Production of goats by somatic cell nuclear transfer

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In this study, we demonstrate the production of transgenic goats by nuclear transfer of fetal somatic cells. Donor karyoplasts were obtained from a primary fetal somatic cell line derived from a 40-day transgenic female fetus produced by artificial insemination of a nontransgenic adult female with semen from a transgenic male. Live offspring were produced with two nuclear transfer procedures. In one protocol, oocytes at the arrested metaphase II stage were enucleated, electrofused with donor somatic cells, and simultaneously activated. In the second protocol, activated in vivo oocytes were enucleated at the telophase II stage, electrofused with donor somatic cells, and simultaneously activated a second time to induce genome reactivation. Three healthy identical female offspring were born. Genotypic analyses confirmed that all cloned offspring were derived from the donor cell line. Analysis of the milk of one of the transgenic cloned animals showed high-level production of human antithrombin III, similar to the parental transgenic line.

Keywords: nuclear transfer, oocyte, transgenic, antithrombin III, goat

The production of human recombinant pharmaceuticals in the milk of transgenic farm animals[1,2] solves many of the problems associated with microbial bioreactors (lack of post-translational modifications, improper folding, high purification costs) or animal cell bioreactors (high capital costs, expensive culture media, low yields). Dairy goats are ideal for transgenic production of therapeutic recombinant proteins. At concentrations of recombinant protein of 1–5 g/L that have been reproducibly achieved with various animal models by this and other groups[1–3], herds of transgenic goats of manageable size could easily yield 1–300 kg of purified product per year. Moreover, the much lower incidence of scrapie in goats (only seven cases reported by the US Department of Agriculture through 1998) relative to sheep (1,117 US cases through 1992[4]) adds to the goats' attractiveness for recombinant protein production.

To date, the only reliable method available to produce transgenic goats has been pronuclear microinjection. While successful, this approach has had limited efficiency. Transgene integration into the genome of founder animals is low, with only 0.5–3% of the microinjected embryos producing transgenic offspring[5]. Moreover, the frequent generation of mosaic founder animals resulting from pronuclear microinjection[6–8] complicates the expansion of transgenic herds.

The application of nuclear transfer technology using blastomeres of early caprine embryos has been reported[9]. This approach is limited by the small numbers of available embryonic blastomeres and by the inefficiency of introducing foreign genetic material into such cells. In contrast, the discoveries that differentiated embryonic[10–12], fetal[11,13–15], or adult somatic cells[10,15–17] can function as karyoplast donors for nuclear transfer have provided a wide range of possibilities for germline modification. The use of recombinant somatic cell lines for nuclear transfer allows the introduction of transgenes by traditional transfection methods, increases the efficiency of transgenic animal production to 100%, and overcomes the problem of founder mosaicism.

In this report, we describe the application of somatic cell nuclear transfer to the propagation of transgenic goats. Three transgenic female goats carrying a transgene targeting the expression of recombinant human antithrombin III (rhAT) to the mammary gland were generated following two activation protocols. Southern blotting, fluorescence in situ hybridization (FISH) and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analyses verified that these animals were clones of the female hAT cell line. One of the resulting transgenic females was hormonally induced to lactate, and rhAT expression in its milk was consistent with rhAT expression detected in the milk of other transgenic does from the same line obtained by natural breeding[3].

Results

Caprine fetal somatic cells. Six fetal somatic cell lines were generated from four 35-day and two 40-day fetuses resulting from the mating of a founder BC6 (goat β-casein–hAT cDNA transgene[3]) transgenic buck with two nontransgenic does. The BC6 transgenic line was chosen for these experiments because it provides a well-characterized genetic marker to the somatic cell lines (BC6 transgene), and it targets high-level expression of a complex glycosylated protein (hAT) in the milk of lactating transgenic does. Following PCR analysis of the six primary cultures, two male and four female lines were identified. One transgenic female cell line (CFF6, derived from a 40-day fetus) was used in all nuclear transfer experiments described in this study. In CFF6 cell cultures, as well as in the other primary caprine fetal somatic cell lines, two morphologically distinct cell types were noted. Larger "fibroblast-like" cells (Fig. 1A, center) and smaller

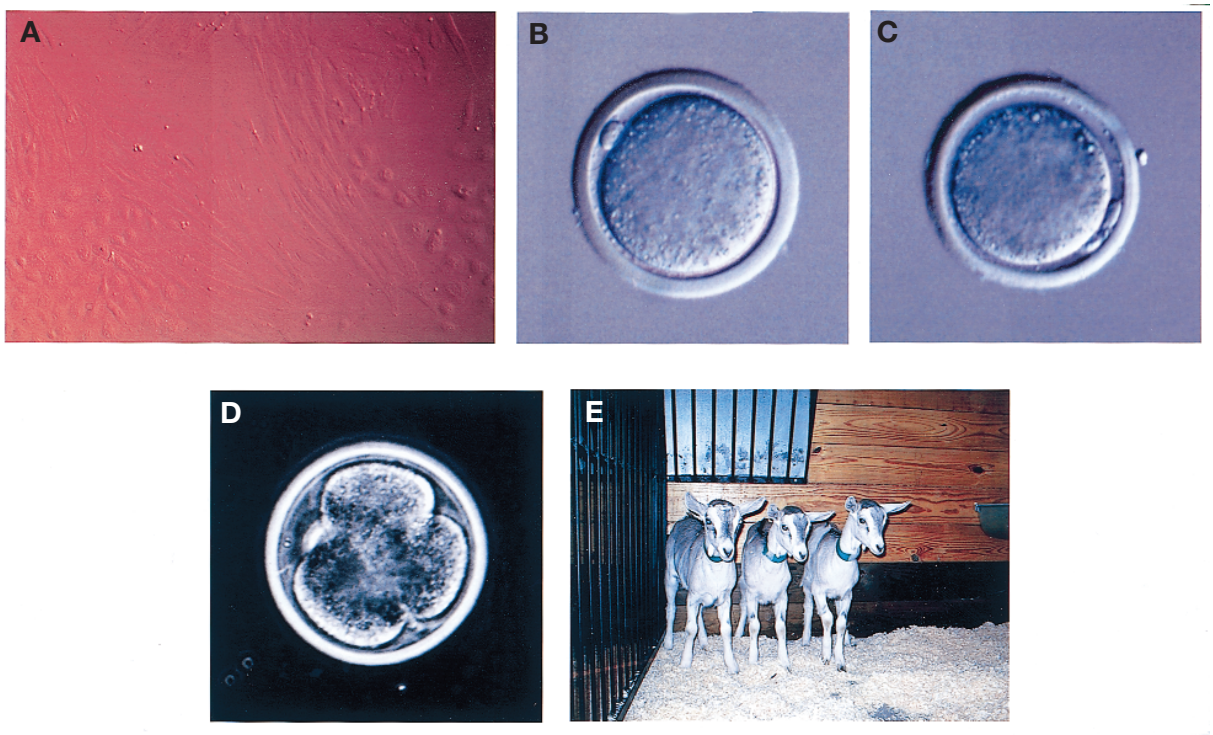

Figure 1. (A) In vitro-cultured caprine fetal somatic cells at Passage 6, magnification ×200. (B) In vivo-matured Metaphase-II stage caprine oocyte, magnification ×400. (C) In vivo-matured, activated Telophase-II stage caprine oocyte, magnification × 400. (D) A reconstructed 4-cell stage caprine embryo 48 h after fusion of a CFF6 cell to an in vivo-matured, activated Telophase II stage enucleated cytoplast, magnification ×400. This reconstructed embryo was transferred to recipient 1169 (along with one 2-cell stage and another 4-cell stage reconstructed embryo) that delivered the nuclear transfer offspring CFF6-2 and CFF6-3. (E) Three cloned goats at 8 weeks (CFF6-1) and 5 weeks (CFF6-2 and CFF6-3) that were produced by nuclear transfer with fetal somatic cells from the CFF6 transgenic cell line. From left to right, CFF6-1, CFF6-2 and CFF6-

"epithelial-like" cells (Fig. 1A, bottom left and right sides) coexisted in primary cultures. At first, larger cells dominated (>80%), but with increasing number of passages the proportion of epithelial-like cells increased to >50% of the total cell population.

Production of nuclear transfer embryos. Donor fetal somatic cells were synchronized by seven days of serum deprivation, followed by exposure to medium containing 10% fetal bovine serum (FBS) for 1–3 h before the nuclear transfer procedure. A total of 14 rounds of oocyte collection and embryo transfer procedures were performed. In an effort to optimize the use of oocytes collected, three enucleation/activation protocols were employed: arrested metaphase II oocytes (Fig. 1B), calcium-activated telophase II oocytes (Telophase-II-Ca; Fig. 1C), or ethanol-induced calcium-activated telophase II oocytes (Telophase-II-EtOH). Following fusion/activation, reconstituted embryos were cocultured with primary goat oviduct cells for 48 h, until 2- to 16-cell stage embryos had developed. The majority of embryos were transferred to progestin-synchronized recipient does, at chronologically correct two- and four-cell stages (Fig. 1D). Rates of development were slightly greater when using cytoplasts from Telophase-II-Ca (45%) and Telophase-II-EtOH (56%) oocytes when compared with arrested metaphase II (35%) oocytes (Table 1).

Pregnancy and cloned transgenic offspring. Recipient does were examined by ultrasonography starting at 25 days after embryo transfer. For all three oocyte enucleation/activation protocols, a high proportion of does (55–78%) exhibited vesicle formation very similar to that seen in does at 30 days of gestation (Table 1). In most cases, fetal heartbeats could not be detected at this early stage. The echogenic patterns suggested the presence of trophoblastic vesicles, in the absence of normal embryonic structures. Weekly examinations, between day 25 and day 40 post-transfer, failed to reveal normal embryonic development. By days 55–70, vesicular structures had been resorbed, and the recipient does had returned to estrus.

Table 1. Development of caprine embryos reconstructed by nuclear transfer using CFF6 transgenic fetal somatic cells.

Nuclear transfer protocols	Metaphase-II	Telophase-II-Ca	Telophase-II-EtOH
Oocytes reconstructed	138	92	55
Oocyte lysis (%)	67 (48.5)	38 (41.3)	23 (41.8)
Embryo cleavage (%)	48 (34.8)	41 (44.6)	31 (56.4)
Embryos transferred (%)	47 (34)	38 (41.3)	27 (49.1)
Recipients	15	14	9
Ultrasound positive (%)			
30 days	9 (60)	11 (78.6)	5 (55.5)
40 days	1 (6.7)	1 (7.1)	0
60 days	1 (6.7)	1 (7.1)	0
Term pregnancy (%)	1 (6.7)	1 (7.1)	0
Offspring	1	2	0
% of embryos transferred	2.1	5.2	0
% of embryos reconstructed	0.7	2.2	0

Three enucleation protocols were used (see text for details): Metaphase-II (Metaphase-II arrested), Telophase-II-Ca (calcium-activated Telophase-II oocytes), Telophase-II-EtOH (ethanol-induced calcium-activated Telophase-II oocytes). In all cases, simultaneous electrical fusion and activation were induced.

In two recipients, fetal heartbeats were confirmed by day 40 post-transfer. In both of these cases, ultrasound examination between day 25 and day 40 also revealed the development of recognizable fetal structures. One of these two pregnancies resulted from the transfer of embryos (four four-cell stage) that were produced using the arrested metaphase II nuclear transfer protocol. Karyoplasts were from passage 6 of the CFF6 cell line. A healthy transgenic female kid, CFF6-1 (Fig. 1E, left), was delivered on day 154 of gestation after induction of parturition on day 152. The

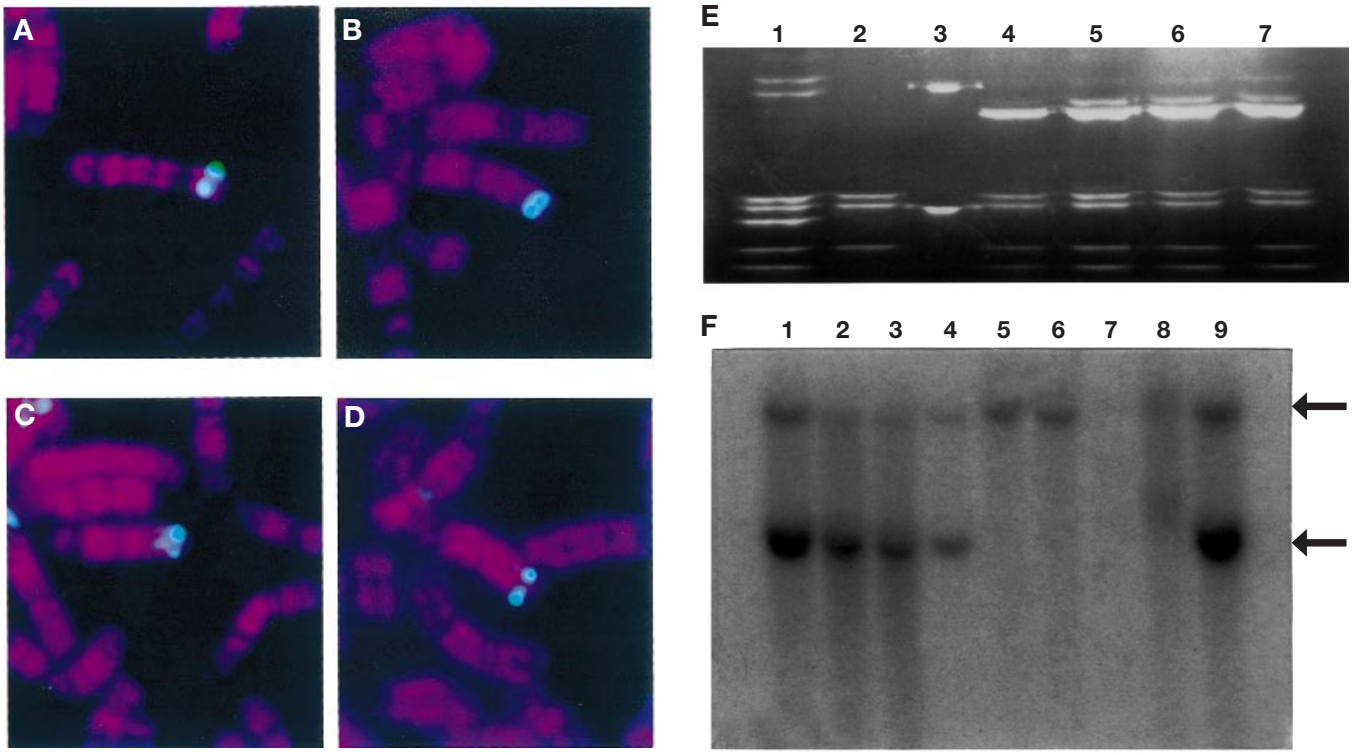

Figure 2. Genotypic analysis of nuclear transfer goats. FISH analysis of BC6 transgene integration for (A) the CFF-6 cell line, (B) the CFF6-1, (C) the CFF6-2 and (D) the CFF6-3 kid. The BC6 integration (green dots) is located close to the telomere of caprine chromosome 5 (C 5q). (E) *Rsa* I PCR-RFLP analysis of the second exon of the caprine MHC class II DRB gene. Lane 1: recipient 1034, lane 2: recipient 1169, lane 3: molecular weight marker (Gibco BRL 100 bp ladder, 100b and 200 bp fragments are visible), lane 4: CFF6 cell line, lane 5: CFF6-1, lane 6: CFF6-2, lane 7: CFF6-3. (F) Southern blot analysis of *Eco*RI restriction digests of genomic DNAs. Lane 1: CFF6-1, lane 2: CFF6-2, lane 3: CFF6-3, lane 4: CFF6, lane 5: recipient 1034, lane 6: recipient 1169, lane 7: molecular weight marker (λ phage DNA digested with *Bst* EII restriction enzyme), lane 8: negative goat, lane 9: BC6 transgenic control. The top arrow shows the hybridization signal (14 kb band) of the hAT cDNA probe to the endogenous caprine AT locus and serves as an internal loading control. The bottom arrow shows the hybridization signal (4.1 kb band) of the hAT cDNA probe to the BC6 transgene.

weight at birth was 2.35 kg, which is within the normal range for newborns of this breed. The second pregnancy (twins) resulted from the transfer of embryos produced by the activated Telophase-II-Ca nuclear transfer protocol with karyoplasts originating from passage 5 of the CFF6 cell line. In this case, three nuclear transfer embryos (one two-cell stage and two four-cell stage) were transferred to a recipient doe. Two healthy female kids, CFF6-2 and CFF6-3 (both 3.5 kg at birth, Fig. 1E center and right), were born following a natural delivery (151-day gestation). The coat color was similar in all three kids and reflected the phenotype of the BC6 transgenic buck.

All pregnancies that reached 60 days were carried to term, and no postpartum morbidity was noted. No term pregnancy was produced in this study with embryos generated from oocytes activated with ethanol.

Genotyping of cloned offspring. Genomic DNA was isolated from blood and ear tissue of the cloned animals and compared with genomic DNA samples isolated from the recipient does (1034 and 1169) and from the transgenic CFF6 donor somatic cell line. Southern blot analysis (Fig. 2F) demonstrated the presence and integrity of the BC6 transgene. Hybridization to a diagnostic 4.1 kb *Eco*RI fragment was detected for all three cloned animals, the CFF6 cell line, and a transgenic positive control (BC6 buck), but not for the two recipient does. As expected, cross-hybridization of the hAT cDNA probe to the endogenous goat AT locus caused a 14 kb band to be detected in all samples.

Analysis of blood cultures from each transgenic kid with FISH probes for the BC6 transgene showed that all three carry a chromosome 5 (C5) transgene integration identical to that found in metaphase spreads derived from the CFF6 cell line (Fig. 2A–D). Moreover, analysis of more than 75 metaphase spreads for each cloned offspring confirmed that they are not mosaic for the C5 transgenic integration.

Table 2. rhAT expression in CFF6-1 goat milk from an induced lactation.

CFF6-1 induced lactation day	hAT concentration (g/liter)	h AT activity (U/ml)
Day 3	4.7	N/D
Day 5	5.8	20.5
Day 6	5.0	18.3
Day 7	4.7	15.6
Day 8	4.1	14.4
Day 9	3.7	14.6

N/D: not determined.

As final confirmation that all three kids are derived from the transgenic CFF6 cell line, PCR-RFLP analysis for the very polymorphic[18] major histocompatibility complex (MHC) class II *DRB* gene was undertaken (Fig. 2E). As illustrated by the *Rsa*I digests of the *DRB* gene second exon, the three cloned females are identical to each other and identical to the CFF6 donor cell line, whereas the recipient does carry different alleles.

Expression of rhAT. At two months-of-age, the first cloned goat CFF6-1 was subjected to a two-week hormonal lactation-induction

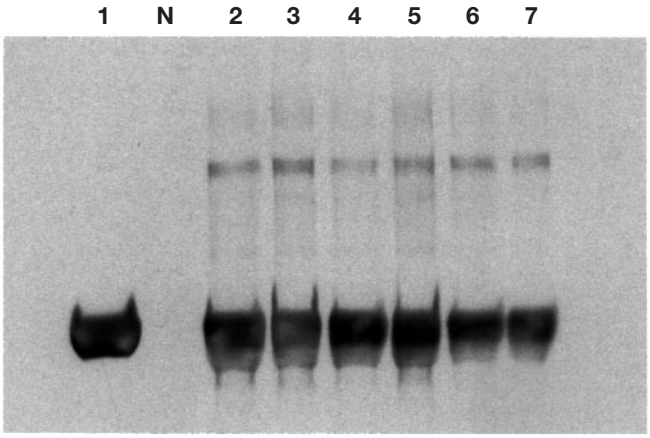

Figure 3. Western blot of rhAT expressed in the milk of the cloned transgenic doe CFF6-1. Five micrograms of protein were applied to the standard hAT lane. The Western blot was developed with a sheep anti-hAT HRP conjugated antibody (SeroTEC) and color development was performed using the ECL system (Amersham). Lane 1: purified hAT standard (5 µg), Lane N: negative goat milk (10 µl), Lanes 2 to 7: samples (9–13 µl) from lactation day 3, 5, 6, 7, 8 and 9, respectively.

protocol. At the end of treatment, milk samples (0.5–10 ml) were collected daily for 33 days. The volumes had increased to 10 ml/day by the time lactation was stopped, with a total of 159 ml produced over the 33 days of milking. The concentration and activity of hAT in representative samples was evaluated (Table 2). As had been noted[3] with does from this specific BC6 transgenic line, high-level expression of the rhAT was detected by western blot analysis (Fig. 3). The concentration of rhAT in the milk of the CFF6-1 animal (Table 2) was 5.8 g/L (20.5 U/ml) at day 5, and 3.7 g/L (14.6 U/ml) by day 9.

Discussion

Three cloned transgenic goats were generated by nuclear transfer of fetal caprine somatic cells to in vivo-derived caprine oocytes. All cloned offspring born in this study were healthy with birthweights within the normal range for their breed. This contrasts with the perinatal morbidity/mortality observed with other demonstrations of cultured cell nuclear transfers in ovine[10–13] and bovine[14,15,17] systems. It is not clear whether this is due to a lesser susceptibility of caprine embryos to as yet ill-defined perturbations caused by the nuclear transfer process, in vivo sourcing of mature oocytes, or a reflection of the relatively low number of clones produced in the caprine nuclear transfer system. It is also noteworthy that, in this program, embryos were transferred to the oviduct of recipient does at the two- or four-cell stage, with minimal in vitro culture.

In this study, nonviable early pregnancies were found in two-thirds of the embryo transfers performed irrespective of the nuclear transfer protocol that was used. Vesicle-like structures were detected by ultrasonography at 25 days post-transfer, and these "embryonic" structures had been resorbed by 55 days post-transfer. It is possible that the vesicular structures were a result of aberrant placental development and were of trophoblast origin. This may reflect the failure of blastocyst differentiation and inner cell mass development as a consequence of faulty genome activation. Alternatively, some of the vesicular structures may have resulted from the transfer of parthenogenetic, aneuploid, or polyploid embryos, an artifact of incomplete enucleation of recipient oocytes.

Nuclear transfer was performed on oocytes that were enucleated either at the arrested metaphase II stage or the activated telophase II stage. Subsequent studies of the cell cycle status of fetal caprine somatic cells has suggested that the starved cells were likely at the G0/G1 transition following restoration of 10% serum. Immunofluorescence screening revealed that after seven days of serum starvation, fetal somatic cells were negative for G1-stage cyclins D1, D2, D3, and proliferating cell nuclear antigen (PCNA).

Previous successful efforts to produce cloned animals by somatic cell nuclear transfer have used either quiescent (G0) donor karyoplasts[11—13,16,17] or actively dividing (G1) donor karyoplast cells[14,15] with arrested metaphase II oocyte-derived cytoplasts. The use of an activated telophase II cytoplast may have several practical and biological advantages. Enucleation of telophase II oocytes is technically easier and removes considerably fewer cytoplasmic factors and organelles. Moreover, telophase II enucleation provides a synchronous population of activated recipient cytoplasts that, when used for embryo reconstruction, show a higher rate of embryonic development in vitro (ref. 19 and the present study).

The ability to use preactivated oocytes could be important in the context of a commercial operation involved in the generation of transgenic animals for the production of therapeutic proteins. Regulatory guidelines recommend the use of closed herds of well-characterized scrapie-free goats as embryo donors and recipients. This then requires an efficient use of such limited valuable animals as donors and recipients. It should not be overlooked that gonadotropin-treated donors most frequently yield heterogeneous populations of in vivo-matured oocytes. The option of using both metaphase II and telophase II-stage oocytes for nuclear transfer will permit a more efficient use of donor goat herds.

Oocytes that were activated by ethanol treatment did not give rise to term pregnancies, in spite of a good cleavage rate (56%) following reconstruction. It could be that these oocytes were developmentally compromised from the beginning, that the ethanol treatment was too harsh and caused irreversible damage to the embryo, or merely that a low number of transfers was performed using the Telophase-II-EtOH protocol.

Our data suggest that nuclear transfer of transgenic somatic cells is at least as efficient as microinjection for generating transgenic animals. Overall, 2.6% of the nuclear transfer embryos transferred to recipients (3.5% if one does not consider the ethanol activation protocol) gave rise to nonmosaic transgenic offspring. This is in comparison with 0.5–3% of the embryos transferred in a typical caprine microinjection program[5] (data not shown). However, in a founder production program, nontransgenic fetal somatic cell lines would need to be transfected with appropriate transgenes and clonal selection applied. Although it is obvious that caprine fetal somatic cell cultures have a finite life span, it has been possible to transfect and select these cells before the onset of senescence (data not shown). Work is ongoing to verify that transfected caprine cell lines can efficiently serve as karyoplast donors for nuclear transfer. Previous work has demonstrated that transfected bovine and ovine primary fibroblasts are capable of producing cloned transgenic animals[13,14].

The generation of transgenic animals that have completely identical genetic backgrounds enhances the possibility of studying the expression and secretion characteristics of recombinant proteins by the mammary gland. For example, the availability of several completely identical transgenic females producing rhAT will help determine the extent of variation in the carbohydrate structure of this protein, as it is produced by the mammary gland. Thus, it may be feasible either to improve the characteristics of the recombinant proteins produced in the transgenic animal system by varying environmental factors such as nutrition, or to increase the milk yield from lactation-induction protocols to diminish further the time necessary to obtain adequate amounts of recombinant protein for preclinical or clinical programs.

The high-level expression of rhAT detected in the milk of the CFF6-1 cloned goat illustrates one of the most important aspects of

this technology. By combining nuclear transfer with lactation-induction in prepubertal goats, it may be possible to characterize transgenic animals and the proteins they secrete in eight to nine months from the time of cell line transfection to milk expression. The amount of milk collected in an induced lactation is not only sufficient to evaluate the recombinant protein yield, but when expression levels in milligrams per milliliter are obtained, is adequate for such qualitative analyses as glycosylation, preliminary pharmacokinetics, and biological and pharmacological activities. The continued availability of the transfected donor cell line also insures that genetically identical animals can be quickly produced, in order to generate a ready supply of therapeutic proteins (with predictable characteristics) for clinical trials.

Experimental protocol

Goats. The herds of pure- and mixed-breed, scrapie-free, Alpine, Saanen, and Toggenburg dairy goats used for this study were maintained under Good Agricultural Practice (GAP) guidelines at the Genzyme Transgenics Farm in Massachusetts.

Isolation of caprine fetal somatic cell lines. Six primary caprine fetal fibroblast cell lines to be used as karyoplast donors were derived from 35- and 40-day fetuses produced by artificially inseminating two nontransgenic does with fresh-collected semen from the transgenic BC6 founder buck[3]. Fetuses were surgically removed and placed in equilibrated phosphate-buffered saline (PBS, Ca^{2+}/Mg^{2+}–free). Single-cell suspensions were prepared by mincing fetal tissue exposed to 0.025% trypsin, 0.5 mM EDTA at 38 °C for 10 min. Cells were washed with fetal cell medium (equilibrated Medium 199 [M199]; Gibco, Gaithersburg, MD) with 10% FBS supplemented with nucleosides, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, and 1% penicillin/streptomycin (10,000 IU/ml each), and were cultured in 25 cm^2 flasks. A confluent monolayer of primary fetal cells was harvested by trypsinization after four days of incubation and then maintained in culture or cryopreserved.

Sexing and genotyping of donor cell lines. Genomic DNA was isolated from fetal tissue[20] and analyzed by PCR for the presence of hAT sequences, as well as, for sequences useful for sexing. The hAT sequence was detected by amplification of a 367-bp sequence with oligonucleotides GTC 11 and GTC 12. Sexing was performed[21] using a zfX/zfY primer pair and *Sac*I restriction enzyme digest of the amplified fragments. Oligonucleotide sequences are, for GTC 11, 5′-CTCCATCAGTTGCTGGAGGGTGTCATTA-3′; for GTC 12, 5′-GAAGGTTTATCTTTTGTCCTTGCTGCTCA-3′; for zfX, 5′-ATAATCACATGGAGAGCCACAAGC-3′; for zfY, 5′-GCACTTCTTTGGTATCTGAGAAAG-3′.

Preparation of donor cells for embryo reconstruction. The transgenic female line (CFF6) was used for all nuclear transfer procedures. Fetal somatic cells were seeded in four-well plates with fetal cell medium and maintained in culture (5% CO_2, 39°C). After 48 h, the medium was replaced with fresh low-serum (0.5% FBS) fetal cell medium. The culture medium was replaced with low-serum fetal cell medium every 48–72 h over the next seven days. On the seventh day following the first addition of low-serum medium, somatic cells (to be used as karyoplast donors) were harvested by trypsinization. The cells were resuspended in equilibrated M199 with 10% FBS supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (10,000 IU/ml each) 1–3 h before fusion to the enucleated oocytes.

Oocyte collection. Oocyte donor does were synchronized and superovulated as described[22] and were mated to vasectomized males over a 48 h interval. After collection, oocytes were cultured in equilibrated M199 with 10% FBS supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin (10,000 IU/ml each).

Cytoplast preparation and enucleation. Oocytes with attached cumulus cells were discarded. Cumulus-free oocytes were divided into two groups: arrested metaphase II (one polar body) and telophase II protocols (no clearly visible polar body or presence of a partially extruding second polar body). The oocytes in the arrested metaphase II protocol were enucleated first. The oocytes allocated to the activated telophase II protocols were prepared by culturing for 2–4 h in M199/10% FBS. After this period, all activated oocytes (criterion: presence of a partially extruded second polar body) were grouped as culture-induced, calcium-activated telophase II oocytes (Telophase-II-Ca) and enucleated. Oocytes that had not activated during the culture period were subsequently incubated 5 min in M199, 10% FBS containing 7% ethanol to induce activation[19], and then cultured in M199 with 10% FBS for an additional 3 h to reach telophase II (Telophase-II-EtOH protocol). All oocytes were treated with cytochalasin-B (5 µg/ml in M199 with 10% FBS; Sigma, St. Louis, MO) 15–30 min before enucleation. Metaphase II-stage oocytes were enucleated with a 25–30 µm glass pipette by aspirating the first polar body and adjacent cytoplasm surrounding the polar body (~30% of the cytoplasm) to remove the metaphase plate. Telophase-II-Ca and Telophase-II-EtOH oocytes were enucleated by removing the first polar body and the surrounding cytoplasm (10–30% of cytoplasm) containing the partially extruding second polar body. After enucleation, all oocytes were immediately reconstructed.

Nuclear transfer. Donor cell injection was conducted in the same medium used for oocyte enucleation. One donor cell was placed between the zona pellucida and the ooplasmic membrane using a glass pipette. The cell–oocyte couplets were incubated in M199 for 30–60 min before electrofusion and activation procedures. Reconstructed oocytes were equilibrated in fusion medium (300 mM mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, 1 mM K_2HPO_4, 0.1 mM glutathione, and 0.1 mg/ml bovine serum albumin) for 2 min. Electrofusion and activation were conducted at room temperature in a fusion chamber with two stainless-steel electrodes (500 µm gap; BTX-Genetronics, San Diego, CA) filled with fusion medium. Fusion and activation were simultaneously induced with an initial alignment/holding pulse of 5–10 V a.c. for 7 s, followed by a fusion pulse of 1.4–1.8 kV/cm d.c. for 70 µs using an Electrocell Manipulator 200 (BTX-Genetronics). Reconstructed embryos were washed in fusion medium for 3 min, then incubated at 39°C in M199 containing 5 µg/ml cytochalasin-B and 10% FBS for 1 h before culture.

Nuclear transfer embryo culture and transfer to recipients. All nuclear transfer embryos were cocultured on monolayers of primary goat oviduct epithelial cells in 50 µl droplets of M199 with 10% FBS overlaid with mineral oil. Embryo cultures were maintained in a humidified 39°C incubator with 5% CO_2 for 48 h before transfer of the embryos to recipient does. Recipient embryo transfer was performed as described[22].

Pregnancy and perinatal care. Pregnancy was determined by ultrasonography starting on day 25 after the first day of standing estrus. Does were evaluated weekly until day 75 of gestation, and once a month thereafter to assess fetal viability. For the pregnancy that continued beyond 152 days, parturition was induced with 5 mg of prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) (Lutalyse; Pharmacia & Upjohn, London). Parturition occurred within 24 h after treatment. Kids were removed from the dam immediately after birth, and received heat-treated colostrum within 1 h after delivery.

Genotyping of cloned animals. Shortly after birth, blood samples and ear skin biopsy specimens were obtained from the cloned female goats and the surrogate dams for genomic DNA isolation[20]. Each sample was first analyzed by PCR using hAT-specific primers, and then subjected to Southern blot analysis using the hAT cDNA[3]. For each sample, 5 µg of genomic DNA were digested with *Eco*RI (New England Biolabs, Beverly, MA), electrophoresed in 0.7% agarose gels (SeaKem; FMC BioProducts, Rockland, ME), and immobilized on nylon membranes (MagnaGraph; MSI, Westboro, MA) by capillary transfer following standard procedures[23]. Membranes were probed with the 1.5 kb *Xho*I to *Sal*I hAT cDNA fragment labeled with α-^{32}P-dCTP using the Prime-It kit (Stratagene, La Jolla, CA). Hybridization[24] was executed at 65°C overnight. The blot was washed with 0.2× SSC , 0.1% SDS, and exposed to X-OMAT AR (Kodak, Rochester, NY) film for 48 h.

PCR-RFLP typing. Typing for the second exon of the caprine MHC class II *DRB* gene was performed as described[25]. A 15 µl aliquot of nested PCR product was digested with 20 units of *Rsa*I (New England Biolabs, Beverly, MA). Following digestion, restriction fragments were separated at room temperature in a 4–20% nondenaturing polyacrylamide gel (MVP precast gel; Stratagene) in the presence of ethidium bromide.

FISH analysis. For typing of the cloned goats, whole blood was cultured for lymphocytes harvest[26]. Fibroblast cells and lymphocytes were pretreated[27] and hybridized[28], as described. A digoxygenin-labeled probe containing the entire 14.7-kb BC6 transgene was used in this procedure. The TSA-Direct system (NEN Life Science Products, Boston, MA) was used to amplify the signal. R-bands were visualized using DAPI counterstain and identified as in ref. 29. A Zeiss Axioskop microscope mounted with a Hamamatsu digital camera was used with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) to capture and process images.

Milk protein analyses. Hormonal induction of lactation[30] for the CFF6-1 female was performed at two months-of-age. The CFF6-1 kid was hand-milked once daily to collect milk samples for hAT expression analyses. Western blot and rhAT activity analyses were performed, as described[3].

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