—Original—

Effect of aging on telomere lengths in bovine oocytes and granulosa cells

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Abstract: Age-associated telomere shortening in oocytes and granulosa cells is considered a sign of age-associated decline in oocyte quality. The present study examined the effect of aging on telomere lengths (TLs) in bovine oocytes, embryos, and granulosa cells, as well as the relationship between the TLs in oocytes and granulosa cells. TL was directly assessed by real-time PCR, using a telomeric standard of 84 bp length TTAGGG, repeated 14 time). TLs in immature oocytes derived from early antral follicles (EAFs) and antral follicles (AFs) as well as for in vitro matured oocytes derived from aged cows (>120 months) were shorter than their respective counterparts in younger cows (20–70 months, 0.45-, 0.82-, and 0.84- fold, respectively, $P < 0.05$). Telomeres elongate extensively during embryo development until the blastocyst stage (4.2-fold, $P < 0.05$); however, TLs in the blastocysts did not differ between the two age groups. TLs in the granulosa cells of both AFs and EAFs were shorter in aged cows than in younger cows, and showed a positive correlation with TLs in oocytes (r=0.66, $P < 0.05$). In conclusion, aging affects TL in oocytes, and the TLs in granulosa cells and oocytes are correlated.

Key words: Telomere, Oocyte, Granulosa cells, Aging

Introduction

Telomeres are repetitive sequences of TTAGGG at the 3′ end of eukaryotic chromosomes [1]. Telomeres shorten in length with every cell division because of the replication mechanism and/or exposure of their guanine-rich parts to oxygen radicals [2]. Therefore, telomeres in the cells of aged animals are generally shorter than those in their younger counterparts [2]. An age-associated decline in oocyte quality is a common phenomenon in mammals, and telomere shortening is one of the casual factors of chromosomal disjunctions [3] and blastomere aneuploidy [4]. In addition, recent evidence shows that telomere length (TL) in granulosa cells or cumulus cells reflects oocyte fertilization ability [5]. However, to the best of our knowledge, the exact relationship between the TLs in oocytes and granulosa cells is unclear.

Oocytes emerge in the ovaries of the early fetus after active division of primordial germ cells, and await ovulation for a long time without undergoing cellular division. During this period, they are exposed to oxygen radicals. As noted above, this is expected to shorten telomeres, and in support of this, shorter TLs have indeed been observed in the oocytes of aged mice [6]. Interestingly, TLs are shorter in oocytes than in somatic cells [7], but after fertilization or parthenogenetic activation, telomeres elongate to a great extent, leading to much longer telomeres in blastocysts than in oocytes [7, 8]. Despite the self-elongation of telomeres in early preimplantation embryos, it has been shown that the TL in offspring is affected by the mother's TL [9]. Studies like this one should help determine whether aging affects the TL in embryos. The present study addresses the following issues: (1) whether aging affects TLs in bovine oocytes and granulosa cells, (2) whether TLs in granulosa cells correlate with TLs in oocytes, and (3) whether aging affects TLs in blastocyst-stage embryos.

Materials and Methods

Chemicals

All chemicals were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. Medium 199 supplemented with 10% fetal calf serum (FCS; 5703H; ICN Pharmaceuticals, Costa, Mesa, CA, USA) and 5 mM taurine was used for *in vitro* maturation (IVM). *In vitro* fer-

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tilization (IVF) medium and *in vitro* culture (IVC) medium were based on synthetic oviduct fluid (SOF) [10], with minor modifications, as reported previously [11]. The IVF medium consisted of SOF containing 5 mg/ml fatty acid-free bovine serum albumin (BSA) and 10 IU/ml heparin (Sigma-Aldrich, St. Louis, MO, USA). The IVC medium used for culture during the first 48 h after fertilization consisted of SOF containing amino acids (Sigma-Aldrich), 1.5 mM glucose and 1% FCS. From 48 h to seven days after fertilization, the FCS concentration in the culture medium was increased from 1% to 5%.

Oocyte collection and in vitro maturation (IVM)

In Japan, the breed and age of a cow can be identified at slaughterhouses, because each animal has individual identification number for the traceability of all Japanese beef. Ovaries of Japanese Black Cows (Bos Taurus) with their ages identified in months were collected from a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at approximately 25 °C. Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (AFs, 3–6 mm in diameter) of individual cows using a 21 gauge needle connected to a 5 ml syringe. The cohort COCs collected from each donor cow were cultured in 100 *μ*l of IVM medium (10 oocytes/drop) under paraffin oil (Tissue Culture Grade, Nacalai Tesque, Kyoto, Japan) for 21 h at 38.5 °C in an atmosphere of 5% CO2 in air with maximum humidity.

In vitro fertilization and in vitro culture of embryos

Frozen thawed semen from a Japanese black bull was washed with a 30–60% discontinuous Percoll gradient solution (Amersham Co., Ltd., Uppsala, Sweden) by centrifugation for 10 min (800×g). After centrifugation, sperm (2 × 10^6 cells/ml) were coincubated with oocytes for 5 h in 100 *μ*L of IVF medium (10–15 oocytes/drop). After fertilization, these COCs were cultured in 100 *μ*l of IVC medium (10–15 oocytes/drop). Fertilization and culture were performed at 38.5 °C in an atmosphere of 5% CO_2 in air with maximum humidity, for two days after fertilization. The surrounding cumulus cells were then removed to obtain cleaved embryos (>eight-cell stage). The cleaved embryos were cultured in 10 *μ*l IVC medium (3–5 embryos/drop) for five days at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 with maximum humidity. At day 6.5 after fertilization, blastocyst-stage embryos were stained with Hoechst 33342 to determine the cell numbers under a fluorescence microscope (IX71; Olympus, Tokyo, Japan). The blastocysts were then retrieved from the slide and treated with an acidic solution (pH 2, 0.2% PBS-polyvinyl alcohol solution) to remove the zona pellucida. After several rounds of washing, each blastocyst was transferred to a PCR tube for DNA extraction followed by telomere measurement.

Measurement of TLs in oocytes

DNA of oocytes and blastocysts was extracted in 12 *μ*l of lysis buffer (20 mM Tris, 0.4 mg/ml proteinase K, 0.9% Nonidet P-40, and 0.9% Tween 20) and incubated at 55 °C for 30 min, followed by incubation at 98 °C for 5 min. TL was measured in duplicates using real-time PCR, following a protocol reported earlier [12, 13]. The primer set was 5′-cggtttgtttgggtttgggtttgggtttgggtttggg tt-3′ and 5′-ggcttgccttaccttaccttaccttaccttaccct-3′ (HPLC grade), respectively, and the concentration used was 0.5 *μ*M. The standard was a serial-diluted synthesized oligonucleotide (84 bp in length; TTAGGG repeated 14 times). The PCR enzyme SsoFast^{TM} Eva Green (BIO-RAD) was used. PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C (15 sec) and 60 °C (60 sec). To obtain TLs in oocytes, the predicted copy number of PCR products targeting the telomere was divided by the oocyte number in a template and 240 (chromosome number is 60 in cows), and multiplied by 84. To determine the TLs in blastocysts, the copy number of the PCR product was divided by the cell number of the blastocyst and 120, and then multiplied by 84.

Measurement of TLs in granulosa cells

Granulosa cells were collected from follicles and washed twice in PBS. DNA was extracted from the cellular pellets using an extraction kit (QIAamp DNA mini kit, QIAGEN). The estimated TL was obtained by real-time PCR as described above, and the cell number of the sample was measured by real-time PCR targeting one copy gene (IL13, forward primer: 5′-CCCTTACTG-GTTGTGGCAT-3′, and reverse primer: 5′-CTTCAGT-GTTCCCGAGCTTC-3′, 106 bp). The conditions for the PCR targeting one copy gene were: 95 °C for 3 min, followed by 40 cycles of 98 °C (5 sec) and 59 °C (60 sec). As an external standard, the PCR product of the corresponding gene was cloned into a vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA), the product of which was sequenced for confirmation before use. For the telomere measurement, extracted DNA of the granulosa cells was diluted until the cell number in each PCR reaction tube was 20. To determine the TLs in the cells, the copy number of the PCR products targeting telomeres was divided by the cell number of the samples and 120, and multiplied by 84.

Statistical analysis

The TLs of the young and aged cows were compared using Student's t-test. Percentages were arcsine-transformed prior to analysis. Correlation was calculated using Pearson's method and all analysis were conducted using SPSS (Version 17.0, Chicago, IL, USA). Values of $P < 0.05$ were considered significant.

Results

TLs in oocytes shortened as donor cows aged

First, we compared the TLs in oocytes collected from AFs (3–5 mm in diameter) between young and aged cows. To adjust the sample DNA content, 10 oocytes were used for one PCR reaction which was duplicated (20 oocytes were subjected to analysis). Real-time PCR analysis revealed that the average TLs in oocytes derived from 12 young cows (average age: 26.2 ± 1.9 months) was 10,309.7 ± 484.1 bp, which was significantly longer than that of aged cows (n=18, average age 171.2 ± 24.3 months, TL: 8,494.5 ± 607.4 bp, Fig. 1). In addition, we investigated the TL in more immature, germinal vesicle-stage oocytes, which were collected from early antral follicles (EAFs, 0.5–0.7 mm in diameter). It was difficult to collect 20 oocytes from the EAFs of each donor, and therefore, 10 oocytes per donor were collected. The TLs in oocytes of EAFs were measured by a single PCR, and it was found that the TLs in oocytes derived from small follicles were shorter in aged cows than in their younger counterparts (aged cows: n=10, average age 171.4 ± 8.4 months, TL 5,136.5 ± 840.8 bp vs. young cows: n=11, average age 28.4 ± 1.6 months, TL 11,452.8 ± 2399.2 bp, Fig. 1).

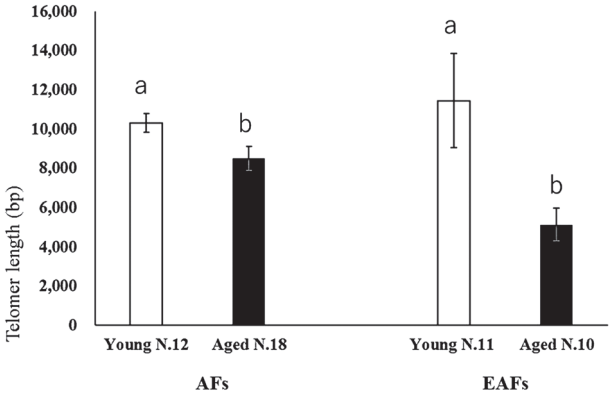

Fig. 1. Telomere lengths in oocytes collected from antral follicles (AFs; 3–6 mm in diameter) and early antral follicles (EAFs; 0.5–0.7 mm in diameter). The Y-axis is the predicted telomere length (bp) determined by real-time PCR. Telomere measurement was conducted using a group of 10 oocytes in a 20-μl reaction solution. Twelve (N.12) and 18 (N.18) young, and 11 (N.11) and 10 (N.10) aged cows were used for measurement of telomere length in oocytes derived from AFs and EAFs, respectively. a-b, $p<0.05$.

TLs in in vitro matured oocytes from aged cows were short and the fertilization ability of these oocytes was low

In this experiment, 20 cohort oocytes were collected from individual cows and cultured for 22 h for *in vitro* maturation, after which the average TL in the matured oocytes was determined by real-time PCR. As shown in Table 1, the average TL in matured oocytes derived from aged cows was significantly shorter than that in their younger counterparts (6,706 ± 513bp vs. 7,996 ± 277 bp, $P < 0.05$). To confirm whether or not there is an age-associated decline in oocyte quality, 20 oocytes were collected from individual young and aged cows, and their fertilization rates were determined. Normal fertilization, in which two pronuclear and polar bodies were observed, showed a higher rate in the oocytes collected from young cows than in those collected from older cows (Table 1, $P < 0.05$).

No difference was found in the average TLs in blastocysts from the two age groups

We examined the effect of aging on the average TL in blastocysts. Oocytes were collected from 10 young (26.1 ± 0.5 months of age) and 12 aged cows (147.8 ± 5.0 months of age), and oocytes from each cohort were matured, fertilized and cultured for 6.5 days. Subsequently, all blastocysts were subjected to telomere measurement following cell counting, as described above. The aver-

Table 1. Comparison of telomere lengths and fertilization abilities of oocytes between young and aged cows

	No. of Cows	Average months	TL (bp)	No. of Cows	Average months	Fertilization (%)	
						Normal	Abnormal
Young	28	33.3 ± 3.4	7,996 ± 277.8a	17	37.5 ± 5.1	66.4 ± 4.9a	14.4 ± 2.9a
Aged	25	143.8 ± 3.5	6,706 ± 513.0b	16	138.8 ± 3.2	48.9 ± 4.5b	26.5 ± 5.0b

Data are presented as Mean ±SEM. a-b; $p<0.05$. TL: telomere length.

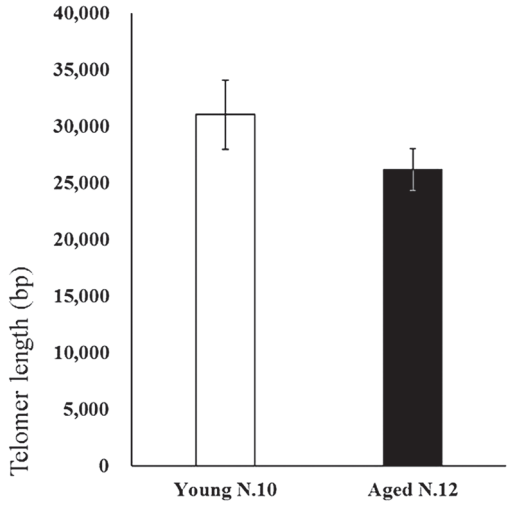

Fig. 2. Telomere lengths in blastocysts derived from the oocytes collected from the antral follicles of young and aged cows. The Y-axis is the predicted telomere length (bp) determined by real-time PCR. Telomere measurement was conducted individually for each blastocyst in a 20 μl reaction solution, and the average telomere length in blastocysts was calculated for each donor. Ten (N.10) young and 12 (N.12) aged cows were used for this experiment. a-b, $p<0.05$.

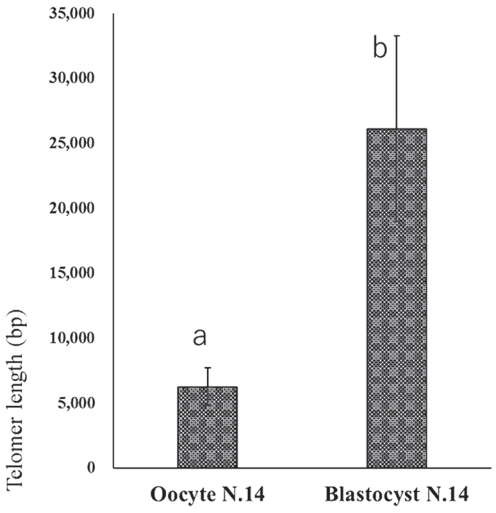

Fig. 3. Comparison of telomere lengths between oocytes and blastocysts derived from a cohort of oocytes collected from the same cows. The Y-axis is the predicted telomere length (bp) determined by real time PCR. Telomere measurement was conducted using a group of 10 oocytes or a single blastocyst in a 20 μl of reaction solution, and the average telomere length in blastocysts was calculated for each donor. Fourteen (N.14) donors were used in this experiment.

age developmental rates to the blastocyst stage of the young and aged donor cows at 6.5 days after insemination, were 26.5 ± 5.4% and 20 ± 2.6%, respectively, and the total cell numbers of blastocysts were comparable between the two groups (46.8 ± 2.8 vs. 49.3 ± 3.4 cells, respectively). The TLs in blastocysts derived from 28 young cows and 24 aged cows were successfully measured. The average TL in the blastocysts derived from young and aged cows were 31,015.8 ± 3,064.2 bp and 26,152.9 ± 1,877.2 bp, respectively, but this difference was not significant (Fig. 2, $P = 0.19$).

TL elongated during early embryo development

The large difference in TL between oocytes and blastocysts prompted us to compare the TL between the two stages within oocytes from the same donor. Over 30 oocytes were collected from 14 individual donor cows (6 young cows and 8 aged cows): 20 oocytes were used to determine the average TL in germinal vesicle-stage oocytes, and the remaining oocytes were subjected to IVM, IVF, and IVC, and the average TL in the blastocysts was examined. TLs in 33 blastocysts were successfully determined, from which the average TL in blastocysts was determined for each donor cow. The average TL in blastocysts was 26,115.7 ± 7141.7 bp, which was significantly longer than that of the cohort oocytes (6,239.5 ± 1,461.7 bp, $P < 0.05$, Fig. 3).

TL in granulosa cells from aged cows is short

We examined the TLs in granulosa cells that were collected from EAFs (0.5–0.7 mm in diameter) and AFs (3–5 mm in diameter). Firstly, granulosa cells were collected from 5 oocyte-granulosa cells complexes of EAFs which had been collected from 11 young and (26.6 ± 0.6 months) and 13 aged cows (156.5 ± 5.3 months). Average TLs in the granulosa cells of young and aged cows were 21,945.1 ± 899.3 bp and 19,074.2 ± 513.7 bp, respectively (Fig. 4, $P < 0.05$). Granulosa cells were also collected from AFs (3–5 mm in diameter) of young (n=25, 25.8 ± 0.4 months) and aged cows (n=14, 148.2 ± 7.4 months) and the TLs in the granulosa cells from the young cows were significantly longer than those of the aged cows (Fig. 4, 21,551.3 ± 1842.9 bp vs. 12,884.9 ± 1,931.5 bp, $P < 0.01$).

TLs in granulosa cells correlated with TLs in oocytes

We examined the relationship between the TLs in granulosa cells and in oocytes. Ten and twenty oocytes were

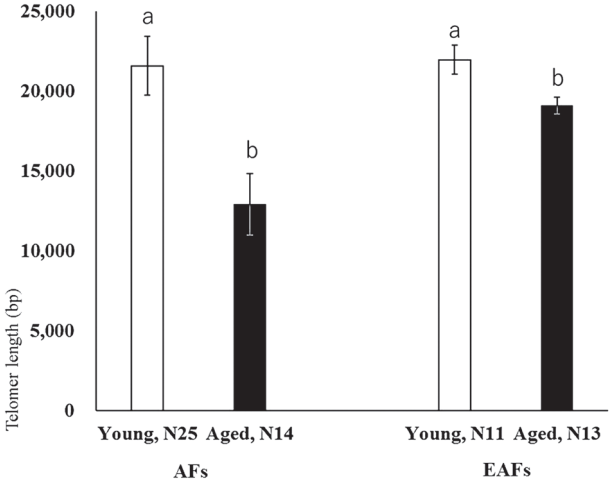

Fig. 4. Telomere lengths in granulosa cells derived from antral follicles (AFs) and early antral follicles (EAFs) of young and aged cows. The Y-axis is the predicted telomere length (bp) as determined by real-time PCR. The concentration of granulosa cells was measured using real-time PCR targeting one copy gene. Telomere measurement was conducted using 20 cells in a 20 μl of reaction solution. Twenty-five (N.25) and 14 (N.14) young and 11 (N.11) and 13 (N.13) aged cows were used for measurement of telomere lengths in granulosa cells derived from AFs and EAFs, respectively. a-b, $p<0.05$.

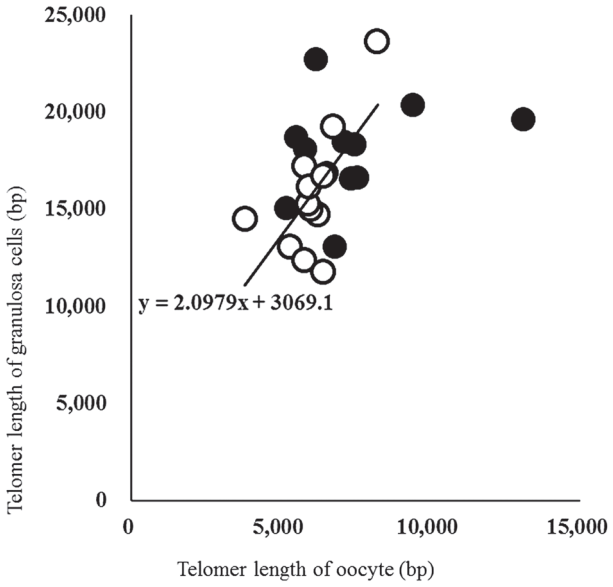

Fig. 5. Comparison of telomere lengths between oocytes and granulosa cells. Oocytes and granulosa cells were collected from early antral follicles (white circle) and antral follicles (black circle) of individual cows. The X-axis is the telomere length in oocytes and the Y- axis is the telomere length in granulosa cells (bp). A significant correlation was observed for antral follicles ($y=2.0979\times+3069.1$, $r=0.66$, $p<0.05$).

collected from EAFs and AFs of 12 donor cows (8 aged and 4 young cows), and granulosa cells of EAFs and AFs were collected from the same donor cows. Comparison of the TLs in oocytes and granulosa cells of AFs and EAFs from the same donors revealed that TLs in AFs were slightly shorter than in EAFs, although the difference was not significant (oocytes: AFs vs. EAFs, 7,350.0 ± 616.5 bp vs. 6,065.8 ± 292.6 bp, $P = 0.07$; granulosa cells: AFs vs. EAFs, 17,860.4 ± 723.7 bp vs. 15,794.3 ± 935.0 bp, $P = 0.09$). As shown in Fig. 5, TLs in oocytes and granulosa cells collected from AFs significantly correlated ($r=0.66$, $P < 0.05$) but not those collected from EAFs ($r=0.29$, $P > 0.05$).

Discussion

The present study found there is an age-associated shortening of telomeres in germinal vesicle-stage oocytes collected from EAFs (0.5–0.7 mm in diameter) and medium-size AFs (3–5 mm in diameter), and in *in vitro* matured oocytes. In addition, the telomere length was shorter in granulosa cells of EAFs and AFs derived from aged cows than their younger counterparts, and the TL correlated positively with that of oocytes. Furthermore, although the TLs elongated during early embryo development, no significant difference was observed in the TLs in blastocysts collected from the two age groups.

TLs in oocytes has been estimated by real-time PCR [3] and q-FISH [13, 14]. In most real-time PCR trials, the TL is estimated as the relative TL, the ratio of two PCRs targeting a telomere sequence and a single copy genes which is normalized to that of a randomly selected sample. The number of steps involved in the estimate might bias the results of telomere measurement. In the present study, we measured TL using real-time PCR, with a synthesized telomere sequence of TTAGGG repeats as a standard. In this method, the telomere sequence is estimated in a simple manner (Fig. 6). However, it should be noted that the amplification efficiency was 0.8–0.9, and the cell numbers subjected to PCR were within a small range (10 oocytes, 20 granulosa cells, 20–35 blastomere/20 μl reaction solution) in all reactions, and data were compared within one reaction group.

The first experiment revealed that the TLs in GV oocytes collected from both AFs (3–5 mm in diameter) and EAFs (0.5–0.7 mm in diameter) were shorter in the oocytes derived from aged cows than in their counterparts from young cows. Age-associated shortening of telomeres has been reported in fully grown oocytes of mice

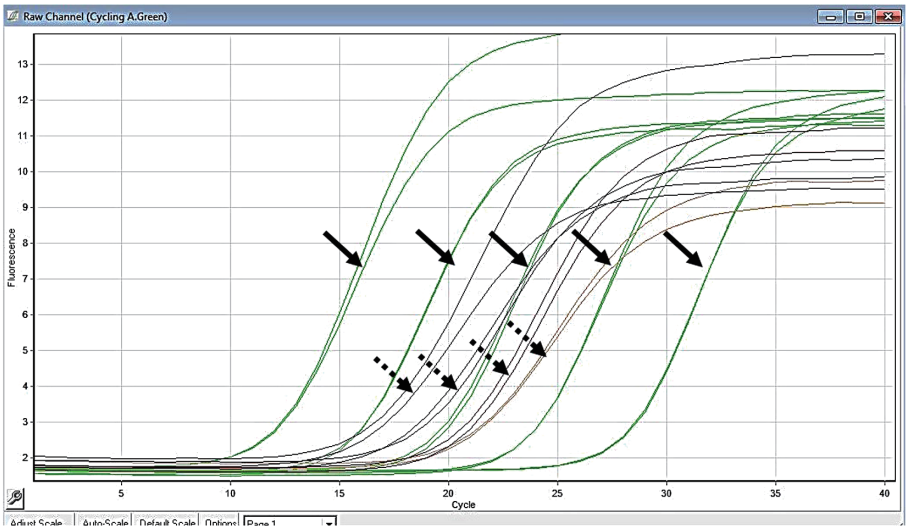

Fig. 6. Representative standard curve (indicated by solid arrows) created by serial dilution of an oligonucleotide (84 bp long TTAGGG repeated by 14 times), and the amplification curve for granulosa cells and oocytes. From the right, the dotted arrow indicates 10 oocytes, and 20, 400 and 8,000 granulosa cells, respectively.

and humans [3, 6]. However, to the best of our knowledge, this is the first report of age-associated shortening of telomeres in earlier stage oocytes. In addition, after maturation, the TLs in oocytes from aged cows were shorter than in their younger counterparts. Telomeres are vulnerable to oxidative stress due to their proximal location to the membrane and the guanine rich sequence [15], and we suggest that the oxidative stresses faced by oocytes in ovaries during their lifetime adversely affects oocyte TL. The fertilization ratio showed an age-associated decline in oocyte fertilization ability: the normal fertilization rate of oocytes derived from aged cows was significantly lower and the abnormal fertilization ratio was significantly higher. This result is in agreement with our previous reports [16, 17].

The present study showed that TLs in granulosa cells collected from EAFs and AFs of aged cows were shorter than those of young cows. Length of TL in cumulus cells and granulosa cells is related to oocyte and embryo quality [5], but whether or not the TL in oocytes correlates with that in granulosa cells had not been clarified. Therefore, we examined the relationship between TLs in oocytes and granulosa cells, and found a positive correlation between them. This is the first report to show a relationship between the TLs in oocytes and granulosa cells in a large mammal. However, this relation was not observed in EAFs. This was either due to small sample size or related to some or all of the following reasons. EAFs take approximately 2 weeks to develop to the AF stage in cows, and during this period, active cellular proliferation and oocyte growth occurs. Thus, we speculate that this phase strongly affects TL in both granulosa cells and oocytes in a similar manner, and our results support earlier speculation that TLs in granulosa cells are marker of oocyte quality. Note that this result was obtained from randomly selected cows and this relationship should be further examined within animals of the same age and genetic background, which are maintained under the same environment.

We suggested that the TL becomes longer during early embryo development in cows, and this trend has been observed in humans, mice, and cows [8, 14, 18]. Although the TL in oocytes from aged cows is shorter, the difference in TL at the blastocyst stage was not significant. On the basis of these results, it is possible to make the following assumptions: (1) TL is restored by active telomere extension during early embryo development; (2) due to high polyspermic fertilization in oocytes of aged cows, the TL in blastocysts was over-estimated using the formula: TL of embryo/cell number; (3) because TLs in sperm are longer than in oocytes and profoundly influence the TL of zygotes [19], a greater number of sperm incorporated into the oocytes of aged cows may affect the TL of blastocysts and; (4) embryos having excessively low TL may be eliminated during embryo development. These points should be addressed in future experiments.

In conclusion, aging affects TLs in oocytes and granu-

losa cells in a similar way, and although oocyte quality declines with donor age, a difference in TL in blastocysts collected from the two age groups was not observed in the present study.

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