into an FFF instrument (Model F-1000-FO, FFFractionation; LLC, Salt Lake City, UT) operating with 0.007% Triton X-100 in water mobile phase at 2 ml min^{-1} and a cross-flow rate of 0.5 ml min^{-1}.

17. Electrodeposition was performed by placing 20 μl of the nanotube suspension on the surface of a freshly cleaved HOPG substrate (Advanced Ceramics, Cleveland, OH), confining the droplet within a viton O-ring (4-mm outer diameter, 1.7 mm thick), capping the trapped suspension with a stainless steel electrode on top of the O-ring, and applying a steady voltage of 1.1 V for 6 min. When suspended in water, the nanotubes are negatively charged and are therefore driven by the electric field onto the HOPG surface. After deposition, the HOPG-nanotube surface was washed with methanol on a spin coater to remove the water and Triton X-100 surfactant.
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Cloned Transgenic Calves Produced from Nonquiescent Fetal Fibroblasts

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An efficient system for genetic modification and large-scale cloning of cattle is of importance for agriculture, biotechnology, and human medicine. Here, actively dividing fetal fibroblasts were genetically modified with a marker gene, a clonal line was selected, and the cells were fused to enucleated mature oocytes. Out of 28 embryos transferred to 11 recipient cows, three healthy, identical, transgenic calves were generated. Furthermore, the life-span of near senescent fibroblasts could be extended by nuclear transfer, as indicated by population doublings in fibroblast lines derived from a 40-day-old fetal clone. With the ability to extend the life-span of these primary cultured cells, this system would be useful for inducing complex genetic modifications in cattle.

Research has been in progress for more than a decade to develop a system for genetic modification and large-scale cloning in cattle (*1*), an important species in agriculture, biotechnology, and human medicine. In the initial work on cloning, embryonic blastomeres were used as donor nuclei because they were thought to be relatively undifferentiated, readily reprogrammed, and likely to support full-term development of the fetus (*2*). Initial efforts at refining the methodology of nuclear transfer resulted in significant, but limited, improvements in efficiency, and at most, only a few identical calves could be produced from a single donor embryo because of the limited number of cells in the early embryo (*3*). The next step toward expanding the potential of cloning was the development and use of embryonic stem cells as a source of donor nuclei. Embryonic stem cells are derived from the inner cell mass of an early embryo and are thought to be relatively undifferentiated. In addition, mouse embryonic stem cells divide indefinitely in culture without differentiation and can be readily genetically modified (*4*). Embryonic stemlike cells have been developed in the bovine (*5*) and have been used as a source of donor nuclei in nuclear transfer, but they only supported development of fetuses to 60 days in vivo (*6*). To date, a source of cells that can be used for genetic modification and large-scale cloning in cattle has not been found.

Other research in nuclear transplantation has shown that the cell cycle stage of the donor cell affects the extent of development of the embryo after nuclear transfer. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated (*7*). This condensation phase has been shown to cause chromosomal defects in donor cells that are undergoing DNA synthesis (*7*). Donor cells in the G_1 phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development (*7*).

In previous work in the sheep, it was suggested that arrest in G_0 (by serum starvation) was the key in allowing donor somatic cells to support development of embryos to term (*8*).

Our rationale in selecting an optimal donor cell for nuclear transplantation was that the cell should not have ceased dividing (which is the case in G_0) but be actively dividing, as an indication of a relatively undifferentiated state and for compatibility with the rapid cell divisions that occur during early embryo development. The cells should also be in G_1, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long G_1 phase. We chose fibroblasts from fetuses because they can grow rapidly in culture and have an inherently long G_1 phase (*9*).

Fetal fibroblasts were isolated from a day 55 male fetus (Fig. 1A), cultured in vitro, and passaged twice before being transfected with a marker construct consisting of a β-galactosidase–neomycin resistance fusion gene driven by a cytomegalovirus (CMV) promoter (pCMV/β-GEO) (*10*). Cells were selected with neomycin for 2 weeks, and five neomycin-resistant colonies were isolated and analyzed for stable transfection by polymerase chain reaction (PCR) amplification of a segment of the transgene (*11*) and by assay of β-galactosidase activity. Colony CL1 was chosen for nuclear transfer experiments. These fibroblast cells

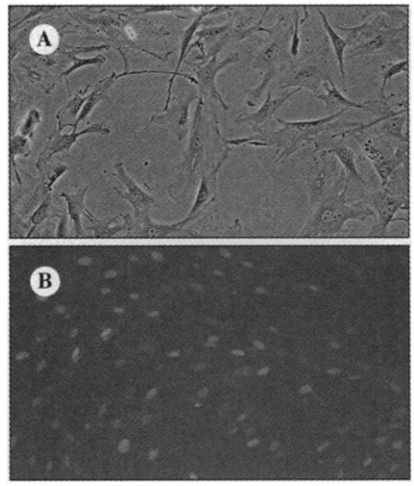

Fig. 1. Transgenic fetal fibroblast CL1-5 used for nuclear transplantation (**A**) phase contrast (×100). (**B**) Labeling of CL1-5 fibroblast cell line with PCNA monoclonal antibody (Sigma, St. Louis, MO) and FITC-conjugated secondary antibody (magnification × 200).

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were characterized as negative for cytokeratin, positive for vimentin (mesoderm origin), and negative for a human fibroblast cell surface marker.

A total of 276 nuclear transfer embryos were produced and 33 blastocysts (12%) were obtained after a week in culture (*12*). Twenty-eight of the blastocysts were nonsurgically transferred into 11 synchronized recipients (day 7 after onset of heat). Six cows were detected pregnant by ultrasound 40 days after nuclear transfer (55%), and five cows remained pregnant by day 60 of gestation (45%). No multiple pregnancies were produced. One cow aborted at day 249 of gestation. Its placenta was characterized as having hydroallantois, enlarged placentomes, and an edematous chorioallantois and amnion. Upon necropsy, the fetus was oversized (54 kg at month 8 of gestation), lung lobes were edematous, umbilical vessels were twice normal size, and the right heart ventricle was enlarged. The remaining four calves continued development to term. As controls, 122 in vitro–matured oocytes were parthenogenically activated, and after a week in culture 21 (18%) blastocysts were obtained.

Calves ACT2, ACT3, ACT4, and ACT5 were delivered at 277, 286, 287, and 289 days of gestation, respectively. Calf ACT2 died 5 days after birth as a result of pulmonary hypertension leading to insufficient pulmonary perfusion. Along with this pathology, the animal exhibited a dilated right ventricle, a patent ductus arteriosus, a pulmonary artery greater than the size of the aorta, and umbilical vessels three times normal size. The placenta also manifested abnormalities such as hydroallantois and a reduced number of enlarged placentomes. Calves ACT3 and ACT5 were delivered by cesarean section, and ACT4 was born vaginally. These three animals were phenotypically normal, and no abnormal placentation was revealed (Fig. 2). All five calves were screened by PCR amplification of a segment of the transgene (*11*), which confirmed that the cells were transgenic with the pCMV/βGEO gene (Fig. 3).

A restriction enzyme digest and Southern blot of genomic DNA (*13*) from calves ACT3, ACT4, and ACT5 demonstrated that these animals had an identical gene integration site and therefore were derived from the same fibroblast clone (Fig. 4). Also, the neomycin resistance gene was demonstrated to be functional in fibroblasts obtained from dermis of the calves. When cultured under selection with Geneticin, cells were able to survive for more than 10 days in culture (Fig. 5).

Developmental problems exhibited by the nonsurviving calves could be attributed to abnormal placentation. It remains to be determined whether the origin of this pathology can be connected to the nuclear transfer procedure itself or to the culture conditions in which the embryos were maintained during the first week of development. Previous data on cattle have indicated an association between in vitro culture conditions and calf abnormalities (*14*). In sheep, when nuclear transfer embryos were grown in vivo, perinatal loss occurred; however, no common factor could be attributed as to the cause of death (*8*).

Because it has been observed in rabbits that chemical synchronization can result in fewer successful pregnancies (*15*), CL1 bovine cells were not synchronized in G_1 but were constantly cultured with 10% fetal bovine serum and used at 70 to 80% confluence. Immunohistochemical analysis showed that 82% (279/340) of the cells were positive for proliferating cell nuclear antigen (PCNA) (Fig. 1B). Fluorescence-activated cell sorting (FACS) analysis revealed that, even though the cells were actively dividing, 56% of the cells were in G_1, providing a large population that could support development and precluding the need for an artificial synchronization procedure.

Our results indicate that an actively dividing population of cells can support development to term after nuclear transfer and that serum starvation is not a necessary treatment. Although our population of cells were actively dividing, we cannot determine which subpopulation (G_1, S, G_2, or M) may have produced the offspring. Certainly, more work will be necessary to fully understand the properties of somatic cells required to allow for successful reprogramming and full-term development of offspring.

The fibroblasts used in this study have a finite life-span in culture, which could limit the types of transgenic modifications that could be made. When cultured until senescence, fibroblasts derived from 6-week-old fetuses undergo 30 population doublings, with an average cell cycle length of 28 to 30 hours. As shown with the previous data, this number of population doublings is sufficient to generate clonally derived transgenic cell lines. However, many uses of genetically modified fetuses and animals will require gene targeting by homologous recombination. For homologous recombina-

Fig. 2. Normal cloned calves ACT3, ACT4, and ACT5, at 3 weeks of age.

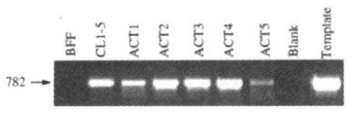

Fig. 3. Gel of PCR-amplified segment of the pCMV/βGEO construct (*15*) of DNA obtained from the original cell line and calf ear samples. BFF, nontransgenic fetal fibroblasts; CL1-5, original clonal cell line used for nuclear transfer; ACT1, fetus aborted at 249 days of gestation; ACT2, calf dead at 5 days after birth; ACT3, ACT4, and ACT5, normal calves. Size marker (in base pairs) is on the left.

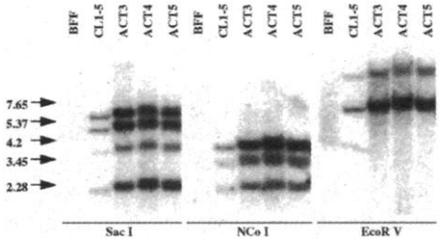

Fig. 4. Southern blot of genomic DNA (*16*) obtained from ear notches of live calves. DNA was cut with three different restriction enzymes: (**A**) Sac I, (**B**) Nco I, and (**C**) EcoR V. Size markers (in kilobase pairs) are on the left.

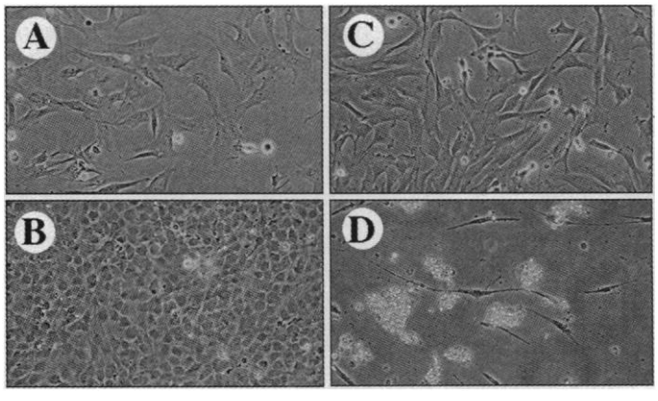

Fig. 5. Fibroblast from ACT4 calf and nontransgenic fetal fibroblast cultured with Geneticin (300 μg/ml, Sigma). (**A**) ACT4 calf fibroblasts isolated from dermis at day 1 and (**B**) day 10. (**C**) Nontransgenic fetal fibroblast at day 1 and (**D**) day 10.

tion, transgenic cells are first selected, but then a second round of selection is necessary to identify the correctly targeted cells. The two rounds of selection will require a greater number of cell divisions, and the cells could easily become senescent by the time the correctly targeted cells are identified, and they would certainly be senescent before a homozygous mutant is produced. To address this problem, we generated a 40-day-old fetus using the CL1 cell line at 0.8 population doubling from senescence. The fetus was removed from the uterus, and fibroblasts were derived from it. The number of population doublings until senescence was 31 and 33 for the nuclear transfer and same-age nonmanipulated fetal fibroblasts, respectively. These data suggest that fibroblast life-span can be enhanced by nuclear transfer. This approach could enable us to generate as many gene targeting events as needed by subjecting the cell line to the successive rounds of nuclear transfer.

This somatic cell nuclear transfer procedure could improve the efficiency of producing transgenic cattle and broaden the scope of applications for transgenic cattle. With previous microinjection techniques, about 500 embryos would have to be injected and transferred to recipient cows to get one transgenic offspring (*16*). For the nuclear transfer technique with transgenic somatic cells, the transfer of nine embryos to four cows produced a transgenic offspring, greatly reducing the time and costs involved. With the nuclear transfer approach, an entire herd of the appropriate sex transgenic cattle could be produced in one generation, whereas the traditional microinjection approach would require at least two generations, and likely more, to obtain a production herd. This is a savings of 2 years for each generation. Finally, the somatic cell nuclear transfer approach could broaden the scope of use of transgenic cattle because it allows the targeting of DNA inserts to specific sites in the genome. This is important for deleting or replacing bovine genes that might interfere with human protein isolation or cause rejection of grafted tissues. Inserting genes into a selected site could be used to ensure tissue-specific and consistent expression levels of transgenes. Furthermore, insertion of genes into the same site in multiple lines of animals could be used to quickly generate homozygous lines of animals while avoiding inbreeding.

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10. The plasmid pCMV/βGEO was constructed by inserting the βGEO fusion fragment {Xho I to Xba I [G. Fiedrich *et al.*, *Genes Dev.* **5** (no. 9), 1513 (1991)]} into pcmvβ (Clonotech) between Not I sites.
11. The transgene was detected in transfected cells, fetuses, and tissue from adult animals by PCR with a 21-base sense primer (ACT3βGEO) 5′-CGCTGTGGTACACGCTGTGCG-3′, and a 22-base antisense primer (ACT4βGEO) 5′-CACCATCCAGTGCAGGAGCTCG-3′ (Amitof Biotech). Reactions were run for 35 cycles, with denaturation at 95°C for 30 s, annealing at 65°C for 1 min, followed by extension for 2 min at 72°C. A final extension for 10 min at 72°C was included after the last cycle. The amplified product was a 782–base pair (bp) fragment. The samples were analyzed by separating them by size in a (1%) tris-borate EDTA agarose gel containing ethidium bromide.
12. For nuclear transfer, bovine oocytes were aspirated from slaughter-collected ovaries, put in maturation media [P. Damiani *et al.*, *Mol. Reprod. Devel.* **45**, 521 (1996)], and shipped to the laboratory overnight at 38.5°C. Oocytes were mechanically enucleated at 18 hours after maturation, and chromosome removal was assessed with bisBENZIMIDE (Hoechst 33342; Sigma, St. Louis, MO) dye under ultraviolet light. Successfully enucleated oocytes were fused with actively dividing CL1 fibroblasts by using one electrical pulse of 180 volts/cm for 15 μs (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 2 to 4 hours, oocytes were then chemically activated with calcium ionophore (5 μM) for 4 min (catalog number 407952; Cal Biochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 with bovine serum albumin (BSA) (3 mg/ml) for 3 hours (fatty acid free, Sigma) [J. L. Susko-Parrish *et al.*, *Devel. Biol.* **166**, 729 (1994)]. After activation, eggs were washed in hamster embryo culture medium (HECM)–Hepes five times and placed, for culture, in a 500-μl well of CR2 (Specialty Media, Lavallette, NJ) with BSA (3 mg/ml) (fatty acid free) along with 1×10^6 mouse embryonic fibroblasts (MEFs) per milliliter. Incubation was performed for 6.5 days at 38.5°C and 5% CO_2 in air, and selected embryos were shipped overnight in a portable incubator to the embryo transfer facility.
13. Calf tissue samples (peripheral ear tissue) were prepared for Southern blot analysis according to P. Laird *et al.* [*Nucleic Acids Res.* **19**, 4293 (1991)]. Ten micrograms of genomic DNA was run in a 0.75% agarose gel in tris-acetate buffer, transferred onto Zetabind (Cuno, Meriden, CT), cross-linked by ultraviolet light, prehybridized for 4 hours, then hybridized at 42°C for 8 hours. The blot was washed and exposed to Biomax film (Kodak). A probe of 3881 bp, EcoR I–EcoR I DNA fragment of βGEO was labeled with ^{32}P-deoxycytidine triphosphate (dCTP) by using a random primed labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).
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17. We thank J. Balise, R. Duby, J. Morris, N. Kieser, E. Dickinson, and N. Ward Mackall for their technical assistance; G. Morgan for cell cycle studies; J. Hill, S. R. Bumgardner, and A. J. Russel for attending calves at birth; and C. Looney for embryo transfer work. Supported by a grant from Advanced Cell Technology to the University of Massachusetts.

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A Signaling Complex of Ca^{2+}-Calmodulin–Dependent Protein Kinase IV and Protein Phosphatase 2A

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Stimulation of T lymphocytes results in a rapid increase in intracellular calcium concentration ($[Ca^{2+}]_i$) that parallels the activation of Ca^{2+}-calmodulin–dependent protein kinase IV (CaMKIV), a nuclear enzyme that can phosphorylate and activate the cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB). However, inactivation of CaMKIV occurs despite the sustained increase in $[Ca^{2+}]_i$ that is required for T cell activation. A stable and stoichiometric complex of CaMKIV with protein serine-threonine phosphatase 2A (PP2A) was identified in which PP2A dephosphorylates CaMKIV and functions as a negative regulator of CaMKIV signaling. In Jurkat T cells, inhibition of PP2A activity by small t antigen enhanced activation of CREB-mediated transcription by CaMKIV. These findings reveal an intracellular signaling mechanism whereby a protein serine-threonine kinase (CaMKIV) is regulated by a tightly associated protein serine-threonine phosphatase (PP2A).

Cellular responses to external signals require coordinated control of protein kinases and phosphatases; multiple complexes containing both intracellular signaling enzymes are likely to be important for the regulation and specificity of signal transduction pathways. The targeting of protein kinases and phosphatases to specific subcellular compartments, through association with scaffold proteins such as A-kinase anchoring proteins, may contribute to the specificity of cellular signaling (*1*). However, the enzymes are retained by the anchoring protein in their inactive state (*2*), and potential regulatory interactions within these multiprotein complexes remain unknown. Preexisting com-