Norikazu MIYASHITA1), Yasuaki KUBO2), Miharu YONAI3), Kanako KANEYAMA4), Norio SAITO5), Ken SAWA5), Akira MINAMIHASHI6), Toshiyuki SUZUKI7), Toshiyuki KOJIMA8) and Takashi NAGAI9)

1)Laboratory of Reproduction and Development, National Institute of Agrobiological Sciences, Ibaraki 305-0901, 2)Livestock Research Support Center, National Institute of Livestock and Grassland Sciences, Ibaraki 305-0901, 3)Grazing and Meat Production Research Team, National Agricultural Research Center for Tohoku Region, Iwate 020-0198, 4)Department of Technology, National Livestock Breeding Center, Fukushima 961-8511, 5)Department of Animal Science, Iwate University, Iwate 020-8550, 6)Reproductive Biotechnology Laboratory, Hokkaido Animal Research Center, Hokkaido 081-0038, 7)Animal Industry Research Institute, Iwate Prefectural Agriculture Research Center, Iwate 020-0173, 8)Veterinary Theriogenology Laboratory, Kagoshima University, Kagoshima 890-0065 and 9)Research Manager, National Institute of Livestock and Grassland Sciences, Ibaraki 305-0901, Japan

Abstract. Dolly, the first mammal cloned from a somatic cell, had shorter telomeres than age-matched controls and died at an early age because of disease. To investigate longevity and lifetime performance in cloned animals, we produced cloned cows with short telomeres using oviductal epithelial cells as donor cells. At 5 years of age, despite the presence of short telomeres, all cloned cows delivered multiple healthy offspring following artificial insemination with conventionally processed spermatozoa from noncloned bulls, and their milk production was comparable to that of donor cows. Moreover, this study revealed that the offspring had normal-length telomeres in their leukocytes and major organs. Thus, cloned animals have normal functional germ lines, and therefore germ line function can completely restore telomere lengths in clone gametes by telomerase activity, resulting in healthy offspring with normal-length telomeres.

Key words: Aging, Cattle, Mammalian cloning, Germ line, Telomere

In mammals, most somatic cells undergo a finite number of cell divisions and ultimately enter a nondividing state known as replicative senescence [1] as a consequence of structural changes that take place in chromosomes over time. Chromosomes terminate in a nuceloprotein complex termed a telomere, which consists of repetitive sequences of G-rich noncoding DNA (TTAGGG), and specific proteins. Telomeres are attached to the nuclear matrix and protect chromosome ends from degradation, fusion and recombination [2]. However, conventional DNA polymerases cannot replicate the extreme 5’ ends of chromosomes because removal of the most terminal RNA primer in the lagging strand leaves a small region of un coppied DNA; therefore, telomeres are incrementally eroded with each cell division [3]. On the basis of this mechanism, it has been proposed that progressive telomere shortening during each cell division eventually yields a threshold telomere length beyond which additional normal cell divisions are not possible. Thus, telomere length acts as a mitotic clock that accounts for the limited lifespan of cells [4].

In recent years, animal production by somatic cell nuclear transfer (SCNT) has offered a range of opportunities in basic and applied research, agriculture, genetic conservation and human medicine [5]. Somatic cell-cloned animals are produced using donor cells that have variably aged in vivo and in vitro, raising interesting questions about possible foreshortening effects on the lifespan and reproductive potential of these animals. In some cases, particularly with respect to cows and sheep, there is also the question of potential reductions in overall milk production. In this context, the telomere lengths in Dolly, the first mammal produced by SCNT, have been reported to be significantly shorter than those in age-matched controls, and this was consistent with the age of the donor mammary epithelial cell culture, which was derived from a 6-year-old donor sheep [6]. Dolly died at 7 years of age, well short of the natural lifespan of a normal sheep (≥12 years). However, it is unclear whether short telomeres were responsible for her death because the development of serial progressive lung disease forced her to be euthanized, and no other such cases were available for comparison. In contrast, cloned cattle [7–10], mice [11], pigs [12], and goats [13] produced from fibroblasts and cumulus cells have normal telomere lengths relative to age-matched controls. However, regardless of their normal telomere lengths, some of these animals died shortly after birth [8, 10]. Additionally, in cloned mice produced from the same donor cell culture, a population that is likely to exhibit little variation in telomere length, some lived normal life spans and others died earlier than control animals [14].

To answer some of the questions raised and address the discrepancies concerning lifetime and telomere lengths in cloned animals, we have used a standardized protocol to produce numerous cloned cows. Moreover, this study revealed that the offspring had normal-length telomeres in their leukocytes and major organs. Thus, cloned animals have normal functional germ lines, and therefore germ line function can completely restore telomere lengths in clone gametes by telomerase activity, resulting in healthy offspring with normal-length telomeres.
cattle from various somatic cell types derived from donor animals of different ages [15]. Moreover, we have generated offspring from these cloned cows by artificial insemination with conventionally processed spermatozoa from noncloned bulls [16]. Although some cloned cattle died soon after birth regardless of telomere lengths, all cloned cows that grew to puberty (~6 months) demonstrated the ability to reproduce. Even in cloned cows with short telomeres, milk production was comparable to that of the donor cow [16]. Here, we address the question of whether short telomeres affect the lifespan and lifetime performance of cloned animals. Importantly, from a comparison of telomere lengths and bodily features between the cloned cows and their offspring, we infer that germ-line function in the clone is normal, showing that telomere length is restored during gametogenesis in the clones and that offspring of clones do not inherit cloned animal-specific aberrations.

Materials and Methods

Preparation of donor cell cultures

Oviducal epithelial cells were collected by squeezing oviducts of slaughtered cows with scissors. Uterine epithelial cells were collected by perfusing PBS (139 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·7H2O, 1.48 mM KH2PO4) into the uterus of a live cow. Cumulus cells were collected by gently pipetting cumulus oocyte complexes obtained from the ovaries of live cattle by the transvaginal ovum pick-up method or through controlled ovarian hyperstimulation. Fibroblasts were obtained by culturing pieces of ear skin or muscle biopsies as explants. The cells were cultured in Dulbecco’s minimum essential medium (D-MEM) containing 10% fetal calf serum (FCS) at 38 C in a humidified 5% CO2 atmosphere until reaching confluence. These cells were passaged three times.

Immunocytochemical staining

Donor cell cultures were typed by immunocytochemical staining with monoclonal antibodies directed against cytokeratin (for epithelial cells) or vimentin (for fibroblasts). In brief, part of the donor cell cultures was grown to confluency in Lab-Tek chamber slides. The cells were washed with PBS and fixed in methanol at –20 C for 5 min. They were then washed in PBS and blocked with 3% BSA in PBS for 1 h. After removing the blocking solution, 100 μl of anti-pan cytokeratin clone-11 antibody (Sigma, St. Louis, MO, USA; 1:400 dilution) or anti-vimentin clone V9 antibody (Sigma; 1:40 dilution) was added, and slides were incubated for 1 h at 37 C. The cells were washed with PBS and then incubated for 1 h with 100 μl Texas red-labeled anti-mouse IgG (Sigma; 1:300 dilution). The cells were then washed in PBS, covered with 50% glycerol in PBS under a coverslip and observed by fluorescence microscopy. Immediately before nucleus transfer, donor cells were trypsinized, washed by centrifugation and resuspended in PBS supplemented with 0.5% FBS.

Measurement of telomerase activity

Telomerase activity of tissues and somatic cells was detected using a TRAPeze telomerase detection kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer’s instructions. In brief, minced tissues and cell pellets were extracted in CHAPS lysis buffer for 30 min on ice. The suspension was centrifuged at 12,000 g for 20 min at 4 C. After determination of the protein concentration, 0.02–1.5 μg of protein in tissues and cell extracts was incubated with TRAP reaction buffer at 30 C for 30 min, and PCR was performed at 94 C for 30 sec and then 55 C for 30 sec for 33 cycles in a thermocycler. PCR samples were run on a 12.5% (w/v) nondenaturing PAGE gel in 0.5x TBE buffer for 1 h at 500 V. After electrophoresis, the gel was stained with ethidium bromide. In positive samples, the ladder of products with six base increments starting at 50 nucleotides and a 36 bp internal control band should be observed. In negative samples, only the 36 bp internal control band is observed.

Preparation of recipient cytoplast

Ovaries with an unknown genetic background (cows slaughtered at a local abattoir) were transported to the laboratory while immersed in physiological saline at 25 C. Cumulus-oocyte complexes (COCs) were aspirated from the pooled ovaries and incubated in TMC199, supplemented with 5% calf serum (CS), for 20 h for in vitro maturation. In vitro matured COCs were treated in the M2 medium supplemented with 0.5% hyaluronidase for 5 min, and the cumulus cells were removed by gentle pipetting. The zona pellucida of the denuded metaphase II oocytes were cut near the polar body and enucleated by pushing out the cytoplasm including the nucleus with a glass needle in PBS supplemented with 20% CS and 5 μg/ml of cytochalasin B. Enucleation was confirmed for each removed cytoplast by fluorescent dyeing with Hoechst 33342.

Nucleus transfer

Nucleus transfer was carried out as described previously [15]. Briefly, the donor cells were introduced into the perivitelline space of the enucleated oocytes with a glass microinjection pipette. Fusion of the cell-oocyte complexes was accomplished by applying a single 25-V pulse for 50 μsec. Fused complexes were exposed to 5 μM calcium ionophore (A23187, Sigma) for 5 min and then incubated for 6 h in TCM-199 medium supplemented with 5% calf serum and 10 μg/ml cycloheximide (Sigma). After the activation treatment, nucleus-transferred oocytes were cultured to the blastocyst stage (8 day) in CR1aa medium [17] supplemented with 5% calf serum.

Embryo transfer

Candidate recipient cows were synchronized, and the status of their corpora lutea was evaluated by rectal palpation 1 day before the day of embryo transfer on day 7 or day 8 (day 0 = day of estrus). Optimal recipients were selected, and blastocysts were nonsurgically transferred into the uterine horn. Pregnancy was assessed between 40 and 60 days after transfer using ultrasonography. Par- turition was induced using 20 mg dexamethasone (Nihon Zenyaku Kogyo, Fukushima, Japan) and 1 mg prostaglandin-F2α analogue (Cloprostenol, Sumitomo Chemical, Osaka, Japan) at 280 and 285 day of gestation, respectively. Calves were delivered by normal expulsion, except in cases where uterine cervical dilation was inadequate, in which case the calves were delivered by cesarean section.
Feeding management

Growth period: The cloned calves were reared in individual calf huts for the first 45 days after birth. After 45 days, the cloned calves were reared together with other calves produced by AI or embryo transfer. During the weaning period, they were held in a large pen in mixed groups of 3 (total) cloned and age-matched calves (produced at the National Livestock Breeding Center). After weaning, they were moved into a pen in groups of 10 to 20 animals. At 12 months of age, they were moved to a free-stall barn for heifers. The calves were given pasteurized colostrum twice a day for the first 5 days after birth. During the next 40 days, they were given milk replacer twice a day. They were also given calf starter pellets, hay and water ad libitum during this period. They were weaned from milk replacer 45 days after birth. Their main feed was changed gradually from calf starter pellet to formula feed over 2 weeks. From 60 days to 12 months of age, each cow was given 2.0 to 3.0 kg/day of formula feed, and hay and water ad libitum. From 12 months of age to 2 months before their estimated day of parturition, they were fed TMR consisting largely of grass silage. The feed volume given per day was determined by the roughage content of each lot of feed and by the monthly change in average BW of the cows. After 8 months of age, they were grazed for approximately 5 h/day between May and October.

Prepartum period: Pregnant cloned heifers were moved to a free-stall barn 2 months before their estimated day of parturition. They were given grass haylage and hay ad libitum. Dry cows were fed individually using an auto feeder. Two weeks before the expected day of parturition, each heifer was moved to a calving pen and held there until parturition. Pregnant heifers were given corn silage and formula feed individually during this period. Feed volume was determined according to BW. Rectal temperature was measured every day. If a decrease in rectal temperature was recorded compared with the previous day, more frequent observations were carried out.

Lactation period: After parturition, cloned cows were moved back to a free-stall barn. They were given a mixed ration consisting largely of corn silage and were fed formula feed using an auto feeder. Individual formula feed volumes were based on the total milk yield of the previous day and were given separately at least 4 times a day. The electrical conductivity of the milk was monitored ad libitum during this period. The electrical conductivity of the milk was monitored ad libitum during this period. The electrical conductivity of the milk was monitored ad libitum during this period.

Briefly, genomic DNA was extracted from leukocytes or major organs and then digested with the restriction enzymes, AluI and HinfI. Equal amounts of genomic DNA (1 μg) and 1- and 5-kb ladder markers were loaded onto a 1% agarose gel (15 × 15 cm). Long telomeric DNA was separated by pulse-field electrophoresis on a FIGE Mapper electrophoresis system (Bio-Rad, Hercules, CA, USA). The DNA was transferred to nylon membranes using a standard Southern blotting procedure and then cross-linked to the membranes with 1200 μl of UV light. The telomere-specific oligonucleotide (TTAGGG), was end-labeled at 37 C for 15 min using terminal deoxynucleotidyl transferase from a DIG Oligonucleotide 3'-End Labeling Kit (Roche, Mannheim, Germany). The blotted nylon membranes were prehybridized in 40 ml of DIG Easy Hyb (Roche) for 2 h at 37 C, and then hybridized in 10 ml of DIG Easy Hyb containing 50 pmol of end-labeled telomere-specific probe for 16 h at 37 C. Membranes were washed three times in 100 ml of 0.5 × standard saline citrate (SSC; 1 × SSC: 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min at 37 C. Detection was by chemiluminescence using a DIG Luminescent Detection Kit (Roche) and exposure to X-ray film (RX-U; Fujifilm, Minamiashigara, Japan).

Calculation of telomere length

The chemiluminescence signals were acquired using a Model GS-710 Imaging Densitometer (Bio-Rad) at the highest resolution (0.01 mm). The acquired 16-bit images were then processed using Molecular Analyst software (Bio-Rad). Once the distance calibration was obtained, all distances were converted to telomere lengths, and the dependence of signal intensity on telomere length at each point was analyzed. The mean terminal restriction fragment (TRF) size was calculated as \[ \Sigma (OD_i) \] where \( OD_i \) is the telomere length at each point \( i \) (0.01 mm) within the lane. Sums were calculated over the range of 3 to 50 kb.

Statistical analysis

Mean TRF analyses in cloned cattle were repeated at least twice; the average is reported. The differences between samples, including leukocytes, donor cells, cloned cattle and control cattle, were analyzed using the Student’s t-test.

Results

Telomere lengths in cloned cattle produced from epithelial cells

To generate donor cells, we first prepared epithelial cell cultures derived from the oviduct and uterus of normal adult cows. The identity of cells was confirmed by immunostaining with antibodies against the epithelial cell and mesenchymal cell markers, cytokeratin and vimentin, respectively (Fig. 1). Incidentally, telomerase activity was not detected in any cell cultures (Table 1). Consistent with the relatively long mean TRF sizes in the uterine epithelial cell culture, significant telomerase activity was detected in the uterine epithelium (data not shown). On the other hand, oviductal epithelial cells had relatively short telomeres based on the weakness of telomerase activity in the oviduct epithelium regardless of rapid cell turnover (data not shown).

Using the epithelial cells as donor cells, we produced three...
TELOMERE LENGTHS IN CLONE OFFSPRING

At birth, cloned cows produced from an oviductal epithelial cell culture derived from a 13-year-old cow (COE13 group) exhibited remarkably smaller mean terminal restriction fragment (TRF) sizes in leukocytes (13.31 ± 0.24 kb, \( P<0.01 \)) than we had previously observed in age-matched control calves (19.97 ± 0.41 kb; Miyashita et al., 2003; \( P<0.001 \)). Significantly smaller leukocyte TRF sizes were also observed at birth in cloned cows produced from oviductal epithelial cells derived from a 6-year-old cow (COE6 group; 15.32 ± 0.37 kb, \( P<0.01 \)). In contrast, the mean TRF size (19.0 kb) in leukocytes at 2 years of age in a cloned cow produced from a uterine epithelial cell culture derived from an 8-year-old cow (CUE8) was normal (i.e., within the variation among age-matched control cows). Overall, a comparison of mean TRF sizes in donor epithelial cell cultures with those in leukocytes of the resulting cloned cows showed that the mean TRF sizes in leukocytes from all cloned cows were approximately 2 kb smaller than the mean TRF sizes in the donor cell cultures (Table 1). Incidentally, the success rates in terms of surviving cloned calves from the SCNT embryos transferred were 4.8, 10.8 and 25.0% for COE13, COE6 and CUE8, respectively.

In marked contrast to these results, previous studies have reported that the telomere lengths of cloned calves produced from cumulus cells and fibroblasts are the same as or longer than those in the donor cells and within the variation among age-matched controls [7–10]. To confirm that these apparently contradictory

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**Table 1.** Telomere lengths in donor cells, the resultant cloned cows and their offspring

<table>
<thead>
<tr>
<th>Origin</th>
<th>Donor cell culture</th>
<th>Cloned cows at birth</th>
<th>Their offspring at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
<td>Mean TRF size (kb)</td>
<td>Telomerase activity</td>
</tr>
<tr>
<td>Oviduct</td>
<td>13</td>
<td>15.2</td>
<td>Not detected</td>
</tr>
<tr>
<td>Oviduct</td>
<td>6</td>
<td>16.9</td>
<td>Not detected</td>
</tr>
<tr>
<td>Uterus</td>
<td>8</td>
<td>21.2</td>
<td>Not detected</td>
</tr>
<tr>
<td>Cumulus cell b)</td>
<td>Not detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblast b)</td>
<td>Not detected</td>
<td></td>
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</tbody>
</table>

*: Represent significantly shorter telomeres than those in age-matched control calves (19.97 ± 0.41 kb; Miyashita et al. 2003; \( P<0.001 \)). a The leukocytes of CUE8 were sampled not at birth, but at 2 years of age. b Donor cell cultures were derived from different cattle.

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**Fig. 1.** Typing of donor cell cultures from the oviduct and uterus. Donor cells were collected from the oviduct (A, B) and uterus (C). They were cultured and immunostained with anti-cytokeratin or anti-vimentin primary antibody and Texas Red-labeled secondary antibody. Positive cytokeratin signals were detected throughout the cell population, but vimentin-positive cells were absent (data not shown), demonstrating epithelial cell types. The COE13 group cows, COE6 group cows and CUE8 cow were produced by SCNT using A, B and C cells, respectively.

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**Fig. 2.** Geriatric features observed in cloned cows with short telomeres. A: Poor wound healing in the forehead. B: Alopecia on the nape of the neck. These features were found in one group of clones with the shortest telomeres (COE13 group), but were absent in another group with short telomeres (COE6 group).
observations are attributable to real differences in donor cell types and not due to differences in cloning protocols, we measured mean TRF sizes of cloned cattle derived from oviductal epithelial cells shown to be short (Lanes 1, 3 and 5), but those of their offspring were restored to normal lengths (i.e., within the variation among age-matched controls; Lanes 2, 4 and 6). Because uterine epithelial cells had relatively long telomeres, a cloned cow produced from these cells showed normal telomere lengths (Lane 7). Its offspring also had normal-length telomeres in leukocytes (Lane 8). Telomeres in organs of their offspring were found to be not short (e.g., lung, heart, liver, spleen and kidney; Lanes 1–5, respectively).

**Bodily features in cloned cows with short telomeres**

The remarkably short telomeres in the leukocytes of the COE13 (n=6) and COE6 (n=4) groups prompted us to monitor the progress of these animals after birth. We previously reported that at 3 years of age, the growth, behavior and reproductive traits—including age of puberty onset, age at first conception, gestation period, calf weights and interval from parturition to the next conception—were normal for all ten animals; all also lactated as much as their donor cows [16]. Now 5 years old, all are still alive and healthy. They have delivered three times following artificial insemination with normal bull spermatozoa and have maintained normal reproduction and lactation (personal communication). However, all cloned cows (6/6) in the COE13 group appeared older, exhibiting alopecia, rough hair, wrinkled skin and poor wound healing (Fig. 2). Although none of the animals in the COE6 group appeared older, all four showed a slight limp. None of these characteristics were observed in the CUE8 cow.

**Telomere lengths in clone offspring**

The offspring of our cloned cows showed none of the bodily features described above, and with the exception of two instances of stillbirth, all were completely healthy and normal (n=30). The mean TRF sizes of the leukocytes of the COE13 and COE6 offspring were 19.95 ± 0.38 and 19.52 ± 0.24 kb, respectively, within the variation among age-matched control calves (Figs. 3 and 4, Table 1). In one offspring, the mean TRF sizes determined in different organs were 17.3 kb (lung), 17.8 kb (heart), 21.8 kb (liver), 20.9 kb (spleen), 20.9 kb (kidney), 19.5 kb (small intestine), 22.7 kb (muscle) and 23.0 kb (testis), none of which was below the 9.7-kb threshold of replicative senescence in bovine cells [10]. The mean TRF size (21.7 kb) for offspring of the CUE8 cow was normal (Figs. 3 and 4, Table 1).

**Discussion**

At 5 years of age, all cloned cows with short telomeres (COE13 and COE6 groups) had normal growth, reproduction and lactation characteristics, and all delivered three times. These observations are similar to those for Dolly, which also exhibited short telomeres, but grew normally and had four lambs. Unfortunately, because Dolly had to be euthanized due to serial progressive lung disease, the relationship between short telomeres and lifespan and lifetime performance in cloned animals remained unclear. In this study, the number of samples was limited because the production of somatic cell cloned cows is an inefficient process. Nonetheless, long-term observation of these animals can be expected to provide useful information.
The finding that all cows of the COE13 group exhibited bodily features, including alopecia, rough hair, wrinkled skin and poor wound healing (Fig. 2) at only 5 years of age was notable, given that the normal lifespan of a cow under optimal feeding conditions is 10–15 years. Similar findings are often associated with human premature senility, such as in patients with Hutchinson-Gilford progeria [19, 20], which characterized by extremely short telomeres, reduced cell-division potential and premature death (13 years, on average). Hence, the bodily features shown in our cloned cows might also occur as a result of short telomeres. If so, cells in vivo might approach a threshold for replicative senescence in the near future, and the cloned cows might then lose functions in many organs, resulting in early death. However, it is probable that the bodily features were triggered by abnormal gene expression induced by epigenetic errors associated with SCNT technology. Moreover, because the cloned cows that exhibited the bodily features were derived from one donor cow, the effects may be due to genotype rather than the short telomeres.

There is no evidence from the first 5 years of life suggesting that our cloned cows with short telomeres are undergoing a gradual involution in reproduction or lactation. Because there is a close link between reproductive involution and the timing of natural death in most animal species, except humans, our cloned cows could have a relatively long future lifetime. The most likely explanation for the discrepancy between shorter telomeres in our cloned cows and youthful reproductive performance is that the lifespan of a cow may not be closely linked to telomere length. Normal cattle have longer telomeres than humans and appear to die a natural death despite having sufficient telomere lengths. Our previous work has shown that telomere lengths in the leukocytes of normal 18-year old cattle, which are regarded as very old, were approximately 15 kb (Fig. 4); this contrasts with 9.7 kb in senescent bovine fibroblasts, which is regarded as a threshold telomere length of replicative senescence [10]. In mice lacking the mouse telomerase RNA (mTR) gene, phenotypic changes occurred during successive generations. First generation mTR−/− mice lacked detectable telomerase activity, but had relatively long telomeres and exhibited no geriatric features. With each generation, the telomeres became shorter such that by the sixth generation, the telomere length approached the threshold of replicative senescence and caused geriatric features, infertility and premature deaths [22–24]. Accordingly, our cloned cows, with short telomeres but no evidence of involution, may be linked to first-to-fifth generation mTR−/− mice, which had sufficient telomere lengths until reaching the threshold of replicative senescence. However, because telomeres derived from donor epithelial cells shorten from SCNT to birth (Table 1), the use of epithelial cells that are more senescent than those used in this study as donor cells for SCNT would yield cloned animals with innately shorter telomeres than our cloned cows and a greater likelihood of dying earlier deaths, as is the case with sixth generation mTR−/− mice. In particular, transgenic cloned animals produced using transgenic mammary epithelial cells as donor cells might have fatally short somatic cell telomeres and would thus be at risk of premature death because the donor cells undergo numerous divisions through a series of experimental steps prior to SCNT.
animals have short telomeres (Fig. 5). Restoration of telomere length in the clone germ line closely resembles a phenomenon observed in cloned mice, in which epigenetic errors and pathological abnormalities are lost in the next generation [30], even among pairs of male and female cloned mice [31]. Although some cloned animals have short telomeres and epigenetic errors resulting in geriatric features, pathological abnormalities and premature deaths, we conclude that 1) cloned animals have normal functional germ lines, 2) germ line function can completely normalize genomic errors (i.e., short telomeres and epigenetic changes derived from aged and differentiated donor somatic cells) in gametes, and 3) the offspring resulting from normalized gametes are themselves normal. This work provides striking support for a 2-step model of telomere length regulation in cloned animals and their offspring [32].

We have previously expressed concern that if cloned animals with short telomeres are selectively reproduced for their high performance attributes, their short telomeres may be transmitted to the next generation, causing an entire species to become short-lived and ultimately face extinction [10]. However, this study reveals that even cloned animals with very short telomeres bear offspring with normal-length telomeres. Hence, SCNT technology provides the practical benefit of reproducing elite animals and could contribute to the preservation of endangered animals.

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