Questions have been raised as to whether cells or organisms created by nuclear transfer will undergo premature senescence. Normal somatic cells display a finite replicative capacity when cultured in vitro (1, 2). The germ line appears to maintain an immortal phenotype in part through expression of the ribonucleoprotein complex telomerase, which maintains the telomeres at a long length. However, nuclear transfer technologies use embryonic, fetal, and adult somatic cells that often do not express telomerase from a range of mammalian species (3–10). A recent report (11) suggests that nuclear transfer may not restore telomeric length reflecting a DNA checkpoint arrest (22–26), possibly indicating the age of the transferred nucleus, which could limit the utility of the cloning of replacement cells and tissue for human transplantation (12, 13).

Wilmot et al. (3) have reported that arrest in the G0 phase of the cell cycle is required to obtain normal development of animals cloned from differentiated cells. Replicative senescence is a physiological state distinguishable from quiescence achieved by either serum starvation or density-dependent inhibition of growth of young cells (14–18) and appears to involve blocking the G1/S boundary in the cell cycle (19–21), possibly reflecting a DNA checkpoint arrest (22–26). Here we investigate whether the production of live offspring is possible by nuclear transfer of late-passage somatic cells and whether the epigenetic changes seen in the donor cells, such as telomere shortening and loss of replicative life-span, are reflected in the resultant organism.

A somatic cell strain was derived from a 45-day-old female bovine fetus (BBF) and transfected with a PGK-driven selection cassette. Cells were selected with G418 for 10 days, and five neomycin-resistant colonies were isolated and analyzed for stable transfection by Southern blotting with a full-length cDNA probe. One cell strain (CL53) was identified as 63% (total nuclei) positive for the transgene by fluorescence in situ hybridization (FISH) analysis and was chosen for our nuclear transfer studies. These fibroblast cells, which were negative for cytokeratin and positive for vimentin, were passaged until greater than 95% of their life-span was completed, and their morphology was consistent with cells close to the end of their life-span (Fig. 1A).

A more detailed ultrastructural analysis by electron microscopy demonstrated that these cells exhibited additional features of replicative senescence, including prominent and active Golgi apparatus, increased invaginated and lobed nuclei, large lysosomal bodies, and an increase in cytoplasmatic microfibrils as compared with the young cells (Fig. 1B) (27). In addition, these late-passage cells exhibited a senescent phenotype in showing a reduced capacity to enter S phase (Fig. 1C) and a significant increase in the staining of β-galactosidase (28, 29). Furthermore, these cells exhibited a reduction in EPC-1 (early population doubling level cDNA-1) (30) mRNA levels as compared with early-passage bovine BFF cells in a manner analogous to the changes observed during the aging of WI-38 cells (Fig. 1D).

A total of 1896 bovine oocytes were reconstructed by nuclear transfer with senescent CL53 cells (4). Eighty-seven blastocysts (5%) were identified after a week in culture. The majority of the embryos (n = 79) were transferred into progesterin (SYNCROMATE-B)-synchronized recipients (2 to 6 years old), and 17 of the 32 recipients (53%) were pregnant by ultrasound 40 days after transfer. One fetus was electively removed at week 7 of gestation (ACT99-002), whereas nine (29%) remained pregnant by 12 weeks of gestation. Two of these aborted at days 252 (twins) and 253, and one was delivered stillborn at day 278. The remaining six recipients continued development to term. The rates of blastocyst formation (5%) and early (3%) and term (19%) pregnancies with senescent CL53 cells were comparable to those of control embryos produced with nonsenescent donor (CL57) cells from early-passage cells (5, 45, and 13%, respectively).

Six calves were delivered by elective cesarean section (Fig. 2). Genomic analyses confirmed the presence of the transgene in two of the animals (CL53-1 and CL53-12), as well as in the fetus that was removed electively at day

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43. The combined probability expression $P = 1 - \{1 - P_1\} \cdot \{1 - P_2\} \cdot \{1 - P_3\}$ for faults through c assumes independent sources of hazard, since we cannot include future interactions and, for all but the most recent earthquakes, we cannot include past interactions.

49. At birth, the presentation of the cloned calves was consistent with previous published reports (4, 6, 31, 32). In general, birth weights (51.6 ± 3.6 kg) were increased, and several of the calves experienced pulmonary hypertension and respiratory distress at birth, as well as incidence of fever after vaccinations at 4 months. After the first 24 hours, the calves were vigorous with minimal health problems. However, we noted a moderate incidence of polyuria/polydypsia and lowered dry matter intake during the first two months. The occurrence of these complications was linked neither to the donor cell population (isolate 53 or 57) nor to the presence or absence of transgene integration. After about 2 months, all of the calves resembled healthy control calves generated from both in vitro fertilization and in vivo embryo transfers, and they remained alive and normal 7 to 12 months after birth. Messenger RNA from dermal fibroblasts of the cloned calves was isolated (Fig. 1D). The cells from the cloned animals expressed about threefold higher EPC-1 mRNA levels than the early-passage fetal bovine cells. Furthermore, these dermal fibroblasts also expressed a 3.5- to 5-fold higher level of EPC-1 mRNA than comparable lines derived from age-matched control animals. This suggests that the fibroblasts derived from the cloned animals are potentially younger than the control fibroblasts.

To confirm that results from the cloned calves were not due to variations in the donor cell population, we produced dermal fibroblasts from three adult Holstein steers. Single-cell clones were isolated, and population doublings were counted until senescence. Nuclear transfer was performed with those fibroblast cells that were at or near age-matched control. Birth weights of the cloned calves was consistent with previous studies (51.6 ± 3.6 kg) and were not due to variations in the donor cell population (isolate 53 or 57) nor to the presence or absence of transgene integration. After about 2 months, all of the calves resembled healthy control calves generated from both in vitro fertilization and in vivo embryo transfers, and they remained alive and normal 7 to 12 months after birth. Messenger RNA from dermal fibroblasts of the cloned calves was isolated (Fig. 1D). The cells from the cloned animals expressed about threefold higher EPC-1 mRNA levels than the early-passage fetal bovine cells. Furthermore, these dermal fibroblasts also expressed a 3.5- to 5-fold higher level of EPC-1 mRNA than comparable lines derived from age-matched control animals. This suggests that the fibroblasts derived from the cloned animals are potentially younger than the control fibroblasts.

Table 1. Population doublings in fibroblasts derived from normal fetuses and fetuses generated from clonal populations of adult senescent cells.

<table>
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<th>PDs left at time of nuclear transfer in original adult cells</th>
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Fig. 1. Characterization of cell senescence in nuclear transfer donor cells. (A) Cells were observed by phase contrast microscopy. The donor cells (CL53) displayed an increased size cell and cytoplasmic granularity as compared with the early-passage BFF cells. (B) Representative electron micrographs of BFF and donor CL53 cells. Note the convoluted nucleus (n) of CL53 cells. CL53 cells are larger than BFF cells, and their cytoplasm contains abundant lysosomes (arrows) and thick fibrils. Both pictures are at the same magnification. Bar, 2 μm. Mitochondria (m). (C) Entry of early- (BFF, a) and late-passage (CL353, b) cells into DNA synthesis as determined by 3H-thymidine incorporation during a 30-hour incubation (40). The cells were processed for autoradiography and then observed microscopically and scored for labeled nuclei. At least 400 nuclei were counted (40). (D) The cells were measured for autoradiography and then observed microscopically and scored for labeled nuclei. At least 400 nuclei were counted (40). The donor CL53 cells exhibit reduced EPC-1 mRNA levels as determined by Northern blot analysis. Human fibroblasts (WI-38) at early passage (Y) and late passage (O), bovine fibroblasts at early passage (Y; BFF) and late passage (O; donor CL53), RNAs isolated from cloned cattle (animals CL53-1, CL53-10, CL53-11, and CL53-12), and age-matched control (animals 1 and 2) dermal fibroblast strains are indicated. Total RNA was extracted from the cells after they were grown to confluence and growth-arrested in serum-free medium for 3 days (41). Equal amounts of RNA were treated with glyoxal, separated by electrophoresis on agarose gels, ethidium bromide transferred to positively charged nylon membranes, and hybridized with the full-length EPC-1 cDNA (42).

Fig. 2. Normal heifers cloned from senescent somatic cells. (A) CL53-8, CL53-9, CL53-10, CL53-11, and CL53-12 (nicknamed Lily, Daffodil, Crocus, Forsythia, and Rose, respectively) at 5 months of age and (B) CL53-1 (Persephone) at 10 months of age.
two population doublings, respectively, whereas none of the cells divided more than three times (Fig. 3C). These data are consistent with a second experiment that was performed in which 250 cells were seeded at clonal densities (none of the cells underwent more than four population doublings). In contrast, early-passage (pretransfection) BFF cells underwent $58.7 \pm 1.2$ population doublings, with an average cell cycle length of $17.8 \pm 0.7$ hours during the logarithmic growth phase (Fig. 3A).

To test whether the somatic cell nuclear transfer procedure restored the proliferative life-span of the senescent donor fetal cells, we cultured fibroblasts from an electively removed 7-week-old fetus (ACT99-002). Cell strains from it underwent $96.1 \pm 7.3$ population doublings, with a cell cycle length of $17.7 \pm 0.8$ hours during the logarithmic growth phase (Fig. 3A). One-cell clones ($n = 5$) were generated from the cloned (ACT99-002) and original (BFF) age-matched fetuses, and cultures characterized as fibroblasts by immunohistochemical staining were isolated. These one-cell clones underwent $31.2 \pm 3.4$ and $25.9 \pm 2.9$ population doublings from the cloned and original fetuses, respectively (Fig. 3D).

To further investigate the ability of nuclear transfer to rescue senescent cells, we compared the telomere lengths in nucleated blood cells of the six cloned animals with those of age-matched (5 to 10 months old) control animals, newborn calves (<2 weeks old), and cows of various ages (ranging from 6 months to 19 years old) using flow cytometric analysis after in situ hybridization with directly fluoroscein isothiocyanate (FITC)–labeled (CCCTAA) peptide nucleic acid probe (flow FISH) (Fig. 4, A and B) ($33$, $34$). The results of three separate experiments are indicative of elongation of telomere length in the cloned animals relative to age-matched controls ($63.1 \pm 1.7$ compared with $50.8 \pm 2.9$ kMESF (molecules of equivalent soluble fluorochrome) (mean $\pm$ SD, $P < 0.0001$, experiment 1), $75.4 \pm 1.5$ compared with $60.8 \pm 3.1$ kMESF ($P < 0.0001$, experiment 2), and $73.6 \pm 0.3$ compared with $62.7 \pm 4.0$ kMESF ($P < 0.0001$, experiment 3)). Indeed, in two of three experiments, the telomeres in the cells of the cloned animals were significantly longer than those in cells from the newborn calves ($75.4 \pm 1.5$ compared with $66.8 \pm 5.1$ kMESF ($P < 0.0002$, experiment 2) and $73.6 \pm 0.3$ compared with $62.7 \pm 4.0$ kMESF ($P < 0.0001$, experiment 3)). The mean telomere lengths in nucleated bovine blood cells showed considerable variation at any given age as in human nucleated blood cells ($34$). Nevertheless, a highly significant decline in telomere length with age was observed ($P < 0.001$), corresponding to an estimated 20 to 100 base pairs of telomere repeats per year ($n = 46$). More extensive studies are needed to establish the rate of telomere shortening in the various nucleated blood cells from cattle.

### Reports

Telomere length was also studied with Southern blot analysis of terminal restriction fragments (22). The results (Fig. 4C) obtained with senescent (CL53), control (pretransfection BFF), and cloned (ACT99-002) cells were consistent with the flow FISH analysis of nucleated blood cells. The telomeres were longer in the cells derived from the cloned fetus (20.1 kb, lanes 3 and 6) than in the senescent and early-passage donor cells (15.2 and 18.3 kb, respectively; compare lanes 1 to 6, Fig. 4C). These results were reproduced in two separate experiments and were consistent with flow FISH analysis on the same cells (28).

The telomere length in clonal populations of senescent adult dermal fibroblasts (0.26 to 2.5 population doublings remaining) was compared with that in fibroblasts from cloned fetuses obtained with these cells (Fig. 4C, lanes 7 to 10). In the two cases that could be analyzed, an increase in telomere length was also observed upon cloning from senescent fibroblasts. The increase in telomere length ranged from 14.4 to 16.4 kb for clone 22-1 to from 12.1 to 16.1 kb for clone 25-1. The telomere length in these cloned early-passage (<10 population doublings) fibroblasts with extensive proliferative potential (Table 1) was comparable to that of the senescent fibroblasts that gave rise to the cloned animals with elongated telomeres described in this report. These results highlight the variable terminal restriction fragment length associated with replicative senescence.

High levels of telomerase activity were detected in reconstructed day 7 embryos tested by the telomeric repeat amplification protocol (TRAP) assay (Fig. 5, lanes 5 to 8), whereas the bovine fibroblasts used as donor cells in the nuclear transfer experiments were negative (Fig. 5, lane 9).

Our results differ from the study by Shiels et al. (11), in which telomere erosion did not appear to be repaired after nuclear transfer in sheep. The telomere lengths in cells from three cloned animals, 6LL3 (Dolly, obtained from an adult donor cell), 6LL6 (derived from an embryonic donor cell), and 6LL7 (derived from a fetal donor cell), were decreased relative to those of age-matched control animals. The authors suggested that full restoration of telomere length did not occur because these animals were generated without germ line involvement. They further suggested that the shorter terminal restriction fragment in Dolly was consistent with the time the donor cells spent in culture before nuclear transfer. Our findings show that viable offspring can be produced from senescent...
somatic cells and that nuclear transfer can extend the telomeres of the animals beyond that of newborn and age-matched control animals. It is not known whether the longevity of these animals will be reflected by telomeric measurements, although cells derived from cloned fetuses had an about 50% longer proliferative life-span than those obtained from same-age nonmanipulated fetuses. The ability to extend the life-span of specific differentiated cell types, such as hepatocytes, cardiomyocytes, and islets, an extra 30 population doublings would lead to a billionfold increase in the number of replacement cells generated for tissue engineering and transplantation therapies.

**Fig. 4.** Telomere length analysis. (A) Nucleated blood cells. Peripheral blood samples from cloned and control Holsteins were analyzed by flow FISH (34) in two separate blinded experiments. Duplicate samples (red and blue bars) of nucleated cells obtained after osmotic lysis of red cells with ammonium chloride were analyzed by flow FISH as described (33). The average telomere fluorescence of gated single cells was calculated by subtracting the mean background fluorescence from the mean fluorescence obtained with the FITC-labeled telomere probe. (B) Telomere lengths in nucleated blood cells of 25 normal Holsteins ranging from <2 weeks to 6 years of age, showing the decline in mean telomere lengths against age. (C) Elongation of telomeres in cells upon nuclear transfer. Terminal restriction fragment (TRF) analysis of DNA fragments obtained after digestion with Hinf I–Rsa I was performed on a 0.5% agarose gel run for 28 hours, as described (Telomere Length Assay Kit; Pharmingen, San Diego, California). Lanes 1 and 4, genomic DNA isolated from control cells (pretransfection BFF bovine fibroblasts) (mean TRF length 516.1 kb); lanes 3 and 6, fibroblasts from a 7-week-old cloned fetus (ACT99-002) obtained by nuclear transfer (mean TRF length 16.1 kb). Lane 7, senescent donor fibroblast clone 22-1 (mean TRF length 16.4 kb); lane 9, senescent fibroblast clone 25-1 (mean TRF length 12.1 kb); lane 10, nuclear transfer fetal fibroblasts obtained with senescent 22-1 cells (mean TRF length 20.1 kb) lanes 4 to 6 are longer exposures of lanes 1 and 4; lane 8, nuclear transfer fetal fibroblasts obtained with senescent 22-1 cells (mean TRF length 16.4 kb); lane 9, senescent fibroblast clone 25-1 (mean TRF length 12.1 kb); and lane 10, nuclear transfer fetal fibroblasts obtained with senescent 22-1 cells (mean TRF length 16.1 kb).

The differences between the present study and that reported by Shiel et al. (11) could be due to species differences and/or differences in nuclear transfer techniques or donor cell types. Wilmut et al. (3), for instance, used quiescent mammary cells to produce Dolly, whereas senescent fibroblasts were used in the present experiments. Also, although recent studies have shown that reconstruction of telomerase activity can lead to telomere elongation and immortalization of human fibroblasts (35, 36), similar experiments with mammary epithelial cells did not result in elongation of telomeres and extended replicative life-span (37). Differences between cells in telomere binding proteins (38), the ability of telomerase to extend telomeres, or differences in the signaling pathways activated upon adaption to culture (39) could explain the differences. The elongation of telomeres in the present study suggests that reconstructed bovine embryos contain a mechanism for telomere length regeneration, providing chromosomal stability throughout the events of pre- and postattachment development. The ability of nuclear transfer to restore somatic cells to a phenotypically youthful state may have important implications for agriculture and medicine.

**References and Notes**

Gene Therapy of Human Severe Combined Immunodeficiency (SCID)–X1 Disease

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Severe combined immunodeficiency–X1 (SCID-X1) is an X-linked inherited disorder characterized by an early block in T and natural killer (NK) lymphocyte differentiation. This block is caused by mutations of the gene encoding the γc cytokine receptor subunit of interleukin-2, -4, -7, -9, and -15 receptors, which participates in the delivery of growth, survival, and differentiation signals to early lymphoid progenitors. After preclinical studies, a gene therapy trial for SCID-X1 was initiated, based on the use of complementary DNA containing a defective γc Moloney retrovirus–derived vector and ex vivo infection of CD34+ cells. After a 10-month follow-up period, γc transgene–expressing T and NK cells were detected in two patients. T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit.

In considering diseases that might be ameliorated by gene therapy, a setting in which a selective advantage is conferred by transgene expression, in association with long-lived transduced cells such as T lymphocytes, may prove critical. SCID-X1 offers a reliable model for gene therapy because it is a lethal condition that is, in many cases, curable by allogeneic bone marrow transplantation (1–4). It is caused by γc cytokine receptor deficiency that leads to an early block in T and NK lymphocyte differentiation (1–3). In vitro experiments of γc gene transfer have shown that γc expression can be restored (5–7), as well as T and NK cell development (8–9), while γc− mice can be corrected by ex vivo γc gene transfer into hematopoietic precursor cells (10, 11). Long-term expression of human γc has also been achieved by retroviral infection of canine bone marrow (12). It has been anticipated that γc gene transfer should confer a selective advantage to transduced lymphoid progenitor cells because, upon interaction with interleukin-7 (IL-7) and IL-15, the γc cytokine receptor subunit transmits survival and proliferative signals to T and NK lymphocyte progenitors, respectively (2, 3). This hypothesis received further support from the observation that a spontaneously occurring γc gene reverse mutation in a T cell precursor in one patient led to a partial, but sustained, correction of the T cell deficiency, including at least 1000 distinct T cell clones (13, 14). Spontaneous correction of the immunodeficiency has otherwise not been observed in several hundred γc-deficient SCID patients nor in γc− mice (2–4).

Two patients, aged 11 months (P1) and 8 months (P2), with SCID-X1 met the eligibility criteria for an ex vivo γc gene therapy trial. SCID-X1 diagnosis was based on blood lymphocyte phenotype determination and findings of γc gene mutations resulting either in a tail-less receptor expressed at the membrane (P1) (R289 X) or in a protein truncated from the transmembrane domain that was not expressed at cell surface (P2) (a frameshift causing deletion of exon 6) (15). After marrow harvesting and CD34+ cell separation, 9.8 × 106 and 4.8 × 106 CD34+ cells per kilogram of body weight from P1 and P2, respectively, were pre-activated, then infected daily for 3 days with the MFG γc vector–containing supernatant (16). CD34+ cells (19 × 106 and 17 × 106/kg, respectively) were infused without prior chemoblation into P1 and P2, ~20 to 40% and 36% of which expressed the γc transgene as shown by either semiquantitative PCR analysis (P1) or immunofluorescence (P2). As early as day +15 after infusion, cells carrying the γc transgene were detectable by PCR analysis (17) among peripheral blood mononuclear cells. The fraction of positive peripheral blood mononuclear cells increased with time (Fig. 1). T lymphocyte counts increased from day +30 in P1 (who had a low number of autologous T cells before therapy), whereas γc-expressing T cells became detectable in the blood of P2 at day +60 (Fig. 2). Subsequently, T cell counts, including CD4+ and CD8+ subsets, increased to 1700/μl from day +120 to +150 and reached values of ~2800/μl after 8 months (Fig. 2). Transgenic γc protein expression could not be studied on P1 cells given the presence of the endogenous tail-less protein. However, semiquantitative PCR performed at day +150 showed that a high proportion of T cells carry and express the γc transgene (Fig. 1, A and B). Similar results were observed at day +275. Southern blot analysis of provirus integration in peripheral T cells from both patients revealed a smear indicating that multiple T cell precursors had been infected by the retroviral vector (18). Immunofluorescence studies showed that γc was expressed on the membrane of T cells in P2. The magnitude of expression was similar to that of control cells (Fig. 3A), as found in previous in vitro gene transfer experiments (5, 8, 9). These results indicate that sufficient transgene expression had been achieved and that γc membrane expression is likely to be regulated by the availability of the other cytokine receptor subunits with which γc associates (3). Both αβ and γδ T