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Production of Cloned Cattle from In Vitro Systems

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ABSTRACT

The pregnancy initiation and maintenance rates of nuclear transfer embryos produced from several bovine cell types were measured to determine which cell types produced healthy calves and had growth characteristics that would allow for genetic manipulation. Considerable variability between cell types from one animal and the same cell type from different animals was observed. In general, cultured fetal cells performed better with respect to pregnancy initiation and calving than adult cells with the exception of cumulous cells, which produced the highest overall pregnancy and calving rates. The cell type that combined relatively high pregnancy initiation and calving rates with growth characteristics that allowed for extended proliferation in culture were fetal genital ridge (GR) cells. Cultured GR cells used in nuclear transfer and embryo transfer initiated pregnancies in 40% of recipient heifers (197), and of all recipients that received nuclear transfer embryos, 9% produced live calves. Cultured GR cells doubled as many as 85 times overall and up to 75 times after dilution to single-cell culture. A comparison between transfected and nontransfected cells showed that transfected cells had lower pregnancy initiation (22% versus 32%) and calving (3.4% versus 8.9%) rates.

embryo, in vitro fertilization, oocyte development

INTRODUCTION

Nuclear transfer has been used to clone several mammalian species including sheep, cattle, goats, mice, and pigs [1]. One of the primary goals of established cloning programs is to improve the efficiency of initiating and maintaining pregnancies and reduce the incidence of prenatal and postnatal abnormalities. Considerable effort is also directed toward identifying nuclear transfer (NT) donor cell types and donor cell culture conditions that support genetic modification of the cells prior to NT.

The first cells used to produce mammalian clones were derived from preimplantation embryos [2–7]. However, the low number of cells that could be obtained from these early embryos precluded their use in large-scale cloning efforts [8, 9]. Expansion of embryonic stem (ES) cells in culture, particularly inner mass cells, was pursued as a means of generating a large number of cells that could be genetically altered [10–12]. However, early experiments using ES cells in NT failed at producing cloned animals [10, 11]. In addition, unlike certain lines of mouse ES cells, ES cells from other species such as cattle and pig could not be easily propagated in a manner that allowed for the isolation of transgenic cell lines [11, 13, 14]. A major advance in large-scale cloning was achieved when cultured embryonic cells were used to successfully clone sheep [15]. However, worldwide attention to cloning occurred only after cells from an adult animal, mammary epithelial cells from a ewe, were used to clone ''Dolly'' [16].

Several groups have expanded the use of nonembryonic cells in nuclear transfer to clone goats, mice, cattle, and pigs [17–28]. Several cell types, including adult fibroblasts, fetal fibroblasts, and adult cumulous or granulosa cells have been used to produce these clones. Recent reports have also demonstrated the successful cloning of mice from ES cells [26]. Mouse ES cells are attractive because certain strains of these cells are amenable to transgene integration by homologous recombination. However, since ES cells from other species, particularly cattle and pig, have not been successfully targeted by homologous recombination, other cell types with properties similar to ES cells were sought. Embryonic germ cells derived from fetal genital ridges were studied because they contributed to chimeric animals when injected into developing embryos and their growth characteristics allowed for transgene incorporation in vitro [29–36]. Another cell type that has been successfully targeted is fetal ovine fibroblasts [22]. In contrast, successful targeting of cumulous or granulosa cells has not been reported, probably because the culture lifetime of these cells is limited.

The goal of the studies reported here was to compare the pregnancy initiation and maintenance rates of NT embryos produced from several bovine cell types and to determine which of these cell types produce healthy calves and have growth characteristics that would allow for genetic manipulation prior to NT.

MATERIALS AND METHODS

Isolation and Culture of Genital Ridge Cells

Genital ridges were aseptically removed from bovine fetuses of age 40–80 days. The genital ridges were minced with surgical blades in 1 ml of Tyrode Lactate Hepes (TL-Hepes) medium (Biowhittaker, Inc., Walkersville, MD) containing protease from *Streptomyces griseus* (Sigma, St. Louis, MO; cat. P6991) (3 mg/ml) and incubated at 37°C for 45 min. The minced genital ridges were disaggregated by passing them through a 25-gauge needle several times. The disaggregated genital ridges were diluted with 10 ml of TL-Hepes medium and centrifuged at 300 × *g* for 10 min. A portion of the pellet corresponding to 50 000–100 000 cells was cultured on a mitotically inactivated mouse feeder cell layer (35-mm-diameter culture dish) (see below) in α-MEM (Biowhittaker, Inc.) containing 0.1 mM 2-mercaptoethanol, 4 mM L-glutamine, 100 ng/ml of recombinant human leukemia factor (rhLIF) (R&D System, Inc., Minneapolis, MN), 100 ng/ml bovine basic fibroblast growth factor (bFGF) (R&D System, Inc.), and 10% fetal bovine serum (FBS; HyClone, Logan, UT). All cultured cells were kept in an atmosphere of humidified air/5% CO_2 at 37°C. After 5–7 days in culture, the rhLIF and bFGF concentrations were reduced to 40 ng/ml. After 9–12 days in culture, rhLIF and bFGF were removed from

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the medium. Upon reaching confluence, the cells were passaged using standard procedures [37].

In one experiment, primordial germ cells (PGCs) were isolated from the genital ridge (GR) digest based on their characteristic pseudopodia [33]. Approximately 40%–60% of the cells in the digest displayed this morphology. The cells were drawn into a glass pipette (30-μm inner diameter) attached to a micromanipulation station. Approximately 100 cells were cultured on a mouse feeder cell layer as described above, and another 100 cells were cultured in the same conditions except that neither rhLIF nor bFGF was added to the culture medium.

Isolation and Culture of Cells from Fetal Body Tissue

Fetal bovine tissue corresponding to the outer part of the upper body minus the head and viscera was minced with scalpel blades and then digested in 5 ml of a trypsin-EDTA phosphate-buffered saline (Gibco, Rockville, MD) solution for 45 min at 37°C. The digest was filtered through a 70-μm-mesh cell strainer, and the effluent was centrifuged at 300 × *g* for 10 min. A portion of the pellet corresponding to 50 000–100 000 cells was cultured in 35-mm culture dishes in α-MEM containing 0.1 mM 2-mercaptoethanol, 4 mM L-glutamine, and 10% FBS. The cells were passaged upon confluence. Fibroblast-like cells dominated most cultures of fetal body cells. However, fetal body cell cultures occasionally became dominated with cells that resembled epithelial-like GR cells cultured on mouse feeder layers.

Isolation and Culture of Cells from Bovine Ear Tissue

Small portions of the ear were aseptically removed and washed several times in phosphate buffered saline (PBS). The ear samples were minced with scalpel blades and then digested in 5 ml of a trypsin-EDTA phosphate-buffered saline solution for 45 min at 37°C. The digest was filtered through a 70-μm-mesh cell strainer and the effluent was centrifuged at 300 × *g* for 10 min. The pellet was resuspended and cultured in 35-mm culture dishes in α-MEM containing 0.1 mM 2-mercaptoethanol, 4 mM L-glutamine, and 10% fetal bovine serum. The cells were passaged upon confluence.

Feeder Cell Layer Preparation

A feeder cell layer was prepared from mouse fetuses that were between 15 and 20 days gestation. The head, liver, heart, and alimentary tract were removed, and the remaining tissue was washed and incubated at 37°C in trypsin-EDTA phosphate-buffered saline solution. Cells dissociated from the tissue were cultured in supplemented α-MEM without rhLIF or bFGF but containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). The mouse feeder cells were cultured until confluent and then treated with mitomycin C (10 μg/ml; Calbiochem, La Jolla, CA) for 3 h to block mitosis. The mitotically inactivated feeder cells were cultured for 5–10 days before use.

Transfection Methods

Multiple methods of transfection were used to optimize both for the type of cell used and the design of DNA construct. The methods used were electroporation [38] and commercial reagents such as Lipofectamine (Gibco 8324SA), SuperFect (Qiagen, Valencia, CA; 301305), Effectene (Qiagen 301425), CLONfectin (Clontech, Palo Alto, CA; 8020-1), CalPhos (Clontech K2051-1), pGeneGrip (GTS, Inc., San Diego, CA; G10100K), LipoTAXI (Stratagene, La Jolla, CA; 204110), and TransIT (Mirus, Madison, WI; MIR2500). The manufacturers' recommended transfection protocols were typically used. Most of the DNA constructs contained the aminoglycoside phosphotransferase gene to confer resistance to the neomycin derivative G418 (Gibco 10131-035), and selection was maintained for 12–16 days (600 μM G418) with 0–1 passages.

Nuclear Transfer

Oocytes aspirated from abattoir ovaries were matured overnight in maturation medium (medium 199; Biowhittaker) supplemented with luteinizing hormone (10 IU/ml; Sigma), estradiol (1 mg/ml; Sigma), and FBS (10%; Hyclone, Logan, UT) at 38.5°C in a humidified 5% CO_2 incubator. Typically, after 16–17 h in maturation medium, the cumulous cell layer had expanded and the first polar bodies had extruded in approximately 70% of the oocytes (referred to as young oocytes). The oocytes were stripped of cumulous cells by vortexing in 0.5 ml of TL-Hepes. The chromatin was stained with Hoechst 33342 (5 μg/ml; Sigma) in TL-Hepes solution supplemented with cytochalasin B (7 μg/ml; Sigma) for 15 min. Stained oocytes were enucleated in drops of TL-Hepes solution under mineral oil. Cells used in the NT procedure were prepared by removing a group of confluent cells from the culture dishes using a pipette tip. The isolated cells were incubated in a TL-Hepes solution containing 3 mg/ml *S. griseus* protease at 32°C for approximately 1 h. Once the cells were in a single cell suspension, they were washed with TL-Hepes and used for NT within 2–3 h. Single nuclear donor cells of optimal size (12–15 μm) were inserted into the perivitelline space of the enucleated oocyte. The cell and oocyte plasma membranes were fused by applying an electrical pulse of 90 V for 15 μsec in an isotonic sorbitol solution (0.25 M) containing calcium acetate (0.1 mM), magnesium acetate (0.5 mM), and fatty acid-free bovine serum albumin (BSA) (1 mg/ml; Sigma #A7030) (pH 7.2) at 30°C in a 500-μm chamber. Following 4 h of culture in CR1aa (CR2) medium [39] containing 3 mg/ml BSA, the NT embryos were activated by a 4-min exposure to 5 μM ionomycin (Ca^{2+} salt) (Sigma) in hamster embryo culture medium (HECM) [40, 41] containing 1 mg/ml BSA, followed by a 5-min wash in HECM containing 30 mg/ml BSA. The activated embryos were then incubated in CR2 medium containing 1.9 mM 6-dimethylaminopurine (DMAP; Sigma) for 4 h followed by a wash in HECM and subsequently cultured in CR2 medium with BSA (3 mg/ml) at 38.5°C in a humidified 5% CO_2 incubator for 3 days. The embryos were transferred to CR2 medium containing 10% FBS and cultured for an additional 1–4 days.

Second Nuclear Transfer (Recloning)

Morula stage NT embryos (generally after 4 days in culture) were disaggregated by treatment with cytochalasin B (7.5 μg/ml) in TL-Hepes for 20 min. Single blastomeres were placed into the perivitelline space of enucleated aged oocytes. Aged oocytes were produced by incubating matured young oocytes for an additional time in maturation medium (typically 40–44 h total). The blastomere was fused with the enucleated oocyte via electrofusion in a 500-μm chamber with an electrical pulse of 105 V for 15 μsec. Electrofusion of the aged oocytes with the nuclear donor blastomeres simultaneously activated the oocytes. After blastomere-oocyte fusion, the embryos from the second NT were cultured in CR2 medium supplemented with BSA (3 mg/ml) for 3 days. The embryos were cultured for an additional 4 days in CR2 medium containing 10% FBS. Good quality embryos as determined by morphology were nonsurgically transferred into recipients.

Embryo Transfer

Grade 1 or 2 blastocysts [42] were used for transfer into recipients (1–3 embryos/recipient). Recipients were observed for natural estrus, and blastocysts were transferred into recipients whose predicted ovulation had occurred within 48 h of the time that the nuclear donor cells were fused into the enucleated oocytes. Transfers occurred 5–8 days postfusion.

Parentage Analysis

Parentage of the cloned calves was verified by comparing 20 DNA microsatellite markers from the donor cell line and the recipient with those from the cloned calf [43].

RESULTS

Cell Culture

Genital ridge digests from 40- to 80-day-old bovine fetuses formed small colonies of epithelial-like cells after 3–5 days when cultured on mitotically inactivated mouse feeder cells (Fig. 1A). The size and rate of appearance of these colonies were greater when the growth factors rhLIF and bFGF were included in the medium. Cell shape was irregular (10- to 25-μm horizontal dimensions), but typically the cells did not become elongated. After 7–12 days in culture, the cells became confluent, and thereafter proliferation appeared to slow based on the lack of cell multilayer formation (Fig. 1B). Proliferation rates increased after the first passage (data not shown). Cells cultured individually by diluting dissociated cells at first passage generally continued to divide and doubled up to 75 times after

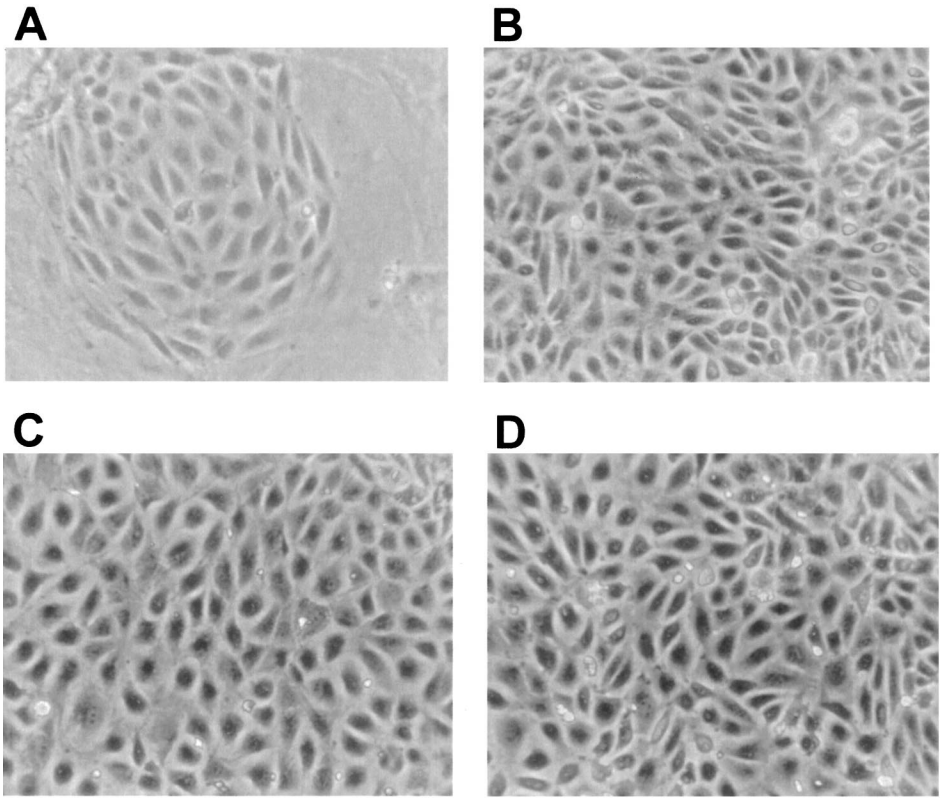

FIG. 1. Genital ridge and epithelial-like body cells used in NT (see text). **A**) A GR cell colony that formed on a feeder cell layer after 4 days. **B**) Confluent GR cells. **C**) Isolated PGCs after 33 days in culture. **D**) Epithelial-like cells from fetal body tissue after 29 days in culture.

isolation (Table 1). After 68 doublings, the cell line BF15c3 maintained a normal karyotype (data not shown).

Genital ridge digests contain several cell types in addition to the characteristic primordial germ cells (PGCs). We manually selected cells that matched the morphology of PGCs [33] and cultured them on feeder cell layers with and without rhLIF and bFGF. Colonies that developed from the presumptive PGCs (Fig. 1C) were similar to those derived from complete GR digests, although their appearance was delayed (>14 days), particularly in the absence of rhLIF and bFGF.

Cells cultured from fetal body digests generally had a fibroblast-like morphology but on occasion developed an epithelial-like morphology similar to cells derived from GR digests (Fig. 1D). These epithelial-like cells eventually dominated the culture.

Nuclear/Embryo Transfer

The first cloned calf produced using nonembryonic cells was a bull named Gene born 6 February 1997. Gene was the result of a NT step using a GR cell (BF5) followed by a recloning step where a blastomere from the first embryo was transferred to an aged enucleated oocyte [44] (see accompanying article [23]). The oocyte used in the first NT step was 20 h postinitiation of maturation at the time of fusion, while the age of the second oocyte was 44 h postinitiation of maturation at fusion. The BF5 NT embryos were transferred into 41 recipients (1–3 embryos/recipient), of which 5 became pregnant (12%). Two pregnant recipients aborted prior to 30 days of gestation, and one pregnancy was voluntarily terminated at 203 days due to hydramnios or hydrallantois. Twin calves in one recipient died at birth due to recipient ketosis, and the fifth pregnant recipient gave birth to Gene (by cesarean section), for an overall calving rate of 5% of all recipients receiving NT embryos.

In general, using young rather than aged oocytes for the first NT step yielded better development to blastocyst. In one experiment, 34/187 (18%) of the oocytes matured for 20 h developed to blastocysts when genital ridge cells were used in NT, but when blastomeres from NT embryos were used, only 2/75 (3%) developed to blastocysts. In contrast, oocytes matured for 44 h yielded 0/77 (0%) to blastocyst when genital ridge cells were used in NT but gave 37/215 (17%) to blastocysts when blastomeres from NT embryos were used. However, the second NT step using blastomeres was generally not used in subsequent experiments because it was found that pregnancy initiation and maintenance rates were similar for both the one-step and two-step NT procedures (data not shown).

The rate of NT embryo development to blastocyst and the results of embryo transfer into recipients are shown in Tables 2–4. These data are from single-step NT embryos produced using young oocytes. Table 2 shows the results for specific cell types from one fetus (BF15). Cultured BF15 cells manually selected from GR digests based on morphological characteristics typical of PGCs [33] initiated pregnancies at a relatively high rate whether or not they were cultured with rhLIF and bFGF (Table 2). Although the calving rate for BF15 GR cells cultured with the growth factors was higher, the low number of embryo transfers

TABLE 1. Cell doublings of BF15 GR cells.

Cell line	Total doublings*	Total passages	Days in culture	Average passage interval (days)
BF15c3	85	35	407	11.6
BF15c2	41	14	65	4.6

* Ten doublings occurred prior to isolation of single cells in culture.

TABLE 2. Efficiency of NT and embryo transfer for different BF15 cell types.

	% Useable embryos*	No. of embryo transfers**	No. of pregnancies	% Initiation	No. calved	% Calved*** (per transfer)
BF15-GR + rhLIF and bFGF	22.2	13	8	62	5	42
BF15-GR	26.4	37	20	54	4	11
BF15-body epithelial-like	19.8	11	6	55	4	40
All nontransfected BF15	21.4	128	53	41.4	19	15.2
All transfected BF15	20.1	768	150	19.5	13	1.7

* Not all useable embryos were transferred into recipients.
** Generally, two embryos were transferred into each recipient.
*** One or more pregnancies were intentionally terminated in each group and were not used to calculate calving percentages.

precludes a statistical comparison. BF15 body cells that developed an epithelial-like morphology similar to the GR cells in culture also produced a high initiation and calving rate (Table 2). Overall, nontransfected BF15 cells (all types) initiated pregnancies at a rate of 41% relative to embryo transfer, and 15% of all recipients maintained the pregnancies to term (Table 2). In contrast, transfected BF15 cells had lower rates of pregnancy initiation (19.5%) and calving (1.7%) than nontransfected BF15 cells (Table 2). The variability in performance among different BF15 cell lines was high, particularly for transfected cells.

A summary of results for all transfected and nontransfected cells from 34 different genetic lines is shown in Table 3. The results represent embryo transfers that occurred between 1 January 1 1998 and 29 February 2000. For all nontransfected cells, 8.9% of the recipients calved as compared with 15% for a subset of the nontransfected cells, the BF15 cells (compare Tables 2 and 3). However, transfected cells overall produced a higher calving rate (3.4%) than transfected BF15 cells (1.7%). The low rate of calving using transfected BF15 cells probably reflects the experimental nature of many of the BF15 cell transfections.

A comparison of overall NT embryo transfer efficiency for different nontransfected cell types indicated that adult cumulous cells produced the highest rate of pregnancy initiation (62%) and calving (15%) (Table 4). Fetal GR cells and fetal body cells that had a fibroblast-like morphology were less efficient than adult cumulous cells but performed better than adult ear fibroblast-like cells (Table 4).

Cloning from Cloned Fetuses

To determine if cells from cloned fetuses could themselves produce a second-generation cloned animal, GR cells from BF15 were used to make a 53-day cloned fetus that was collected from the recipient. Body (fibroblast-like) cells from this second fetus were used to make 28 blastocysts (from 151 NT embryos) that were transferred into 14 recipients. These recloned embryos initiated 9 pregnancies (64%), of which 4 went to term (29%) and produced 5 calves (including 1 set of twins).

Cloning Adult Cattle

One advantage of cloning adult animals is that the phenotype of the animal is known prior to cloning, an advantage absent when cloning from fetal cells. Ear cells from a high genetic-value bull were used to make 32 NT blastocysts that were transferred into 17 recipients, of which 10 became pregnant (59%). Five abortions occurred before 60 days of gestation, and 2 pregnancies were terminated due to hydramnios or hydrallantois. Three of these pregnancies (18%) went to term, producing 3 live births. One calf was killed 11 days after birth due to a heart defect (see [23]).

Another cell type that was used to clone adult cows was cumulous cells obtained from in vivo or in vitro matured oocytes. In one case, cumulous cells disassociated from a single in vivo matured oocyte from a 17-yr-old cow were cultured in α-MEM without added growth factors for 20–30 days prior to NT. Two embryos were transferred into each of 13 recipients, and of these recipients, 11 became pregnant (85%). Three of these recipients delivered 3 healthy calves (23%). Eight pregnancies aborted, 5 before 60 days and one each at 5, 7, and 8 mo.

Parentage Analysis of Cloned Cattle

Microsatellite DNA markers on 20 chromosomes were used to analyze DNA extracted from recipient tissue, the nuclear donor cell line, and the calf. For each cloned calf, the recipient cow was excluded as a possible parent to the calf whereas the nuclear donor cell line could not be excluded as the genetic source for the calf. Parentage analysis of Gene's DNA is shown in Figure 2. This figure shows that two microsatellite markers from Gene match those from the donor GR cells and the placenta but not those from the recipient.

DISCUSSION

The commercial usefulness of cattle cloning depends on the efficiency of nuclear transfer, the rate of pregnancy initiation, and the maintenance of pregnancies to term. The success of cattle cloning as described here certainly justifies

TABLE 3. Overall efficiency of NT and embryo transfer using nontransfected and transfected cells.

	% Useable embryos*	No. of embryo transfers**	No. of pregnancies	% Initiation	No. calved	% Calved*** (per transfer)
Total nontransfected	18.3	530	169	31.9	47	8.9
Total transfected	22.9	1640	366	22.3	56	3.4
Total	21.7	2170	535	24.7	103	4.7

* Not all useable embryos were transferred into recipients.
** Generally, two embryos were transferred into each recipient.
*** Calculated based on embryo transfers that occurred between 1 January 1998 and 29 February 2000.

TABLE 4. Overall efficiency of NT embryo transfer for different nontransfected cell types.

	No. of embryo transfers*	No. of pregnancies	% Initiation	No. of calves	% Calved** (per transfer)
Nontransfected GR	197	78	39.6	17	9.3
Nontransfected fetal body	48	23	47.9	3	9.1
Nontransfected adult ear	142	48	33.8	7	5.1
Nontransfected adult cumulus	34	21	61.8	5	15.2

* Generally 2 embryos were transferred into each recipient.
** Calculated based on embryo transfers that occurred between 1 January 1998 and 29 February 2000. One or more pregnancies were intentionally terminated in each group and were not used to calculate calving percentages.

its use for the production of protein pharmaceuticals in cloned transgenic cattle. However, broad agricultural applications of cattle cloning beyond the duplication of highly valuable animals will require improvements in pregnancy initiation and maintenance to term. It is important to point out that the data presented here reflect early cloning experiments from 1 January 1998 to 29 February 2000 where many cell types from 34 different founders cultured under a variety of conditions were used. In addition, over 75% of the recipients received NT embryos that were produced from transfected cells, which were generally less successful than nontransfected cells.

Genital ridge cells were selected for NT because previous reports showed that they had properties of pluripotent embryonic stem cells [36]. When cultured on mouse feeder cell layers in the presence or absence of added growth factors (rhLIF and bFGF), cells from GR digests from 40- to 80-day-old fetuses formed expanding epithelial-like colonies of cells. Growth factors increased the proliferation rate of nascent colonies but had no obvious effect on the cells after the first passage. In an early experiment, NT of cultured GR cells derived from an abattoir fetus (BF5) produced morula from which blastomeres were mechanically removed and used in a second NT step. The hypothesis was that the second NT step (recloning) would complete the nuclear reprogramming of the cultured GR cell that began in the first NT step. The recloning procedure produced the first cloned bovine calf from a nonembryonic cell. This bull calf was named Gene (born 6 February 1997). From a historic perspective, Gene's gestation overlapped with Dolly's gestation [16]. Gene has matured into a healthy, fertile bull that has sired calves by artificial insemination and in vitro fertilization (data not shown). The second NT step was subsequently found to be unnecessary to produce cloned calves (see below).

To investigate the totipotency of different cell types, GR and body cells from the same fetus (BF15) were compared for the ability to initiate and maintain pregnancies following a single NT step. The GR cells used in this study were derived from presumptive PGCs cultured in the presence and absence of rhLIF and bFGF. The body cells used were those that exhibited an epithelial-like morphology. All three groups of cells initiated pregnancies at a high rate (54%–61%), although pregnancies to term ranged from 11% to 42% (Table 2). The significance of the calving difference between GR cells cultured in the presence and absence of added growth factors (42% and 11%, respectively) cannot be determined due to the low number of total embryo transfers. The advantage of using cultured GR cells is the ability to double many times when cultured singly in isolation (Table 1). Clonal colonies of transfected GR cells can be expanded to a point where transgene copy number and integration sites can be analyzed by fluorescence in situ hybridization prior to NT (data not shown).

Overall, cells from BF15 that were used in these experiments initiated and maintained pregnancies at a higher rate than the average for all nontransfected cells (Table 3). In general, considerable variability of NT/embryo transfer success was observed among cells from different animals and among different cell types from the same animal. The reasons for this variability are not known and are difficult to study because it is not economically feasible to study several cell lines simultaneously using the same batches of oocytes and recipients.

Transfected cells generally exhibited lower pregnancy initiation and maintenance rates following nuclear transfer/embryo transfer compared with nontransgenic cells (Table 3). However, specific transgenic cell lines occasionally had high success rates (data not shown). Factors that could account for the reduced performance of transgenic cell lines include the age, the number of passages, and the number of cell doublings of transgenic cells, which are generally greater than nontransgenic cells at the time of NT. Drug selection of the cells could also reduce totipotency. Finally, the transgenes themselves may interfere with normal fetal development.

Although recloning of preimplantation embryos appeared to have limited usefulness, recloning existing cloned fetuses or animals has the distinct advantage of reproducing unique genetics. For example, transgenic cell populations produced by random gene insertion exhibit many different transgene integration sites and copy numbers. If these transgenic cell populations are used in NT, any pregnancies that are initiated are likely derived from a small subset of

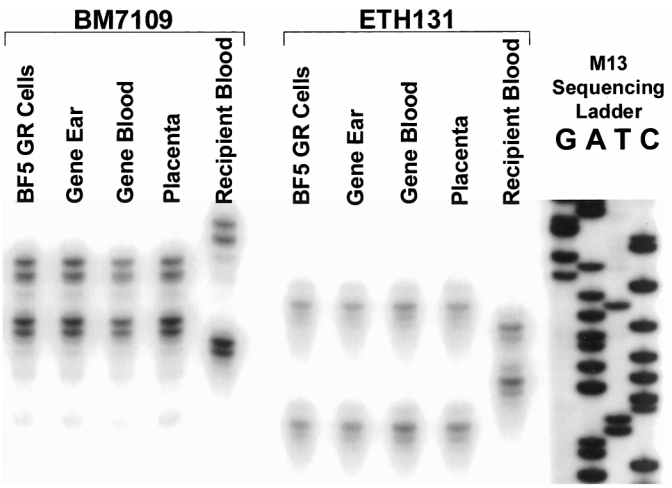

FIG. 2. Analysis of two microsatellite markers. Allele size of bovine DNA microsatellite markers BM7109 (BTA18) and ETH131 (BTA2D) from nuclear donor cells, calf ear, calf blood, placenta, and recipient blood was compared. Analysis of the two microsatellite markers excluded the recipient as a possible parent for the calf Gene.

unique cells that would be difficult to isolate. However, the results presented here demonstrate that, if cells from a NT fetus have desirable transgene characteristics, this cloned fetus can be used in NT to produce healthy calves.

NT using cells from adult cattle was performed to clone cows and bulls of high genetic merit. Cumulous cells from females were chosen because preliminary experiments had shown that NT using cumulous cells yielded a high rate of development to blastocysts after 7 days in culture (data not shown). In one example described here, cumulous cells from a single in vivo matured oocyte from a 17-yr-old cow were used. The high pregnancy initiation (85%) and calving (23%) rates using these cumulous cells reflects an overall high success rate using cumulous cells (Table 4). A disadvantage of cumulous cells is that the limited ability to proliferate in culture makes them refractory to transfection and selection protocols designed to introduce transgenes.

Skin cells are another attractive source of cells for NT since the phenotype of these animals is known. For example, cultured ear cells from a bull of high genetic merit were used in NT and resulted in 3 live calves from 17 recipients. The overall rate of pregnancy initiation and calving using adult fibroblast-like cells was 34% and 5%, respectively (Table 4). These rates were lower than results obtained using fetal fibroblast-like cells (48% for initiation and 9% for calving) (Table 4).

The observation that some cell lines yielded pregnancy initiation rates greater than 60% and calving rates greater than 40% (Table 2; *Results*) indicates that, under the right conditions, cloning can be competitive with other reproductive technologies, including in vitro fertilization, artificial insemination, and embryo transfer. Our goal is to identify conditions that reproducibly yield high cloning efficiencies utilizing a variety of cell types from any genetic background. Many hypotheses to explain the reportedly low efficiency of cloning have been posited, including incomplete reprogramming of donor cell nuclei, improper imprinting, and poor quality oocytes. The common approach to study the causes of low cloning efficiency has been to identify candidate genes or molecular pathways whose disruption in disease or experiments produce abnormalities that resemble those observed in cloning studies. However, because the basic mechanisms of successful cloning are not known, studying candidate pathways that might be causing the low success rate could be fruitless. A more systematic, unbiased approach to studying cloning is needed. The use of DNA microarray technologies may provide a means to compare gene expression patterns in in vivo oocytes, embryos, and fetuses with gene expression patterns in NT oocytes, embryos, and fetuses. In this way, genes and conditions critical in successful cloning can be identified.

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