained. GSH assays were conducted following the manufacturer's protocol (GSSH/GSH quantification kit, Dojindo Molecular technologies, Inc, Kumamoto, Japan). GSH content was compared between the two age groups.

Experiment 4

In experiment 4, we examined the developmental ability of the oocytes collected from EAFs of young and aged cows. Developmental ability was determined by antrum formation of the OGCs during in vitro culture periods (16 days), fertilization ratio of the oocytes grown in vitro, the developmental rate to the blastocyst stage following IVF, and total cell number of the blastocysts. OGCs were cultured using two types of media because there was no information about an optimal culture condition for OGCs of EAFs derived from aged cows. These media were IVC medium containing 0.1 µg/mL estradiol and an IVC medium containing 10 µg/mL estradiol. Concentration of estradiol was set on the basis of the previous reports on estradiol concentration of bovine follicles [23], and that high concentration of estradiol 10 µg/mL enhanced in vitro growth of oocytes of EAFs derived from young cows and gilts [24, 30]. For replicates, about 30–40 oocytes were collected from the group of young or aged cows (Table 1) and the experiment was repeated 10 times. During in vitro culture, the ratio of antrum formation was observed at 4, 8, 12, and 16 days of culture. OGCs showing antrum formation were subjected to IVM and IVF. After IVF, several oocytes were randomly collected, the ratio of fertilization was examined, and remaining oocytes were subjected to IVC. At day 7 after insemination, the ratio of blastulation and total cell number of the blastocysts were examined.

Statistical analysis

Data, such as the rate of antral cavity formation, number of granulosa cells, oocyte diameter, GSH content, and total cell number of the blastocysts obtained from young and aged cows were compared using two-tailed Student's *t*-test. Nuclear maturation, fertilization, and blastulation were compared by χ -square test. All statistical analyses were performed with SPSS (version 17.0) software (SPSS Inc., Chicago, IL, USA). *P* value <0.05 was considered to be significant.

Results

Comparison of gene expression in granulosa cells of young and aged cows

In Experiment 1, we sequenced eight lanes of a cDNA library derived from young and aged cows, and obtained 15.4 Gb of 50-bp reads (7.5 Gb for young cows and 7.9 Gb for aged cows). The sequence data were deposited in the DDBJ Read Archive (DRA) [Accession #: DRA000440]. Out of the 13,953 unique genes from the UCSC database, 88 % of the genes had at least one read aligned (10,046 genes for young cows and 9,964 genes for aged cows), and 9,762 of genes had at least one read aligned for both young and aged cows. In each gene containing an RPKM value greater than one, the normalized number of mapped reads was compared between the young and aged cows. The result of the interactive pathway analysis showed that the top molecular and cellular functions are cell death, cellular growth and proliferation, cellular movement and cell-to-cell signaling and Interactions (Table 2 and Supplementary Table 1). In addition, the top canonical pathways and toxic lists are shown in Table 2, which include recovery from ischemic acute renal failure (rat), glutathione depletion - phase II reactions, and oxidative stress. Of the apoptosis related genes, *CCDC80*, *COL1A1*, *CTGF*, *DNAJB1*, *LGALS9*, *LTF*, *LNM*, *MT2A*, *MTH11*, *SOX4*, and *TIMP2* showed significant differences in expression levels between the two age groups (Supplementary Table 2). Table 3 lists the marker genes for the largest healthy

Table 2 Result of interactive pathway analysis

Molecular and cellular functions	<i>p</i> -value molecules
Cell death and survival	2.24E-11–8.74E-03 74
Cell growth and proliferation	2.06E-10–8.58E-03 78
Cellular movement	5.48E-09–8.40E-03 53
Cellular development	7.73E-07–8.58E-03 73
Cell to cell signaling and interaction	8.58E-07–8.74E-03 49
Top canonical	<i>p</i> -value ratio
Hepatic fibrosis/hepatic stellate cell activation	3.88E-07 10/146 (0.068)
Inhibition of matrix metalloproteases	2.45E-05 5/40 (0.125)
NRF2-mediated oxidative stress response	1.03E-03 7/192 (0.036)
ILK signaling	1.29E-03 7/192 (0.036)
Branched-chain arfa-keto acid dehydrogenase complex	2.62E-03 2/9 (0.222)
Top toxic lists	<i>p</i> -value ratio
Recovery from ischemic acute renal failure (RAT)	5.23E-06 4/14 (0.286)
Hepatic fibrosis	1.7E-05 7/93 (0.075)
Glutathione depletion-phase II reactions	6.68E-04 3/20 (0.15)
Oxidative stress	1.54E-03 4/57 (0.07)
Positive acute phase response proteins	2.23E-03 3/30 (0.1)

Table 3 Expression of marker genes in granulosa cells of aged and young cows

Catogolies ¹	Symbols	RPKM			<i>p</i> -value ^b	References
		Aged	Young	A/Y ^a		
Subordinate	<i>ADAMTSI</i>	5.17	5.06	1.02	1.00	[1]
	<i>CCDC80</i>	19.38	8.98	2.16	0.04	[1]
	<i>CRABP²</i>	456.01	330.46	1.38	0.00	[1]
	<i>GADD45A</i>	28.26	25.14	1.12	0.69	[1]
	<i>KRT8</i>	6.91	4.99	1.38	0.07	[1]
	<i>KRT8</i>	63.02	45.78	1.38	0.07	[1], [2]
	<i>LOXL4</i>	0.71	0.20	3.54	1.00	[1]
	<i>OLR1</i>	1.81	0.12	14.87	0.62	[1]
	<i>OXT</i>	2.20	1.95	1.13	0.68	[1]
	<i>PDK4</i>	4.93	4.70	1.05	1.00	[1]
	<i>PLAUR</i>	0.50	0.24	2.09	1.00	[1]
	<i>SERPINE1</i>	7.16	2.72	2.63	0.14	[1]
	<i>SFRP4</i>	23.34	16.92	1.38	0.28	[1]
	<i>SLC1A1</i>	1.17	1.16	1.01	1.00	[1]
	<i>THBS1</i>	96.17	79.66	1.21	0.15	[1], [2], [3]
	<i>THBS2</i>	4.47	2.83	1.58	0.50	[1], [3]
	<i>TIMP1</i>	20.05	11.88	1.69	0.12	[1]
Largest	<i>AMH</i>	56.98	70.19	−1.23	0.29	[1]
	<i>CYP19A1</i>	41.08	40.20	1.02	0.83	[1]
	<i>FOS</i>	670.35	814.72	−1.22	0.00	[1]
	<i>FSHR</i>	68.18	67.51	1.01	0.86	[1]
	<i>GCLC</i>	339.90	367.61	−1.08	0.50	[1]
	<i>GPX3</i>	63.85	51.45	1.24	0.23	[1]
	<i>GUCA1A</i>	8.15	8.96	−1.10	1.00	[1]
	<i>IGFBP2</i>	9.43	13.31	−1.41	0.54	[1]
	<i>INHA</i>	302.02	356.60	−1.18	0.09	[1]
	<i>INHBA</i>	115.47	130.13	−1.13	0.48	[1], [2], [4], [5]
	<i>PIGF</i>	22.98	23.99	−1.04	1.00	[1]
	<i>PLA2GIB</i>	18.69	19.63	−1.05	1.00	[1]
	<i>SERPINF2</i>	3.05	1.54	1.98	0.45	[1]
	<i>SLC39A14</i>	44.90	44.53	1.01	0.92	[1]
	<i>TMEM20</i>	15.82	20.54	−1.30	0.51	[1]
	<i>TNFAIP8</i>	1.79	1.84	−1.03	1.00	[1]
	<i>CTGF</i>	19.30	7.85	2.46	0.02	[3], [6]
	<i>GSTA1</i>	16.37	30.01	−1.83	0.06	[5]
	<i>ITGA6</i>	275.27	329.79	−1.20	0.07	[2]
	<i>LAPT4A</i>	126.80	130.88	−1.03	1.00	[5]

Largest healthy follicle and second largest or subordinate follicle. marker genes was cited based on the previous reports (1: Hayashi et al. 2010, 2: Mora et al. 2012, 3: Greenaway et al. 2004, 4: Assidi et al. 2008, 5: Chen et al. 2009, 6: Harlow et al. 2007). When the ratio is less than 1.0, it is converted to its negative inverse. *P*-values are calculated by Fisher's exact test

follicles and the subordinate follicles in cows [31–37], and shows the expression levels of these genes in aged and young cows. All genes associated with subordinate follicles were expressed at lower levels in young cows. Genes that are associated with the largest follicles showed a tendency to express at lower levels in aged

cows with 15 of the 21 genes having A/Y rate under 1.0. However, the expression levels of *SERPINF2* and *CTGF* were 1.98 and 2.46 folds relative to the levels seen in young cows.

Interestingly, expression levels of genes related to glutathione appeared to be lower in granulosa cells derived from aged

Table 4 Expression of genes related to glutathione and other antioxidants

Symbol	RPKM			P Value ^b
	Aged	Young	A/Y Ratio ^a	
<i>GCLC</i>	339.90	367.61	−1.08	0.50
<i>GCLM</i>	30.73	31.94	−1.04	1.00
<i>GPX1</i>	208.76	237.57	−1.14	0.30
<i>GPX3</i>	63.85	51.45	1.24	0.23
<i>GPX4</i>	85.25	82.21	1.04	0.70
<i>GPX7</i>	43.42	47.07	−1.08	0.84
<i>GPX8</i>	72.36	72.98	−1.01	0.87
<i>GSR</i>	12.96	14.14	−1.09	0.85
<i>GSS</i>	11.89	11.75	1.01	1.00
<i>GSTA1</i>	16.37	30.01	−1.83	0.06
<i>GSTA3</i>	625.71	745.30	−1.19	0.01
<i>GSTA4</i>	63.38	72.32	−1.14	0.55
<i>GSTA5</i>	49.48	75.91	−1.53	0.03
<i>GSTCD</i>	5.00	5.41	−1.08	1.00
<i>GSTK1</i>	22.71	23.56	−1.04	1.00
<i>GSTM1</i>	11.87	13.11	−1.10	0.85
<i>GSTM3</i>	72.46	137.25	−1.89	0.00
<i>GSTO1</i>	20.13	16.64	1.21	0.52
<i>GSTP1</i>	63.86	66.02	−1.03	0.93
<i>GSTT1</i>	3.26	4.07	−1.25	1.00
<i>GSTZ1</i>	5.66	6.32	−1.12	1.00
<i>IDH1</i>	60.63	80.65	−1.33	0.13
<i>IDH3A</i>	36.19	32.33	1.12	0.63
<i>IDH3B</i>	53.57	53.93	−1.01	0.92
<i>IDH3G</i>	24.01	21.18	1.13	0.66
<i>TXNDC11</i>	13.80	13.93	−1.01	1.00
<i>TXNDC12</i>	43.55	43.93	−1.01	0.92
<i>CAT</i>	55.59	57.04	−1.03	1.00
<i>SOD1</i>	93.81	87.14	1.08	0.55
<i>SOD2</i>	50.06	52.82	−1.06	1.00
<i>SOD3</i>	14.60	15.84	−1.09	1.00
<i>TXN</i>	341.14	343.77	−1.01	0.79
<i>TXN2</i>	28.10	27.97	1.00	0.89
<i>PGD</i>	71.94	80.22	−1.12	0.63
<i>PGP</i>	15.23	18.91	−1.24	0.74

a when the ratio is less than 1, it is converted to its negative inverse, b P-values are calculated by Fisher's exact test

cows than those in young cows, and significantly lower expression was observed in the expression level of *GSTA3* and *GSTA5* (Table 4). In addition, the expression levels of almost all chaperone molecules were lower in OGCs derived from aged cows than those from young cows, and a significant difference was observed in the expression of *HSP90AA1* and *HSP90B1* (Supplementary Table 3).

Comparison of certain characteristic features of oocyte and granulosa cells derived from early antral follicles of young and aged cows

In Experiment 2, the diameters of oocytes were determined immediately after they were collected from EAFs. The average diameter ($\pm$ SE) of oocytes was 105.5 ± 1.0 μ m for oocytes derived from aged cows and 102.7 ± 1.0 μ m for oocytes derived from young cows (Table 5). The difference between the two age groups was significant ($P < 0.05$). The average number ($\pm$ SE) of cells contained in OGCs derived from EAFs of young and aged cows was $10,838 \pm 627$ and $10,522 \pm 517$, respectively, and there was no difference between the groups. Moreover, the average diameter ($\pm$ SE) of full-grown oocytes that were collected from antral follicles (3–6 mm in diameter) of the same donor cows was 134.5 ± 0.6 μ m for those from aged cows and 137.9 ± 0.5 μ m for those from young cows. The difference between the two age groups was significant ($P < 0.05$). In Experiment 3, the average ($\pm$ SE) GSH content of OGCs collected from EAFs of aged cows was 54.8 ± 5.5 pM, which was significantly lesser than that in OGCs of young cows (73.7 ± 4.3 pM, $P < 0.05$).

Growth of OGCs and the developmental ability of oocytes under in vitro conditions

When OGCs were cultured in a medium containing 0.1 μ g/mL E_2 , the rate of antrum formation was higher in OGCs derived from aged cows, whereas the ratio of antrum formation was comparative in a medium containing 10 μ g/mL E_2 (Table 6). In the medium containing lower E_2 , the ratio of polyspermic fertilization was significantly higher in aged cows than in young cows (6.8 and 18.7 % for young and aged cows, respectively), while the ratio was similar in the medium containing higher E_2 (12.0 vs. 11.9 %, respectively). There was no significant difference in the developmental ratio to the blastocyst stage between the two age groups in both media, whereas total cell number of the blastocysts was higher for OGCs derived from young cows compared with that of older counterparts irrespective of the culture conditions (lower E_2 , 99.6 ± 13.7 vs. 67.1 ± 11.1 ; higher E_2 , 68.5 ± 5.4 vs. 49.3 ± 3.4 ; $P < 0.05$).

Discussion

To the best of our knowledge, the present study is the first to demonstrate differential gene expression profiles which were more pronounced in the genes encoding several functional components of the pathways involved in oxidative stress response, in granulosa cells of EAFs of aged cows. In vivo development of the early antral stage follicles of aged cows differ from that of their younger counterparts, as the diameter

Table 5 Comparison of characteristic features of oocyte and granulosa cells derived early antral follicle and antral follicle of aged and young cows

Group	No. of donor cows	AF		No. of oocytes	EAFs		No. of OGCs	GSH content/OGCs (pM)
		No. of oocytes	Oocytes diameter (μm)		Oocytes diameter (μm)	No. of granulosa cell/OGC		
Aged	10	100	134.5±0.6a	91	105.6±1.0a	10,522±517	100	54.8±5.5a
Young	10	100	137.9±0.5b	92	102.6±1.0b	10,838±627	100	73.7±4.3b

Oocytes were collected from early antral follicle (0.5–0.7 mm in diameter, EAF) and middle size follicles (3–6 mm in diameter, MF). a-b, $P<0.05$. All data were presented as mean ± S.E

of oocytes derived from AFs was smaller and the diameter of oocytes from EAFs was larger in aged cows than in young cows. In addition, developmental competence of the oocytes derived from EAFs of aged cows was lower than that of young cows.

So far, no other study has reported the standard gene expression profiles in granulosa cells that are associated with the normal development of healthy EAFs of young cows. Hayashi et al. [36] reported several marker genes related to the largest follicle as the most viable follicle and the second largest follicles as a subordinate follicles in cows. In addition, several studies have reported genes related to healthy large developing follicles or subordinate follicles [31, 34, 35, 37]. First, we compared the expression of marker genes between the two age groups. Interestingly, all genes related to subordinate follicles appear to be expressed at higher levels in granulosa cells of aged cows than in the young cows. In addition, many genes related to healthy large follicles were expressed at lower levels in granulosa cells of aged cows with a few exceptions. These results suggest that the granulosa cells from EAFs showed a similar presentation as those from the subordinate follicles in aged cows.

Gene expression analysis showed that the top toxic list and canonical pathways contained genes involved in oxidative stress response. In aging cells, high amount of reactive oxygen species (ROS) are usually generated due to age-associated decrease in mitochondrial quality. A decrease in the levels of SOD1, SOD2, and catalase mRNA and protein in granulosa

cells derived from antral follicles of older women (≥ 38 years) has been reported [18]. In addition, p38 MAPK was more extensively phosphorylated in the granulosa cells derived from older women (>36) than those derived from younger patients, and the activation and cytoplasmic localization of p38 MAPK in granulosa cells derived from older women was indicative of oxidative stress [20]. In addition, we presented that oocytes collected from antral follicle (3–6 mm in diameter) of aged cows had higher ROS content compared with that of young cows [28]. There has been however, no report regarding the ROS in OGCs derived from EAFs. The gene expression analysis in this study shows that the relative expression of genes related to glutathione was lower in aged cows. In addition, GSH content in OGCs was lower in aged cows than that in young cows. Protein quality is crucial for cellular homeostasis and the presence of mis folding proteins is a common feature of the cells under oxidative stress. Chaperon proteins, including HSP70 and 90, play roles in the degradation and refolding of proteins preventing from protein aggregation and cellular death. Several studies have reported age-associated decline in protein quality and responsiveness of HSP70 and HSP90 in tissues [38–40]. In addition, as the follicle grows, the expression level of HSP 60, HSP70, and HSP90 increases in bovine granulosa cells [41]. These expression levels were affected by follicle conditions such as cystic follicle [42]. Thus, it can be speculated that the HSPs play a crucial role in oocyte development. In the present study, HSP90AA and HSP90B1 were expressed at significantly

Table 6 Growth of OGCs and the development ability of oocyte grown in vitro

E2 µg/ml	Age	No of trials	No. of OGCs	Antrum formation (%)				Fertilization no. (%)		No. (%) of blasturation	Total cell number
				Days of culture							
				4	8	12	16	Norm	Poly		
0.1	Y	10	358	1.1±0.1	39.7±9.8a	46.±13	43.7±16.6a	33/73 (45.2)	5/73 (6.8)a	3/97 (3.1)	99.6±13.7a
	A	10	341	1.0±1.6	51.3±14.0b	56.6±11.9b	58.8±12.6b	24/75 (46.7)	14/75 (18.7)b	10/154 (6.5)	67.1±11.7b
10	Y	10	395	0.3±0.3	45.6±4.0	54.8±3.2	55.1±3.8	20/50 (40.0)	6/50 (12.0)	12/174 (6.9)	68.5±5.4a
	A	10	400	0.3±0.3	49.1±4.4	59.0±4.2	61.5±3.1	25/59 (42.4)	7/59 (11.9)	11/165 (6.7)	49.3±3.4b

Developmental ability was determined by antrum formation of the OGCs during 16 days of culture period, fertilization and developmental rate to the blastocyst stage of oocytes grown in vitro. a:b $P<0.05$

lower levels in granulosa cells of OGCs derived from aged cows than in the young cows. In addition, the relative expression levels of other HSP members were also lower in the aged cows compared with their young counterparts. Thus, these findings suggest that relatively lower anti-oxidative potential and impaired chaperone activities synergistically affect the functions of the granulosa cells in aged cows. Consistent with our results, the top molecular and cellular functions were cell death and survival and cellular growth and proliferations. Genes associated with cell proliferation and cell death are involved in bovine follicular selections [43]. In this context we have provided a list of genes whose expression correlated with these functions in supplementary table 2. Of these genes, *CCDC80*, *CLDN11*, *COL1A1*, *CTGF*, *DNAJB1*, *LGALS9*, *LTF*, *LUM*, *MT2A*, *MYH11*, *NMB*, *SOX4*, and *TIMP2* significantly expressed between the two age groups. *DNAJB1* and *SOX4* are expressed at low level in aged groups and it is reported that these genes contributes to p53 activity and promotes cellular apoptosis, respectively [44, 45]. Similarly, *TIMP2* that is expressed at low levels in aged groups inhibits angiogenesis [46, 47]. Thus, these results suggest that the underlying molecular mechanism of follicular cells survival and follicular development might differ between the two age groups. Oocyte diameter is a critical marker of its development. Among the oocytes of EAFs, the diameter of oocytes collected from medium-sized antral follicles (3–6 mm) of aged cows was smaller than that from young cows, which is in agreement with previous reports in humans [48]. However, the diameter of oocytes derived from EAFs of aged cows was greater than those from EAFs of young cows. These results suggest that in vivo development of EAFs is suspended after some developmental progression of the EAFs and the oocyte growth from EAF to AF is impaired in aged cows; this stagnation may be related to the gene expression profiles in granulosa cells of aged cows.

Oocytes collected from antral follicles (3–6 mm in diameter) of aged cows had evidently lower ability to mature, fertilize, cleave and develop into the blastocyst stage [9–11]. However, there are no reports regarding the in vitro developmental competence of bovine oocytes of EAFs of aged cows. To date, a few pioneer studies have examined the effects of maternal age on pre antral follicle development. Xu et al. [15] reported that pre antral follicles collected from young adult rhesus monkeys grew faster in vitro and secreted more steroids than those derived from older monkeys. In addition, Choi et al. [16] reported that the overall number of follicles in the ovaries of aged mice decreased, and follicle formation of pre antral follicles cultured in vitro was lower in aged mice than in young mice, but the developmental ability to progress to the blastocyst stage was comparable between the two age groups.

In the present study, we cultured OGCs in media containing lower or higher E_2 concentration, based on previous reports. A

recent report showed that E_2 improved antrum formation of bovine OGCs cultured in vitro, and gene expression between OGCs cultured with high E_2 and those with low E_2 . The use of NGS showed that most of the marker genes that are associated with the large healthy follicles were expressed at higher levels in granulosa cells of OGCs cultured with E_2 than in those cultured without E_2 [24]. In the present gene expression analysis, the genes that are associated with healthy large follicles were expressed at lower levels in OGCs of aged cows. Consequently, we deduced that in vitro growth of oocyte derived from EAFs of aged cows is improved by supplementation of the culture medium with high concentration of E_2 . However, in low E_2 condition, the ratio of antrum formation was higher for OGCs derived from aged cows than those for their younger counterparts. In addition, the present gene expression analysis indicated that the expression level (RPKM) of estrogen receptor 1 and 2 did not differ between the two age groups; however HSPA8 and HSP90s, which binds to estrogen receptors were expressed at lower levels in aged group than in young group (Supplementary Table 4). Thus, although we could not identify a direct cause for the high responsiveness of OGCs derived from aged cows to E_2 , our results suggest that post-transcriptional modification of estradiol receptors differs between the two age groups. The responsiveness to E_2 differs among different developmental stages of follicle, and OGCs derived from advanced stage EAFs did not require much E_2 for their in vitro development [30]. In addition, we found that OGCs derived from EAFs of aged cows were at a more advanced stage compared to those of the younger counterparts. Together, these reports and the present results suggest that the differences in the developmental stage of the EAFs contribute to the differential responsiveness to E_2 . However, cellular responsiveness to E_2 is controlled by a complex mechanism that includes binding of ligand and receptors, activity of the receptor including phosphorylation by MAPK and CDC kinase [35, 49], degeneration of ubiquitinated estradiol receptors by proteasome [50], methylation of estradiol receptor genes [51], and estradiol receptor localization [52]. Other possible mechanisms need to be examined.

Recently it is shown that a high ratio of polyspermic fertilization and low total number of the blastocysts were specific features of the oocyte- derived antral follicles (3–6 mm in diameter) from aged cows [9, 28]. In addition, low total cell number of the blastocysts was also observed in mice [53]. When OGCs of EAFs were cultured in medium containing 0.1 $\mu\text{g/mL}$ E_2 , the ratio of polyspermic fertilization of oocytes grown in vitro was higher for aged groups than that for young groups. However, upon culturing the oocytes with high E_2 , the ratio of fertilization outcome was comparative between the two aged groups, which indicate that the modification of steroid concentration may diminish the differential fertilization ability in two age groups. However, total cell

number of the blastocysts developed in vitro still remained lower in aged groups irrespective of E_2 concentration and the developmental ability of oocytes collected from EAFs of aged cows was low.

In conclusion, the developmental ability of oocytes derived from EAFs of aged cows was lower to the extent that the fertilization outcome and total cell number of the blastocysts was low. Furthermore, certain characteristic features of the granulosa cells differed between aged and young cows.

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