Growth, Reproduction, and Lactation in Somatic Cell Cloned Cows with Short Telomeres

M. Yonai,¹ K. Kaneyama,¹ N. Miyashita,² S. Kobayashi,³ Y. Goto,⁴ T. Bettpu,⁵ and T. Nagai⁶

¹National Livestock Breeding Center, Nishigo, Fukushima, 961-8511, Japan

²National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8602, Japan

³National Agricultural Research Center for Kyushu Okinawa Region, Nishigoushi, Kumamoto, 861-1192, Japan

⁴National Livestock Breeding Center Tokachi Station, Otofuke, Hokkaido, 080-0572, Japan

⁵National Livestock Breeding Center Nikkappu Station, Sisonai, Hokkaido, 056-0141, Japan

⁶National Institute of Livestock and Grassland Sciences, Tsukuba, Ibaraki, 305-8602, Japan

ABSTRACT

We previously showed that telomere lengths of 10 somatic cell cloned cows were significantly shorter than normal. In this study, we investigated growth, reproduction, and lactation in these animals to determine if shortened telomeres have any effect on these characteristics. Six Holstein and 4 Jersey cloned cows, derived from oviduct cells, were reared under general group feeding. Body weights were recorded from birth to 48 mo of age. A number of reproductive characteristics were screened during the prepubertal, postpubertal, and postpartum periods. After parturition, milk yields were recorded daily and percentages of milk fat, proteins, and solids-not-fat were measured at monthly intervals. These data were used to estimate production of milk components over a 305-d period. Overall, the cloned heifers exceeded standard growth rates for each breed. The cows were inseminated at the first estrus after they reached 450 d of age, and delivered normal calves except for one stillbirth in the Holstein group. They were inseminated at postpartum estrus to provide second and third parturitions and, again, these pregnancies were normal. Gestational periods and birth weights of the calves were both within the normal range. The average total milk yield per cow in Holstein group clones was less than that of the original cow, whereas Jersey group clones showed a higher average milk yield than the original cow. In both groups of cloned cows, inter-individual variation in milk production was relatively large; however, the coefficient of variation was less than 10%. Our results suggest that the cloned cows have normal growth, reproductive, and lactation characteristics, and thus normal productivity, despite having reduced telomere lengths.

(**Key words:** productivity, cloned cows, short telomeres)

Abbreviation key: CV = coefficients of variation.

INTRODUCTION

The development of the technique for cloning animals by somatic cell nuclear transfer has made it possible to produce copies of agricultural animals with known productivity characteristics. However, one potential concern over the use of somatic cells from adult animals is that telomere lengths in the resulting cloned offspring are sometimes shorter than those of normally generated progeny. The first cloned sheep, Dolly (Wilmut et al., 1997), was euthanized at 7 yr of age because of serious progressive lung disease, although the usual life span of sheep is 12 or more years. Dolly was cloned from a 6-yr-old sheep; if we add the nuclear age of the cells used for cloning, then Dolly could be said to be the equivalent of 13 yr old at the time of death. Examination of telomere lengths in Dolly showed them to be comparable to those of her donor cells (Shiels et al., 1999). This suggests that the presence of short telomeres was not responsible for her illness. However, it is not possible to extrapolate from this single example to conclude that reduced telomere length will not have an adverse effect on health and productivity of cloned domesticated animals. There is a need to demonstrate normal survival times and productivity of cloned domestic animals if they are to be used in the future.

Recently, we reported that 2 sets of cloned cows, derived from a Holstein and a Jersey cow at 13 and 6 yr old, respectively, had shorter telomeres than those observed in ordinary, old cows (Miyashita et al., 2002). These cloned cows will provide information on the potential influence of shortened telomeres on the life span and lifetime performance of cloned animals. Moreover, these animals should also enable us to confirm or disprove the widespread assumption that clones obtained from the same source of cells will have similar pheno-

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Corresponding author: M. Yonai; e-mail: m0yonai@nlbc.go.jp.

types. Information on the performance of cloned animals produced from the same somatic cells will provide insight into the similarity of clones to the original animal. The present study addresses these questions through investigation of growth, reproduction, and lactation of genetically identical cloned cows with short telomeres.

MATERIALS AND METHODS

Clone Production

Donor animals. Oviduct epithelial cells, obtained from a multiparous Holstein (13 yr old) and a multiparous Jersey (6 yr old) cow, were used as donor cells for nuclear transfer. Both cows had high milk performance and had been used as embryo donor cows. The Jersey cow was reared at the National Livestock Breeding Center (Iwate Station) and remained there throughout her life. Records were kept of her growth from birth to 48 mo of age, and of her milk yield during first lactation by the Dairy Herd Improvement test. The Holstein cow was imported from the United States after the first lactation period. No growth records were available but a record was made of milk yield during her first lactation.

Nuclear transfer. Preparation of donor cells and recipient oocytes, and nuclear transfer were all carried out as described by Goto et al. (1999). In brief, cumulus oocyte complexes were aspirated from abattoir-derived ovaries and matured in vitro for 20 h in TCM-199 medium (Gibco BRL, Grand Island, NY) supplemented with 5% calf serum (Gibco BRL). Mature cumulus oocyte complexes were treated with 0.5% hyaluronidase in M2 medium (Fulton and Whittingham, 1978) for 5 min and the cumulus cells removed by gentle pipetting. The oocytes were transferred to PBS supplemented with 20% calf serum and 5 μ g/mL of cytochalasin B (Gibco BRL). The zona pellucida of each denuded metaphase II oocyte was cut near the polar body and the cell enucleated by pushing the nucleus and surrounding cytoplasm out of the cell with a glass needle. Enucleation was confirmed using the nuclear stain, Hoechst 33342 (Gibco BRL). Donor oviduct epithelial cells were introduced into the perivitelline space of enucleated oocytes with a microinjection glass pipette, and then the cell-oocyte complexes transferred to Zimmerman cell fusion medium (Zimmerman and Vienken, 1982). Fusion of the cell-oocyte complexes was accomplished by applying one pulse of 25 V for 50 μ s. Fused complexes were exposed to 5 μ M calcium ionophore (A23187, Sigma, St. Louis, MO) for 5 min, and then incubated for 6 h in TCM-199 medium supplemented with 5% calf serum and 10 μ g/mL cycloheximide (Sigma). After the activation treatment, the nuclear transplanted oocytes were cultured for 8 d in CR1aa medium (Rosenkrans

et al., 1993) supplemented with 5% calf serum to the blastocyst stage. The quality of the blastocysts was assessed and those ranked as good to excellent were used for embryo transfer.

Embryo transfer. Multiparous (from parities 3 to 8) Holstein cows were used as recipients. Candidate recipient cows were synchronized and the status of their corpora lutea checked by rectal palpation 1 d before the day of embryo transfer on d 7 or 8 (d 0 = day of estrus); optimal recipients were selected. Blastocysts were non-surgically transferred into the uterine horn. Ultrasonography was used to check for pregnancy between 40 and 60 d after transfer.

Management of recipients. Pregnant recipient cows were given rations, formulated using the NRC (1989) standards, during the gestation period. Parturition was induced using 20 mg of dexamethasone (Nihon Zenyaku Industries Co., Fukushima, Japan) and 1 mg of prostaglandin- $F_{2\alpha}$ analogue (Cloprostenol, Sumitomo Chemical Co., Osaka, Japan) at 280 and 285 d of gestation, respectively. Calves were delivered by normal expulsion except in cases in which uterine cervical dilation was inadequate; these calves were delivered by cesarean section. Immediately after birth, the calves were given at least 2 L of warmed colostrum. Physiological functions were monitored until they stabilized.

Telomere lengths. Miyashita et al. (2002) described telomere lengths in leukocytes from all the cloned calves. They found that the mean size of the terminal telomere fragments of clones was significantly smaller than those of age-matched controls and 18-yr-old controls. The Holstein and Jersey cloned calves had telomere lengths equivalent to those found in 30- to 35-yr-old and 21- to 27-yr-old animals, respectively (Miyashita et al., 2002).

Feeding Management

All cloned cows were fed in accordance with NRC (1989) standards during the experimental period.

Growth period. The cloned calves were reared in individual calf huts for the first 45 d after birth. After 45 d, 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 National Livestock Breeding Center). After weaning, they were moved into a pen in groups of 10 to 20 animals. At 12 mo 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 d after birth. During the next 40 d, 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 d after birth. Their main feed was changed gradually from calf starter pellet to formula feed over 2 wk. From 60 d to 12 mo of age, each cow was given 2.0 to 3.0 kg/d of formula feed, and hay and water ad libitum. From 12 mo of age to 2 mo 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 mo of age, they were grazed for approximately 5 h/d between May and October.

Prepartum period. Pregnant cloned heifers were moved to a free-stall barn 2 mo 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. Electrical conductivity of milk was monitored individually at every milking; if it rose above the normal level, the cow was given an oral vitamin compound liquid.

Reproductive Management

Cloned heifers were artificially inseminated at the first estrus after 450 d of age. The frozen semen used for insemination in both breeds was from the same lot of the semen from the same sire. After AI, estrus behavior was checked every day; if estrus returned, the heifer was inseminated again. The number of cycles of AI needed for conception was recorded. Pregnancy diagnosis was carried out at 40 d after AI by ultrasonography. Calving was unassisted except when essential. In the first and second postpartum periods, all cloned cows were artificially inseminated again at first estrus, which usually occurred 90 d after parturition. Pregnancy diagnosis was carried out as above.

Observation and Measurement

BW during the growing period. Body weights of cloned animals were recorded every month from birth

to 15 mo of age and every 3 mo between 15 and 24 mo of age.

Observation of puberty. Identification of the onset of estrus behavior could only be determined for 3 of the Holstein heifers as estrus cycles had commenced in the remaining Holstein and Jersey heifers before initiating this part of the study. Ovulation and formation of corpora lutea were monitored 3 times a week by ultrasonography. Plasma samples were collected every 3 d to measure changes in progesterone levels; the samples were stored at -40°C until assayed. Progesterone concentration was measured by enzyme immunoassay as described by Takenouchi et al. (1993). Intra- and interassay coefficients of variation were 1.4 and 7.9%, respectively.

Observation of reproductive performance. After puberty, the estrus behavior of the cloned heifers was monitored twice a day until the animals became pregnant. The lengths of the estrus periods and the occurrence of standing behavior were recorded. Ultrasonography was used 3 times per week to identify follicular waves in the ovaries and to record changes in the numbers of small follicles. Plasma samples were collected every 3 d during this period to determine changes in progesterone concentrations. These data were used to calculate the amount of progesterone secretion in each estrus cycle. The progesterone assay was carried out as described above. Plasma samples were collected daily to measure changes in estradiol-17 β concentrations between d 18 of estrus and the day of ovulation over a period of 17 estrus cycles in the 4 Jersey group cows and 28 estrus cycles in the 5 Holstein group cows. The samples were stored at -40°C until they were assayed. Estradiol-17 β concentration was measured by the enzyme immunoassay method reported by Takenouchi et al. (1997). All samples were assayed at the same time. The intraassay coefficient of variation was 4.1%.

The length of the gestation period and the calf's birth weight were recorded. In the postpartum period, the heifers were monitored twice a day for the reestablishment of estrus behavior from d 7 after parturition until the first estrus. Ultrasonography was used 3 times per week to observe changes in the ovaries. Plasma samples were collected every 3 d to determine changes in progesterone concentration. The progesterone assay was carried out as described above. The interval from parturition to the first ovulation and estrus was determined using a combination of observation and changes in progesterone concentration. For the first and second postpartum pregnancies, the gestation length, calf weight, the interval from parturition to first estrus and first ovulation, and the change of progesterone concentration were recorded in the same manner as described above.

Measurement of milk production. All cows were milked twice per day. Milk yields were recorded every day for 310 d after parturition. Total milk yields for each cloned cow were calculated from these records. Milk fat, protein, and SNF were recorded every month during the lactation period. These monthly records were used to estimate total amounts (and percentages) of fat, protein, and SNF over the lactation period. Eleven half-sib groups of cows that included 3 or more animals were used for comparison of variation in milk production. These cows were reared on our farm during a recent 5-yr period using the same management regimen as for the cloned cows.

Statistical Analyses

Growth, reproduction, and lactation data are presented as means, standard deviations (SD), minimum and maximum values, ranges between minimum and maximum, and coefficients of variation (CV). Differences in milk production were compared using an *F*-test. The milk production records of the cloned cows were also compared with those of 11 half-sib groups of cows. Statistical analyses were carried out using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC).

RESULTS

Production of Somatic Cell Clones

Sixty-three Holstein cows were used as recipients for Holstein group embryo transfer and 22 for the Jersey group embryos. In total, 124 embryos were transferred to Holstein group recipients and 37 embryos to Jersey group recipients. Pregnancy occurred in 18 (28.6%) of the Holstein group recipients and in 7 (31.8%) of the Jersey group recipients. Failure to reach term occurred in 11 (61.1%) pregnancies of the Holstein group and 1 (14.3%) pregnancy of the Jersey group recipients. Overall, calving occurred in 11.1% (7/63) and 27.3% (6/22), respectively, of cows used as Holstein and Jersey group embryo recipients. One of the Holstein group recipients had twin calves. The Holstein group delivered 8 cloned calves and the Jersey group 6 calves. Two of the Holstein group calves and 2 of the Jersey group calves did not survive. Therefore, the overall success rate in terms of surviving calves from the embryos transferred was 4.8% (6/124) for the Holstein group and 10.8% (4/37) for the Jersey group. The production rate of surviving calves from the recipient cows was 9.5% (6/63) and 18.2% (4/22), respectively.

Growth

Changes in BW from birth to 24 mo of age are shown in Figure 1. The averages of birth weight for the Hol-

Figure 1. Mean BW of cloned heifers between birth and 24 mo of age. A) Mean Jersey cloned animals (●, *n* = 4) and original animal (▲); B) Mean of Holstein cloned animals (●, *n* = 6).

stein and Jersey groups were 36.2 ± 7.7 kg (27.0 to 47.0 kg) and 29.4 ± 1.5 kg (27.5 to 31.0 kg), respectively. The range of birth weights was wider in the Holstein than the Jersey group.

The rates of increase in BW were greater in the cloned animals than in the standard of each breed (Figure 1). When compared with the standard growth curves from the Japanese Feeding Standard for Dairy Cows (MAFF, 1999) and Standard Growth of Holstein Heifers (Holstein Cows Association of Japan, 1995), the BW averages of the Holstein group conformed to the standard during the first 3 mo but exceeded the standard after 5 mo of age. The BW averages of the Jersey group exceeded the standard throughout the measurement period. When the growth rates of the Jersey group animals and the nuclear donor Jersey cow were compared, that of the clones exceeded that of the donor from birth to 24 mo of age (Figure 1). The average coefficients of variation in BW from birth to 24 mo for the Holstein and Jersey groups were 7.5 and 4.2%, respectively. The

Table 1. Average daily gain (kg/d) every 3 mo from birth to 24 mo.

		Months of life							
		0 to 3	3 to 6	6 to 9	9 to 12	12 to 15	15 to 18	18 to 21	21 to 24
Holstein clone (n = 6)	Mean	0.72	1.17	0.82	0.85	0.90	0.97	0.68	0.58
	SD	0.14	0.12	0.08	0.11	0.10	0.26	0.11	0.27
	Maximum	0.87	1.30	0.95	0.93	1.05	1.31	0.86	0.88
	Minimum	0.51	1.03	0.73	0.66	0.76	0.65	0.57	0.21
	Variance	0.02	0.01	0.01	0.01	0.01	0.06	0.01	0.06
	CV	19.28	9.94	9.77	12.93	11.44	26.81	15.54	46.16
Jersey clone (n = 4)	Mean	0.49	0.73	0.67	0.53	0.49	0.56	0.51	0.40
	SD	0.02	0.02	0.11	0.06	0.05	0.17	0.16	0.18
	Maximum	0.51	0.76	0.79	0.60	0.51	0.73	0.65	0.64
	Minimum	0.46	0.71	0.56	0.47	0.41	0.39	0.35	0.23
	Variance	0.00	0.00	0.01	0.00	0.00	0.02	0.02	0.03
	CV	4.71	2.90	17.08	12.08	10.81	29.72	31.21	45.87

averages of daily weight gain for each 3-mo period are shown in Table 1. In both groups, the variance of the averages increased as the calves grew. The average weight at 24 mo of age was 637.5 ± 46.3 kg for the Holstein group and 421.9 ± 21.7 kg for the Jersey group.

Puberty

The age of puberty was determined for 3 of the 6 cloned Holstein heifers. Two reached puberty at 323 d of age, the third at 324 d. The other 3 heifers reached puberty at unknown (earlier) ages (Tables 2 and 3, Figure 2).

Reproductive Performance

Our observations on various aspects of reproductive performance in the cloned heifers, from puberty to the third conception, are summarized in Tables 2 and 3.

Postpubertal period. Before the start of AI, 26 and 37 estrus cycles were observed in the Jersey and Holstein group animals, respectively. In the Jersey group, standing behavior was detected in all estrus cycles. In contrast, standing estrus behavior was often absent in the Holstein group. Moreover, in 13 of 37 estrus cycles, we did not detect estrus behavior such as standing, mounting, or roaming. In the Holstein group, the average concentrations of plasma estradiol-17 β 1 d before ovulation were significantly different between heifers with or without clear estrus behavior (6.94 vs. 3.95 pg/mL; $P < 0.05$). The average estradiol-17 β concentration in the Jersey group was constantly high (8.12 pg/mL) at estrus. Follicle waves and corpus luteum formation were observed in all cycles by ultrasonography in both Jersey and Holstein groups. Corpus luteum formation was associated with changes in plasma progesterone concentration. The estimated total amount of progesterone secretion per estrus cycle was estimated from the

areas under the curve of progesterone concentration. The averages of these estimates were 190.6 ng/mL per cycle for the Jersey group and 154.0 ng/mL per cycle for the Holstein group. Average estrus cycle lengths in the Jersey and Holstein groups were 20.2 and 20.3 d, respectively. The average number of follicular waves per estrus cycle for Jersey and Holstein groups was 2.3 and 2.2, respectively.

First conception. One heifer in the cloned and Holstein groups needed 4 and 6 cycles of AI, respectively, to achieve pregnancy; the other heifers conceived at the first or second attempt at AI.

First parturition. One clone in the Holstein group delivered a stillborn calf 2 wk before the predicted parturition day. The average gestational period in the Holstein group, excluding the cow with the stillborn calf, was within the normal range. Two of the 5 Holstein heifers required a limited amount of assistance for delivery of their calves; the other cows delivered unassisted. All the Jersey group heifers delivered calves without any assistance. The average gestational period in the Jersey group also fell within the normal range. The average calf weight was within the normal range. All the calves delivered from the cloned cows looked normal (with the obvious exception of the stillborn case).

Postpartum reproductive function and the second conception. The intervals between parturition and first ovulation and first estrus are shown in Tables 2 and 3. The first postpartum ovulation was observed between 11 and 108 d after parturition in the Jersey group, and between 14 and 118 d in the Holstein group. After ovulation, an increase in plasma progesterone concentration was confirmed in all animals (data not shown). The interval from parturition to first estrus was between 30 and 135 d for the Jersey group and between 62 and 149 d for the Holstein group. Follicular waves were confirmed in all estrus cycles. The average

Table 2. Results of reproductive performance (Jerseys, n = 4).

	Mean	SD	Max	Min	Range
Age at puberty (d)	—	—	—	—	—
Reproductive records from the puberty to the first parturition					
Length of estrous cycle ¹ (d)	20.2	1.4	23	18	5
Follicle waves per cycle ¹ (no.)	2.3	0.8	4	1	3
Plasma estradiol-17 β concentration on estrous day ²					
Detectable (17/17 cycles; pg/mL)	8.12	2.40	12.71	4.68	8.03
Not detectable (0/17 cycles; pg/mL)	—	—	—	—	—
Plasma progesterone area under the curve ³ (ng/mL per cycle)	190.6	59.4	303.9	64.5	239.4
Number of AI for first conception	2.3	1.9	5	1	4
Age at first conception (d)	503	54.9	584	463	121
Gestation period (d)	279	2.5	282	276	6
Calf weight (first parturition) (kg)	22.0	2.1	25.0	20.5	4.5
Reproductive records after first parturition					
Interval from parturition to first ovulation (d)	51.3	42.8	108	11	97
Interval from parturition to first estrus (d)	85.0	52.7	135	30	105
Number of AI for second conception	1.3	0.5	2	1	1
Interval from parturition to second conception (d)	115	16.8	135	94	41
Age of second conception (d)	897	44.8	956	853	103
Calf weight (second parturition) (kg)	26.4	1.1	27.5	25.0	2.5
Reproductive records after second parturition					
Interval from parturition to first ovulation (d)	32.5	19.3	53	15	38
Interval from parturition to first estrus (d)	50.0	27.8	87	20	67
Number of AI for third conception	1.5	1.0	3	1	2
Interval from parturition to second conception (d)	129	49.9	199	88	111
Age of third conception (d)	1304	46.6	1356	1260	96

¹Twenty-six estrous cycles in 4 cloned heifers were included.²Plasma samples were collected from 17 estrous cycles in 4 cloned heifers.³Plasma samples were collected every 3 d during the 26 estrous cycles.**Table 3.** Results of reproductive performance (Holsteins, n = 6).

	Mean	SD	Max	Min	Range
Age at puberty (d)	323	0.6	324	323	1
Reproductive records from the puberty to the first parturition					
Length of estrous cycle ¹ (d)	20.3	1.5	24	18	6
Follicle waves per cycle ¹ (no.)	2.3	0.7	4	1	3
Plasma estradiol-17 β concentration on estrous day ²					
Detectable (19/28 cycles; pg/mL)	6.94	2.64	16.10	4.27	11.83
Not detectable (9/28 cycles; pg/mL)	3.95	1.74	6.18	1.34	4.84
Plasma progesterone area under the curve ³ (ng/mL per cycle)	154.0	58.0	291.1	69.6	221.5
Number of AI for first conception	2.0	2.0	6	1	5
Age at first conception (d)	481	35.0	549	455	94
Gestation period (d)	277	5.8	281	263	18
Calf weight (first parturition) (kg)	37.8	5.0	46.0	32.5	13.5
Reproductive records after first parturition					
Interval from parturition to first ovulation (d)	56.0	41.5	118	14	104
Interval from parturition to first estrus (d)	86.0	33.0	149	62	87
Number of AI for second conception	1.2	0.4	2	1	7
Interval from parturition to second conception (d)	126	41.7	201	90	111
Age of second conception (d)	881	61.7	979	825	154
Calf weight (second parturition) (kg)	44.2	1.9	47.0	42.0	6.0
Reproductive records after second parturition					
Interval from parturition to first ovulation (d)	79.3	18.9	96	47	71
Interval from parturition to first estrus (d)	92.3	19.2	104	68	61
Number of AI for third conception	1.3	0.5	2	1	1
Interval from parturition to second conception (d)	138	34.9	185	102	83
Age of third conception (d)	1297	75.0	1434	1230	204

¹Thirty-three estrous cycles in 5 cloned heifers were included.²Plasma samples were collected from 28 estrous cycles in 5 cloned heifers.³Plasma samples were collected every 3 d during the 33 estrous cycles.

Figure 2. Change in plasma progesterone concentrations of Holstein cloned heifers during pre- and postpubertal periods.

interval to second conception was 115 ± 16.8 d in the Jersey group and 126 ± 41.7 d in the Holstein group. Pregnancy lasted for 102 ± 8 d for the Holstein group, excluding the cow that had a stillbirth in its first pregnancy. The average numbers of cycles of AI required for pregnancy were 1.3 for the Jersey group and 1.2 for the Holstein group.

Second parturition. At the second parturition, 1 of the 6 Holstein group cows needed a limited amount of assistance for expulsion of the calf; the other cows delivered unassisted. All the Jersey group cows delivered their calves without any assistance. The average gestational periods for both the Holstein and Jersey groups fell within the normal range. The average weight of calves in both groups fell in the normal range, although they were heavier than were those of the first parturition. All calves delivered from the Holstein and Jersey group cows appeared normal.

Postpartum reproductive function and the third conception. Various aspects of reproductive function after the second parturition are summarized in Tables 2 and 3. Overall, the data suggest a lower level of variance than seen in the first postpartum period. The first postpartum ovulation was observed between 15 and 53 d after parturition in the Jersey group, and between 47 and 96 d after parturition in the Holstein group. After ovulation, an increased plasma progesterone concentration was confirmed in all animals (data not shown). Follicular waves were confirmed in all estrus cycles. The first postpartum estrus was observed between 20 and 87 d after parturition in the Jersey group, and between 68 and 104 d in the Holstein group. The average interval to conception was 129 ± 49.9 d in the Jersey group, and 138 ± 34.9 d in the Holstein group. On average, 1.5 cycles of AI were required for pregnancy in the Jersey group and 1.3 in the Holstein group.

Lactation Performance

Milk yields and the proportions of various components of the milk, namely milk fat, milk protein, and SNF, were determined over a period of 305 d during the first and second lactation periods (Tables 4 and 5). In the first lactation period, a temporary reduction of milk yield caused by mastitis, bloat, or diarrhea was occasionally observed. However, similar lactation curves were observed in both groups (Figure 3). Mastitis was observed in 2 Holstein group cows, one at 280 d, the other at 304 d after parturition. Bloat was observed in 2 Holstein group cows, one at 131 d, and the other at 133 d after parturition. In the second lactation period, mastitis and bloat, respectively, affected 2 and 1 cows in the Holstein group. The average total milk yields in the first and second lactations were 5896.4 and 7262.8 kg, respectively, for the Jersey group, and 9252.5 and 11,271.4 kg, respectively, for the Holstein group. The expected increase in milk yield between the first and the second lactations was confirmed in both groups. The first- and the second-lactation milk yields in the nuclear donor cows were 5064 and 6087 kg, respectively, for the Jersey cow, and 10,968 and 11,442 kg, respectively, for the Holstein cow. Comparison of the average milk yields showed that the cloned offspring of the Jersey group had a higher yield than the original cow. In contrast, the average milk yield of the Holstein group was lower than that of the original cow. Milk yields varied by 674 kg between Jersey group cows and by 1245 kg between Holstein group animals. The CV of milk yield in the Jersey and Holstein groups were 5.6 and 5.2%, respectively. The CV of milk yields in 11 groups of Holstein half-sibs ranged from 3.9 to 19.0%. Three of the half-sib groups showed a significantly larger variation in milk yield than the Holstein group (Table 6).

The average milk fat percentages in the first and the second lactations were higher than those of their original cows in both groups. The CV of milk fat, protein, and SNF between the cloned cows were less than 5% in first and second lactations. A comparison of total estimated amounts of milk fat, milk protein, and SNF in the original cows and their cloned offspring showed the same trends and reflected the results of milk yields. The CV of the estimated amounts of milk fat, milk protein, and SNF in both the Jersey and Holstein groups were less than 10%, with the exception of the total estimated amount of milk fat in the second lactation in the Holstein group.

DISCUSSION

A low survival rate has been reported for somatic cell cloned animals (Kato et al., 2000; Heyman et al., 2002;

Table 4. Results on milk yield and composition in first and second lactations (Jerseys, n = 4).

		Milk yield	Fat (%)	Fat (kg)	Protein (%)	Protein (kg)	SNF (%)	SNF (kg)
First lactation	Clone 1	5637.4	4.9	275.0	3.6	202.0	9.2	515.0
	Clone 2	6077.9	4.8	305.0	3.7	232.0	9.2	583.0
	Clone 3	6272.6	5.1	331.0	3.9	250.0	9.6	616.0
	Clone 4	5597.7	5.3	290.0	4.0	218.0	9.5	526.0
	Mean	5896.4	5.0	300.3	3.8	225.5	9.4	560.0
	SD	332.0	0.2	23.9	0.2	20.4	0.2	47.8
	CV	5.6	4.4	8.0	4.8	9.1	2.2	8.5
	Donor animal	5064.0	4.9	242.3	4.0	197.1	9.6	477.2
Second lactation	Clone 1	7006.8	5.1	352.0	3.70	255.0	9.30	639.0
	Clone 2	7539.2	5.1	391.0	3.70	282.0	9.30	707.0
	Clone 3	7309.6	5.3	404.0	3.80	284.0	9.30	703.0
	Clone 4	7195.6	5.0	354.0	3.90	278.0	9.50	676.0
	Mean	7262.8	5.13	375.3	3.78	274.8	9.35	681.3
	SD	222.6	0.13	26.2	0.10	13.4	0.10	31.4
	CV	3.1	2.5	7.0	2.5	4.9	1.1	4.6
	Donor animal	6087.0	4.6	280.0	3.67	224.0	9.30	566.0

Renard et al., 2002; Tunoda and Kato, 2002). In the production of cloned cows, abnormalities of fetal development are frequent and they result in either abortion or stillbirth. This has raised the question of their utility for both practical applications and fundamental studies in domesticated animals. However, surviving cloned calves can grow normally (Lanza et al., 2001; Chavatte-Palmer et al., 2002; Pace et al., 2002; Renard et al., 2002; Oback and Wells, 2003). If we plan to use cloned animals as livestock, it is essential to demonstrate that they have normal productive capabilities. Other interesting questions have also been raised: how old are cloned animals in terms of genetic age? Will they have normal life spans? How long will they be able to produce milk and reproduce, particularly when donor cells with short telomeres have been used? Although somatic cell

cloned cows with shortened telomere lengths appear normal (Miyashita et al., 2002), to date there have been no detailed investigations of such cows. To our knowledge, the present study is the first to show normality of growth, reproduction, and milking traits in somatic cell cloned cows with short telomeres.

In the present study, the final production rates of surviving cloned calves in the Jersey and Holstein groups from recipient cows were 18.2 (4/22) and 9.5% (6/63), respectively. Although there was an approximately 2-fold difference in the rates of production between the 2 groups, statistical analysis showed that this difference was not significant due to low power in the experiment. The rates found here fall within the wide range previously reported for the production of somatic cell clones (Kato et al., 2000; Foresberg et al.,

Table 5. Results on milk yield and composition in first and second lactations (Holsteins, n = 6).

		Milk yield	Fat (%)	Fat (kg)	Protein (%)	Protein (kg)	SNF (%)	SNF (kg)
First lactation	Clone 1	8591.2	4.7	368.0	3.3	255.0	9.0	697.0
	Clone 2	9219.5	4.9	466.0	3.4	322.0	9.2	867.0
	Clone 3	9586.5	4.6	444.0	3.2	305.0	8.9	848.0
	Clone 4	9836.0	4.5	449.0	3.2	324.0	8.9	889.0
	Clone 5	9029.1	4.8	448.0	3.1	292.0	8.8	818.0
	Clone 6	9735.6	4.7	467.0	3.3	327.0	9.0	894.0
	Mean	9333.0	4.7	440.3	3.3	304.2	9.0	835.5
	SD	476.4	0.1	36.7	0.1	27.6	0.1	73.4
	CV	5.1	3.0	8.3	3.2	9.1	1.5	8.8
	Donor animal	10,968.0	4.1	452.0	3.3	359.0	—	—
Second lactation	Clone 1	10,678.6	4.4	482.0	3.1	339.0	8.7	940.0
	Clone 2	12,402.6	4.6	531.0	3.2	362.0	8.7	997.0
	Clone 3	11,341.4	4.3	492.0	3.1	356.0	8.6	993.0
	Clone 4	10,376.0	4.7	488.0	3.2	332.0	8.7	909.0
	Clone 5	10,110.2	4.5	461.0	3.1	322.0	8.7	887.0
	Clone 6	12,719.4	4.7	609.0	3.1	410.0	8.8	1146.0
	Mean	11,271.4	4.5	510.5	3.1	353.5	8.7	978.7
	SD	1084.7	0.2	53.4	0.1	31.4	0.1	93.05
	CV	9.6	3.6	10.5	1.6	9.7	0.7	9.5
	Donor animal	11,442.0	3.9	446.2	2.8	320.4	—	—

Figure 3. First-lactation curves of cloned cows. A) Milk yields of individual Jersey cloned animals. B) Milk yields of individual Holstein cloned animals.

2002; Heyman et al., 2002; Renard et al., 2002). Although the same methods and type of donor cells (although from different cows) were used in our study, the production rates varied in each group. This could result from differences in the donor cell lines used, or advantageous factors in the Jersey group. For example, because the incidence of abortion in the Holstein group (68.4%)

was higher than in the Jersey group (31.8%) and dystocia did not occur in Jersey group, the lighter average birth weight of the Jersey group might be related to a higher rate of production. A similar rate of survival (15.0%) was obtained for cloned calves of the Japanese Black breed, which is as small as the Jersey, delivered from Holstein recipient cows (our unpublished data).

Table 6. Distribution of milk performance.

	n	Milk yield (kg)			Milk fat (%)			Milk protein (%)			Milk SNF (%)		
		Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)
Holstein cloned cows	6	9333.0	476.4	5.1	4.7	0.1	3.0	3.3	0.1	3.2	9.0	0.1	1.5
Jersey cloned cows	4	5896.4	332.1	5.6	5.0	0.2	4.4	3.8	0.2	4.8	9.4	0.2	2.2
Holstein half-sib A	13	8746.4	961.7*	11.0	4.1	0.2	5.6	3.3	0.2	6.0	8.9	0.3	2.9
Holstein half-sib B	3	9988.0	583.2	5.8	3.8	0.3	7.9	3.2	0.1	3.1	8.7	0.1	1.1
Holstein half-sib C	3	9522.7	885.6	9.3	3.5	0.2	4.9	3.3	0.2	4.6	9.0	0.3	3.4
Holstein half-sib D	9	9092.8	739.6	8.1	4.0	0.3	7.1	3.2	0.2	5.3	8.8	0.2	2.2
Holstein half-sib E	3	9428.3	589.9	6.3	4.0	0.3	6.2	3.1	0.1	1.8	8.7	0.1	0.7
Holstein half-sib F	4	7925.5	675.6	8.5	4.4	0.4*	8.7	3.3	0.1	2.9	8.9	0.1	1.7
Holstein half-sib G	4	9892.8	1036.0*	10.5	4.2	0.2	5.7	3.4	0.1	1.7	8.9	0.1	0.6
Holstein half-sib H	4	8980.3	627.7	7.0	4.3	0.5*	11.7	3.3	0.3 *	8.9	8.9	0.4*	4.7
Holstein half-sib I	4	9400.3	1705.2*	18.1	4.0	0.4*	9.4	3.0	0.1	3.2	8.6	0.1	1.5
Holstein half-sib J	5	10243.0	396.1	3.9	3.9	0.3	9.0	3.3	0.1	2.7	8.8	0.1	1.3
Holstein half-sib K	6	10487.2	607.3	5.8	4.2	0.3	6.7	3.1	0.1	2.9	8.7	0.1	1.0
Holstein total	58	9407.7	1085.2*	11.5	4.0	0.3*	8.2	3.2	0.2	5.0	8.8	0.2	2.3

*Holstein cloned cows significantly different from each sib ($P < 0.05$).

The birth weights of all the cloned calves were in the normal range for each breed. One of the principal problems often associated with cloned animals is large offspring syndrome (Wilson et al., 1995), which is characterized by an increased size of fetus and by placental and metabolic problems. The normal birth weights in this study may indicate a lower prevalence of problems associated with large offspring syndrome. Four of the 14 cloned calves died soon after birth, a mortality rate that is higher than normal. Nevertheless, the postnatal survival rate of our cloned calves was better than that reported in other studies (Heyman et al., 2002; Obach and Wells, 2003). Kato et al. (2000) reported normal phenotypes (including birth weights) of cloned calves obtained from oviduct cells, the same source of cells as used in this study. The reason for the relatively higher survival rate is not clear. Further studies are required to understand the cause of this effect.

In this study, a large variation in birth weights between individual clones was present in the Holstein group; this was not the case for the Jersey group. Controversial results have been reported on birth weight variability of cloned calves, even when the same cell line was used (Kato et al., 2000; Heyman et al., 2002). It has been suggested that birth weight distributions are variable between experimental series, possibly because of differences in the in vitro culture conditions used for the donor somatic cells. As the donor somatic cells were cultured under identical conditions for both groups in this study, disparities between the 2 groups might be caused by the use of different sources of donor cells. Incidentally, birth weight is influenced by genetic factors, such as the sire, and environmental factors, such as parity and nutritional conditions of the dam (Holland and Odde, 1992). However, in this study at least, we can exclude parity and size of the recipients

as influences on birth weight because there was no correlation between the birth weight and these factors.

In the growth period between birth and 24 mo of age, the monthly CV of BW ranged from 21.6 to 3.1% for the Holstein group, and from 6.1 to 2.0% for the Jersey group. The large CV in the Holstein group may reflect the large variation in birth weights because the CV was greatest at birth and gradually decreased thereafter. The 3-mo CV of average daily gain changed in an inconsistent manner in both groups (Table 1). The cloned calves were born in different seasons (Table 7) and, after 8 mo of age, were grazed between May and October. Therefore, differences in birth season might cause a difference in DM intake. Thus, we concluded that although all the cloned heifers grew normally, they showed differences in their growth characteristics, especially in the Holstein group, an effect that was contrary to our expectation. Similarity in growth rates can only be properly assessed when cloned heifers are raised under a closed, individual feeding regimen.

The changes in plasma progesterone during the pre- and postpubertal periods were consistent with previous

Table 7. The date of birth for each cloned cow in this experiment.

Cloned cow	Date of birth
Jersey group	
Clone 1	8 Dec 1998
Clone 2	8 Dec 1998
Clone 3	13 Apr 1999
Clone 4	13 Apr 1999
Holstein group	
Clone 1	15 Feb 1999
Clone 2	23 Feb 1999
Clone 3	23 Feb 1999
Clone 4	5 May 1999
Clone 5	13 May 1999
Clone 6	22 Nov 1999

reports on puberty in ordinary cows (Gonzalez-Padilla et al., 1975; Suzuki and Sato, 1980; Glencross, 1984). Furthermore, formation of the corpus luteum and appearance of estrus behavior at puberty progressed in the same way as in ordinary heifers (Morrow, 1969; Suzuki and Sato, 1980; Moran et al., 1989). These results indicate that reproductive development at puberty in cloned cows is normal. Enright et al. (2002) reported that age at puberty in 4 cloned heifers was 314.7 ± 9.6 d, and the weight of heifers at puberty was 336.7 ± 13 kg. Compared with these data, the age at puberty in this study was within a narrow range (323.3 ± 0.6 d), whereas weights at puberty were more varied (316.2 ± 25.7 kg). Time of puberty was reported to be under the control of weight or growth rate and to be less related to age (Arije and Wiltbank, 1971; Dufour, 1975; Suzuki et al., 1976; Grass et al., 1982; Schillo et al., 1983, 1992; Moran et al., 1989; Patterson et al., 1992; Sejrsen, 1994). Contrary to this conclusion, although the birth dates of cloned heifers were dispersed over half a year (Table 7) and the growth rates differed, the occurrence of puberty did seem to depend on age in this study. Similarly, Little et al. (1981) reported that in heifers reared under the same nutritional conditions, puberty appeared at the same month of age irrespective of BW. Taken together, these observations suggest that the timing of puberty in cloned cows might be determined by their identical genetic backgrounds and fixed nutritional conditions rather than by growth rate.

In the postpubertal period, clear estrus behavior was absent at ovulation in 32.1% (9/28) of the estrus cycles of the Holstein group heifers (Table 2). The concentration of estradiol-17 β in heifers that did not have clear estrus was significantly lower than that of heifers that had clear estrus behavior. Jersey group heifers showed clear estrus behavior at all estrus cycles and their levels of estradiol-17 β tended to be higher than that of the Holstein group (Table 2). The average duration of estrus in heifers is usually between 14.7 and 18.4 h (Wishart, 1972; Knutson and Allrich, 1988). However, it can be shorter, which can pose problems for detection of estrus. In Holstein heifers, twice-daily observations resulted in detection of estrus in 70% of animals, but missed the remaining 30% (Williams et al., 1981; Stevenson et al., 1996; Van Eerdenberg et al., 1996). Our rate of detection of estrus in the Holstein group was consistent with these reports. The lower level of estradiol-17 β in these animals may cause a shorter duration of estrus, and thereby increase the risk of failure of detection even using twice-daily observations. Estrus cycle lengths of the cloned heifers in our study were comparable to those reported in previous studies (Morrow et al., 1976; Diskin and Sreenan, 2000). In all of the observed cycles, we confirmed the occurrence of follicular waves, ovulation,

and the formation of the corpus luteum. Follicular waves occurred 2.3 times per cycle, a rate consistent with reports on ordinary heifers (Adams, 1999) and cloned heifers (Enright et al., 2002). The levels of progesterone secretion per cycle in the cloned heifers were similar to those of ordinary heifers, suggesting that the cloned heifers have normal corpus luteum function in the postpubertal period. Thus, it can be concluded that the estrus cycles of cloned heifers are normal.

Although 2 heifers (1 clone from each group) needed multiple cycles of AI for conception, the changes in progesterone concentrations between the days of first AI and the day of conception followed a normal pattern. Furthermore, the estrus cycles of these heifers had a regular, repeat pattern during the breeding period. Thus, we suggest that the cause of the occasional need for multiple inseminations might be due to wrong timing of AI. All cloned heifers eventually conceived, suggesting that they were normally fertile. One Holstein cloned cow, however, delivered a stillborn calf 3 wk before the estimated day of parturition. The cause of the stillbirth was not clear, as there was no obvious morphological abnormality in the dead calf. The first estrus appeared around 90 d after parturition in the first and the second postpartum periods; most of the cows conceived after the first AI. All the cloned cows became pregnant 3 times after AI. Thus, it can be concluded that cloned cows with short telomeres are able to conceive and deliver normal calves at least 3 times.

In this study, there was a wide variation in the number of days needed for recovery of reproductive function after the first and second parturitions (Table 3). Reproductive function is influenced by nutritive conditions. In our study, however, the nutritional composition of feeds was constant. If cloned cows have identical digestive capabilities and metabolic characteristics, the wide variation in recovery of reproductive function might be caused by differences in the amount of feed intake under group-feeding conditions. Further research will be needed to clarify whether this is a contributory factor.

Milk yields differed between the cloned cows and the original cows. Total milk yields in first and second lactations were 832.4 and 1175.8 kg higher, respectively, in cloned Jersey cows than in the original cow. Conversely, in Holstein group cows, milk yields were 1635.0 and 170.6 kg lower, respectively, than in the original cow. The reason for these differences is not clear. However, one explanation might be differences in environmental conditions. The higher milk yield in the Jersey group probably resulted from their higher growth rate compared with the original cow. No growth data were available for the original cow of the Holstein group. However, because this animal was reared by a breeder for the purpose of sale, it seems reasonable to assume it was

Figure 4. Photographs of the cloned cows with shortened leukocyte telomeres. A) Four cloned cows of the Jersey group during the second lactation. Donor cells for nuclear transfer were obtained from a 6-yr-old Jersey cow (registration no. 20786; Jersey Cattle Association of Japan). B) Six cloned cows of the Holstein group during the second lactation. Donor cells for nuclear transfer were obtained from a 13-yr-old Holstein cow (registration no. 12787738; Holstein Association USA Inc.).

raised under ideal conditions and this may underlie its higher milk yield. The average CV of milk yield in 11 groups of Holstein half-sibs in our station was 8.6%, greater than that in the Holstein group (5.1%). However, significant differences in milk yield were not detected in all comparisons of cloned cows and half-sibs. Other milking traits showed similar trends as the milk yield. Thus, similarity of milking performance in cloned cows was not proven in our study. Van Vleck (1999) showed that if the heritability of a given trait is 25%, then clones would have a standard deviation of 87% of that of noncloned animals. If heritability is low, the similarity of trait might be below the ideal in the cloned animal. In fact, the heritability of milk production is estimated to be 30%. It follows that indistinct result about similarity of milking performance in our study might be correct. To ensure that cloned cows offer a

useful experimental system, it may be necessary to examine other characteristics that are less susceptible to environmental variables than milk performance; for example, metabolic functions or responses to drugs.

CONCLUSIONS

Our long-term observations on cloned cows with short telomeres suggest they have normal features of growth, reproduction, and lactation (Figure 4). In brief, reduced telomere length did not influence productivity between birth and 3 yr of age. We intend to continue observation of these animals throughout their life spans. Unfortunately, there were considerable inter-individual differences in milk production between cloned cows of both groups. Furthermore, the cloned cows did not show the same milk production as the original cow except for the

percentages of milk fat, protein, and SNF. Cloning may offer a valuable evaluation tool for investigating environmental effects on the development and productivity of commercially important animals. In the future, the similarity of cloned animals may be best assessed when they are raised under standardized, individual feeding conditions.

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