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Production of Cloned Calves Following Nuclear Transfer with Cultured Adult Mural Granulosa Cells[1]

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ABSTRACT

Adult somatic cell nuclear transfer was used to determine the totipotent potential of cultured mural granulosa cells, obtained from a Friesian dairy cow of high genetic merit. Nuclei were exposed to oocyte cytoplasm for prolonged periods by electrically fusing quiescent cultured cells to enucleated metaphase II cytoplasts 4–6 h before activation (fusion before activation [FBA] treatment). Additionally, some first-generation morulae were recloned by fusing blastomeres to S-phase cytoplasts. A significantly higher proportion of fused embryos developed in vitro to grade 1–2 blastocysts on Day 7 with FBA (27.5 ± 2.5%) than with recloning (13.0 ± 3.6%; $p < 0.05$). After the transfer of 100 blastocysts from the FBA treatment, survival rates on Days 60, 100, 180, and term were 45%, 21%, 17%, and 10%, respectively. Ten heifer calves were delivered by elective cesarean section; all have survived. After the transfer of 16 recloned blastocysts, embryo survival on Day 60 was 38%; however, no fetuses survived to Day 100. DNA analyses confirmed that the calves are all genetically identical to the donor cow. It is suggested that the losses throughout gestation may in part be due to placental dysfunction at specific stages. The next advance in this technology will be to introduce specific genetic modifications of biomedical or agricultural interest.

INTRODUCTION

There is intense scientific interest in the field of somatic cell nuclear transfer, principally to enable both the multiplication of elite livestock and the engineering of transgenic animals, for various agricultural and biomedical purposes. The realization of these applications involves the development of embryo and cell manipulation techniques that facilitate the totipotent potential of cultured and/or differentiated cell nuclei to be expressed.

The successful production of offspring derived after nuclear transfer depends upon a wide variety of factors. One factor of importance is appropriate cell cycle coordination. Although there are no definitive reports comparing the effects of cell cycle synchrony between donor nuclei from cultured somatic cells and recipient cytoplasts, there currently appear to be at least two methods that are successful in yielding viable cloned offspring. The first uses donor cells in a quiescent state, in which the cells are presumed to have exited the normal cell cycle and have arrested in what is termed G0. This may be induced in cultured cells by, for example, serum starvation [1, 2] or by using cells that are naturally arrested in this state directly from the animal [3, 4]. It is possible that the reduction in transcriptional activity and chromatin modification associated with cells in G0 [5] may facilitate the reprogramming of nuclei following exposure to oocyte cytoplasmic factors, enabling normal development to occur in some instances [1, 2]. The failure of earlier studies in amphibians to generate viable clones from adults after the use of G0 cells (e.g., [6–8]) may have been due to other limiting facets of the nuclear transfer technique and/or the greater difficulty (or impossibility) to reprogram the nuclei of some terminally differentiated cell types. The latter phenomenon may be the case with Sertoli and neuronal cells in the mouse [3].

The second method that appears successful in somatic cell nuclear transfer involves exploiting the various factors present in the cytoplasm of the metaphase-arrested oocyte that may facilitate the remodeling and reprogramming of somatic cell nuclei [9]. In addition, prolonged exposure to this cytoplasmic environment may aid this process further and appears to have conferred nuclear totipotency to nonquiescent cells [10]. This prolonged cytoplasmic exposure may be achieved by fusing cells before the activation of the reconstructed embryo [9, 11–13]. However, for it to be successful and to avoid chromosomal damage and abnormal ploidy in the resulting embryos, the cell cycle stage of the donor nucleus must be compatible with the high levels of maturation-promoting factor present in the metaphase II (MII) oocyte [14, 15]. For these reasons, only nuclei that have a diploid (2C) DNA content (that is, in either G1 [16] or G0 of the cell cycle [1–3]), or in metaphase [17]) are compatible with nuclear transplantation to enucleated MII oocytes. Although no effect of prolonged exposure of quiescent cells to the MII cytoplasm has been observed in sheep [1], effects have been seen in other species. With unsynchronized bovine cultures of undifferentiated embryonic cells, in which the majority of cells were reported to be in G1, exposure to MII cytoplasm for 4 h before activation significantly increased development to blastocyst, compared to embryos reconstructed by either simultaneous fusion and activation, or with preactivated cytoplasts [18]. Although fetal development did not proceed beyond Day 55 in this study [18], the strategy of fusion before activation has resulted in viable cloned calves using fetal fibroblasts in another study, in which once again the majority of cells were reported to have been in G1 [10]. With quiescent bovine cells derived from either the fetus [12] or adult [13], embryo development was also significantly increased by prolonging the period of exposure of the nucleus to the MII cytoplasm before activation. Similarly in the mouse, embryo and fetal development were both improved with exposure of cumulus cells in G1/G0 to MII cytoplasm for 1–6 h [3].

Although it is now clearly possible to produce cloned offspring from differentiated mammalian cells after nuclear transfer, the overall success rate is currently low in the above-mentioned studies (0.4–1.8%). Furthermore, in most studies, the exact stage of the cell cycle of the successful donor cells, which ultimately yielded the viable cloned offspring, remains uncertain.

The principal objective of the nuclear transfer studies

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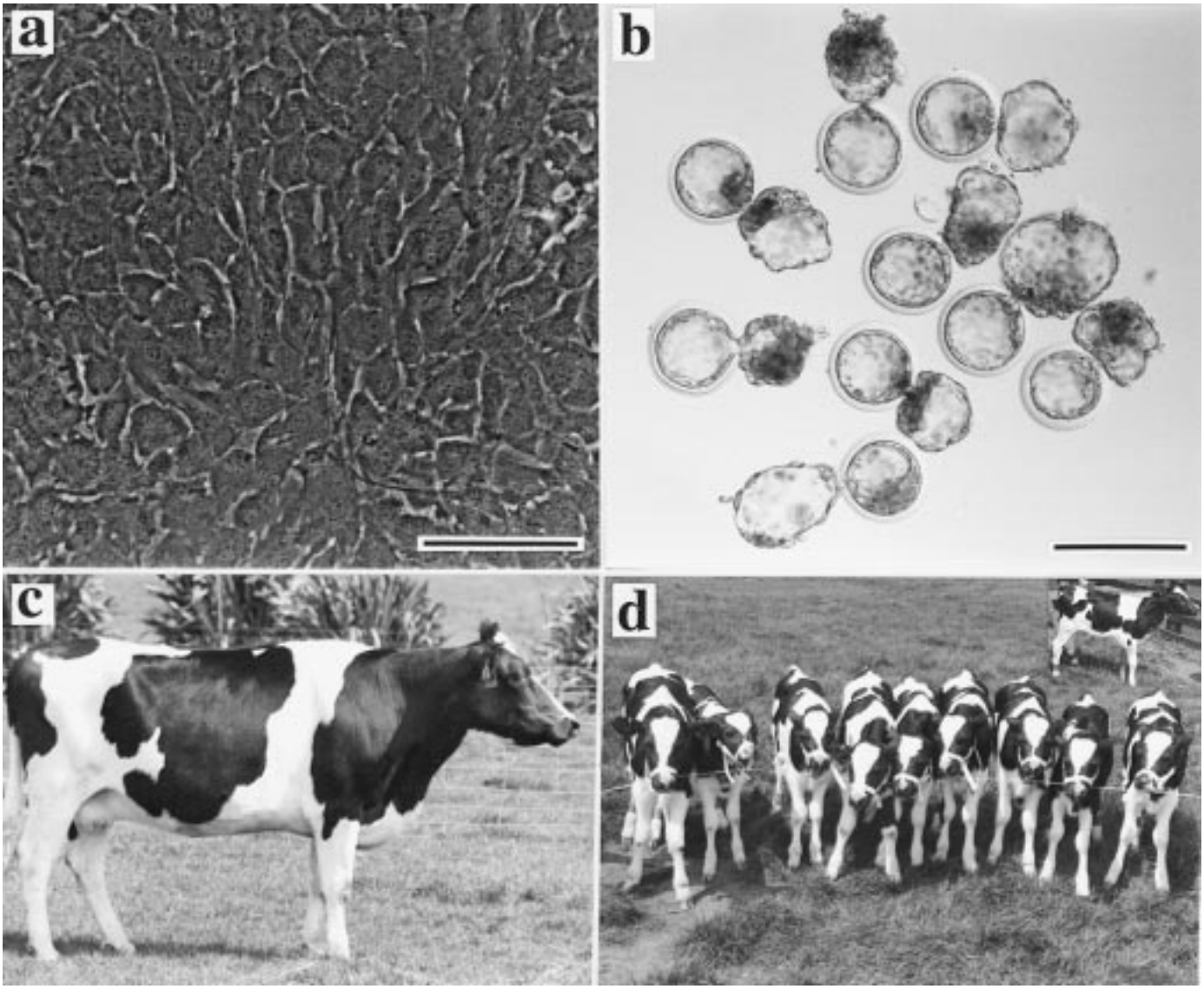

FIG. 1. Demonstration of the totipotency of adult bovine mural granulosa cells after nuclear transfer. **a**) Morphology of the EFC mural granulosa cells at passage five. Bar = 100 μm. **b**) Hatching blastocysts produced seven days postfusion from the FBA treatment with EFC cells. Bar = 200 μm. **c**) The Friesian donor cow. **d**) Ten cloned calves genetically identical to the cow shown in **c.** Insert shows one calf recovering from a broken leg at the time of submission.

described here was to determine the nuclear totipotency of cultured adult bovine mural granulosa cells. We also investigated the effect on embryo and fetal development of prolonged exposure of the transferred nucleus to the oocyte cytoplasm by either fusing quiescent granulosa cells before activation or by recloning the first-generation nuclear transfer embryos. Recloning provides an additional method of allowing a longer period for nuclear reprogramming to occur and has improved development in amphibians [9]. During the course of these experiments, we have established the efficiency of cloning adult females using mural granulosa cells and have begun to identify the important areas of future research needed to improve the success rates beyond the current 10% embryo survival to term reported here.

MATERIALS AND METHODS

Isolation of Mural Granulosa Cells

A primary cell line (EFC) was established from mural granulosa cells collected by aspirating the ovarian antral follicles (3–10 mm in diameter) from a three-year-old Friesian dairy cow of high genetic merit (Fig. 1c), using an ultrasound-guided, transvaginal probe [19]. The collected cells were centrifuged and washed once in culture medium before seeding onto a four-well tissue culture plate (Nunclon, Roskilde, The Netherlands). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium (Life Technologies, Auckland, New Zealand) supplemented with 10% v:v fetal calf serum (FCS; Life Technologies) and sodium pyruvate to a final concentration of 1 mM. During the first seven days, penicillin, streptomycin, and amphotericin B were added. The cell line was routinely passaged using an enzymatic solution (TEG) comprising 0.25% w:v trypsin (porcine pancreas; Life Technologies) and 0.04% w:v EGTA (Sigma, St. Louis, MO) for 7 min at 39°C. Small aliquots of early-passage EFC cells were frozen in 10% dimethyl sulfoxide (BDH, Poole, Dorset, England). The average cell population doubling time was 42.0 ± 1.1 h and the cells were maintained for at least nine passages in culture, representing approximately a total of

15 cell population doublings. The cell morphology is illustrated in Figure 1a. All cells used for nuclear transfer in these experiments had been previously frozen and thawed.

In Vitro Maturation of Oocytes

Slaughterhouse ovaries were collected from mature cows, placed in saline (30°C), and transported within 2 h to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3–6-mm follicles using an 18-gauge needle and negative pressure (40–50 mm Hg). COCs were collected into HEPES-buffered tissue culture medium 199 (H199; Life Technologies) supplemented with 50 μg/ml heparin (Sigma) and 0.4% w:v BSA (Immuno-Chemical Products [ICP], Auckland, New Zealand). Before in vitro maturation, only those COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. They were washed twice in H199 medium + 10% FCS (Life Technologies) before being washed once in bicarbonate-buffered tissue culture medium 199 + 10% FCS. Ten COCs were transferred in 10 μl of this medium and placed into a 40-μl drop of maturation medium in 5-cm Petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). The maturation medium comprised tissue culture medium 199 supplemented with 10% FCS, 10 μg/ml ovine FSH (Ovagen; ICP), 1 μg/ml ovine LH (ICP), 1 μg/ml estradiol (Sigma), and 0.1 mM cysteamine (Sigma) [20]. Microdrop dishes were cultured at 39°C in a humidified 5% CO_2 in air atmosphere for 20 h. After maturation, the cumulus-corona was totally removed by vortexing COCs in 0.1% hyaluronidase (from bovine testis; Sigma) in HEPES-buffered synthetic oviduct fluid (HSOF) [21] for 3 min; this was followed by three washes in HSOF + 10% FCS.

Nuclear Transfer with Granulosa Cells

Media. With embryos that were reconstructed by fusing donor cells and MII cytoplasts before activation (fusion before activation [FBA] treatment), matured oocytes, cytoplasts, and reconstructed embryos were either held or manipulated in HSOF- or SOF-based medium (as appropriate) without calcium for the period following maturation and until 30 min before activation. After this point, calcium was present in all media formulations used.

Enucleation. Oocytes matured for 20–22 h were enucleated with a 30-μm (external diameter) glass pipette, by aspirating the first polar body and the MII plate in a small volume of surrounding cytoplasm. The oocytes were previously stained in HSOF medium containing 10% FCS, 5 μg/ml Hoechst 33342, and 7.5 μg/ml cytochalasin B (Sigma) for 20 min. Enucleation was confirmed by visualizing the karyoplast, while still inside the pipette, under ultraviolet light. After enucleation, the resulting cytoplasts were washed extensively in HSOF + 10% FCS and were held in this medium until injection of donor cells.

Preparation of cells. Donor cells were used for nuclear transfer between passages three and eight of culture. EFC cells were induced to enter a period of quiescence (presumptive G0) by serum deprivation [1]. One day after routine passage, the culture medium was aspirated, and the cells were washed three times with fresh changes of PBS before fresh culture medium containing only 0.5% FCS was added. The cells were returned to culture for a further 8–18 days before they were used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in HSOF + 0.5% FCS and remained in this medium until injection.

Microinjection. Recipient cytoplasts were dehydrated in HSOF containing 10% FCS and 5% sucrose. This medium was also used as the micro-manipulation medium. A 35-μm pipette (external diameter) containing the donor cell was introduced through the same slit in the zona pellucida as made during enucleation, and the cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After injection, the reconstructed embryos were rehydrated in two steps; first in HSOF containing 10% FCS and 2.5% sucrose for 5 min and then in HSOF + 10% FCS until fusion.

Cell fusion. Reconstructed embryos in the FBA group were electrically fused at 24 h poststart of maturation (hpm) in buffer comprising 0.3 M mannitol, 0.5 mM HEPES, and 0.05% fatty acid-free (FAF) BSA (Sigma) with 0.05 mM calcium and 0.1 mM magnesium. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 500 μm apart overlaid with fusion buffer. The reconstructed embryos were manually aligned with a fine, mouth-controlled Pasteur pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 2.25 kV/cm for 15 μsec each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). After the electrical stimulus, the reconstructed embryos were washed in HSOF + 10% FCS. They were then checked for fusion by microscopic examination.

Activation. After fusion, FBA embryos were cultured for a period of 4–6 h in SOF + 10% FCS before chemical activation. Thirty minutes before activation, fused embryos were washed and held in HSOF (containing calcium) + 1 mg/ml FAF BSA. Activation was induced by incubation in 30-μl drops of 5 μM ionomycin (Sigma) in HSOF + 1 mg/ml FAF BSA for 4 min at 37°C. Embryos were then extensively washed in HSOF + 30 mg/ml FAF BSA for 5 min before culture for 4 h in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) in SOF + 10% FCS.

Recloning: Nuclear Transfer with Embryonic Blastomeres

Unlike the FBA treatment, all medium formulations contained calcium. After enucleation, cytoplasts were preactivated at 24 hpm with ionomycin and 6-DMAP, as described above. Donor blastomeres were prepared from compacting morulae 5 days after the fusion of quiescent granulosa cells. Zonae pellucidae were digested using 0.5% w:v pronase (Sigma) for approximately 30 sec. Blastomeres were separated by incubation for 30 min in calcium- and magnesium-free PBS containing 7.5 μg/ml cytochalasin B, with the aid of gentle pipetting. Blastomeres were transferred to the injection chamber in a drop of medium containing cytochalasin B and were individually injected into the perivitelline space of dehydrated cytoplasts around 6–8 h after the activation stimulus. Cell fusion was induced with two DC pulses of 1.20 kV/cm for 80 μsec each in the fusion buffer described above. Successfully fused embryos were then placed into culture as described below.

In Vitro Culture of Nuclear Transfer Embryos

Embryo culture was performed in 20-μl drops of SOFaaBSA (8 mg/ml FAF BSA; Sigma) [22] overlaid with paraffin oil. Whenever possible, groups of five to six embryos were cultured together. Embryos were cultured in a

humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 39°C in a 5% CO_2:7% O_2:88% N_2 gas mix. On Day 5, embryos were transferred to fresh 20-μl drops of SOFaaBSA + 10% charcoal-stripped FCS (csFCS) [23]. On Day 7 postfusion, development to morulae and blastocysts was recorded, and embryos were morphologically assessed using a subjective grading system based on a scale of 1–4, inclusive, representing embryos ranging from excellent to poor quality, respectively. The cell number of some embryos was determined by counting stained nuclei, using an established image analysis method described previously [24].

Oocyte Activation Controls

Oocytes matured for 20 h were stripped of cumulus cells, and those having the first polar body were selected. Before activation at the appropriate time, oocytes were held in calcium-free medium. Oocytes were activated with ionomycin at either 24 or 30 hpm and then cultured in SOF (containing calcium) + 10% FCS and 6-DMAP, as described above for nuclear transfer embryos. After a 4-h incubation, oocytes were washed and transferred to SOF (containing calcium) + 10% FCS until they were whole-mounted and fixed, between 6 and 12 h postactivation, using methods described elsewhere [25]. After staining with 1% orcein in 45% acetic acid, all oocytes were examined by phase-contrast microscopy for the presence of pronuclei formation.

As a negative control for electrical activation at 24 hpm, oocytes were exposed to the electrical stimulation used for fusion as described above. Oocytes were then cultured in SOF + 10% FCS without calcium, exactly as used for the FBA nuclear transfer embryos, but for 10–12 h. Control oocytes were then mounted and fixed, before subsequent examination of chromatin configuration to identify oocytes in either anaphase II or telophase II, or with pronuclei, as evidence of activation.

Embryo Transfer

Recipient cows were synchronized by a single 10-day CIDR-plus (InterAg, Hamilton, New Zealand) treatment. Six days after CIDR-plus insertion, each cow received 250 mg chlorprostenol (1 ml estrumate; Schering-Plough, Union, NJ). The mean onset of estrus was observed 48 h after CIDR-plus withdrawal. Embryo transfer was performed nonsurgically on Day 7 after estrus (estrus = Day 0 = day of fusion). Each cow received two blastocysts of grade 1–2 quality, transferred in HSOF + 5% csFCS medium into the uterine lumen ipsilateral to the corpus luteum.

Determination of Embryonic Survival and Calving

All cows were examined by ultrasonography (Piemed 200 scanner, with a linear 3.5–5 MHz rectal probe; Philipsweg, Maastricht, The Netherlands) on Day 60 of gestation to record fetal development. Pregnant cows were monitored by rectal palpation at regular intervals thereafter. Commencing approximately 2 wk before expected full term, pregnant cows were monitored daily by both rectal and vaginal examination to determine fetal position and cervical dilation. Parturition was induced with an injection of 20 mg dexamethasone (Dexadreson; Intervet, Boxmeer, The Netherlands) administered 17 h before cesarean section between Days 276 and 281.

Neonatal Care

Immediately after delivery, the newborn calves were weighed and transported to a room maintained at 25°C for neonatal monitoring. The calves were dried, rectal temperature was taken, oxygen therapy was provided via a face mask, and the calves were positioned in sternal recumbency. Oxygen therapy was discontinued typically after 30 min, but this depended upon an assessment of general calf demeanor and supplementary analytical data. Blood gas and electrolyte values were determined on some calves by immediately analyzing samples of anaerobic, heparinized blood obtained from the brachial artery, using an i-STAT clinical analyzer (Sensor Devices, Waukesha, WI). The time taken for the calves to stand unaided was recorded, and colostrum was offered via a nipple bottle at this point. It was ensured that calves received a volume of colostrum equivalent to 10% of their live weight in the first 10 h. After their first feed, calves were moved to a recovery room set at 18°C overnight and were then subsequently reared outdoors. All calves received either Zaquilan (Schering-Plough) or Excenel (Upjohn, Kalamazoo, MI) as a precaution against respiratory infection. Jugular venous blood samples were taken 18 h after delivery for routine biochemistry and hematology analyses, and at regular intervals thereafter.

Microsatellite Analyses

Genomic DNA was extracted from the white blood cells collected from the nuclear transfer-derived calves and recipient cows using a guanidine hydrochloride method [26]. Sixteen microsatellite markers (see *Results* for specific loci) were analyzed using methods described elsewhere [27]. The resulting microsatellite alleles for the nuclear transfer-derived calves were compared with those from the donor cow from which the mural granulosa cells were obtained, and contrasted with those from the recipient cows that carried the respective pregnancies.

Animal Ethics

This project was approved by both the AgResearch Ruakura Animal Ethics Committee and the AgResearch Ruakura Biosafety Committee.

Statistical Analyses

The proportional data for cell fusion, in vitro development of embryos, and subsequent survival following embryo transfer were all analyzed by fitting generalized linear models using binomial distributions within the GENSTAT 5 statistical package (Lawes Agricultural Trust, Rothamsted, UK). Embryo cell numbers were analyzed after log-transformation.

RESULTS

Embryo Development

The fusion of embryonic blastomeres to cytoplasts in the recloned group was significantly higher than the fusion of quiescent granulosa cells in the FBA treatment (88.7 ± 3.8% vs. 77.4 ± 2.2%, $p < 0.05$) (Table 1). Electro-fusion with granulosa cells was not affected by either the length of time the cells were in low serum (8–18 days) or passage number (3–8).

Although there was no difference in the proportion of

TABLE 1. Effect of nuclear transfer treatment on the electrical cell fusion rates and the proportion of fused embryos developing to morulae or blastocysts by Day 7.

Treatment	Fusion	Number cultured	Blastocysts (grade 1–2)	Blastocysts (grade 1–3)	Total morulae & blastocysts
FBA	77.4%[a]	552	152 (27.5%)[c]	282 (51.1%)[e]	383 (69.4%)
Recloned	88.7%[b]	146	19 (13.0%)[d]	51 (34.9%)[f]	84 (57.5%)

[ab], [cd], [ef], $p < 0.05$.

fused embryos that developed to the morula or blastocyst stage (grades 1–4) by Day 7, significantly more FBA embryos developed into both grade 1–2 and grade 1–3 blastocysts (27.5 ± 2.5%; 51.1 ± 2.2%) compared to the recloned embryos (13.0 ± 3.6%; 34.9 ± 4.0%, respectively; $p < 0.05$) (Table 1; Fig. 1b). Within the FBA treatment, there was no effect of either granulosa cell passage number or length of time in low serum, on subsequent embryo development rates.

Embryo Cell Numbers

Embryo cell number within each embryo stage and grade category was not affected by FBA or recloning treatments, nor by length of time in low serum or passage number of the granulosa cells. The average cell number for grade 1–2 and grade 3 blastocyst-stage embryos was 132 ± 14 (n = 58) and 82 ± 3 cells (n = 166; $p < 0.05$), respectively. For those embryos that had only developed to morulae or early blastocysts seven days postfusion, the average cell number (26 ± 2) was significantly less ($p < 0.001$; n = 84).

Activation of Control Oocytes

In the FBA and recloning treatments, both the reconstructed embryos and the cytoplasts, respectively, were artificially activated, using ionomycin and 6-DMAP. However, the relative age of the cytoplasm differed, with activation occurring at 24 hpm in the recloning treatment and at 30 hpm with the FBA embryos. Studies with control MII oocytes showed that the activation rate, as observed by pronuclear formation, was not different at either 24 hpm (95.9 ± 1.6%; n = 165) or 30 hpm (93.1 ± 3.4%; n = 131).

With the experimental conditions used here to achieve cell fusion at 24 hpm in the FBA treatment, only 1.0% (n = 97) of control MII oocytes were electrically activated by the stimulus.

Embryo Survival

A total of 100 grade 1–2 blastocyst-stage embryos from the FBA treatment were transferred to 50 recipient cows. Survival rates on Days 60, 100, 180, and term were 45%, 21%, 17%, and 10%, respectively. In contrast, of the 16 embryos transferred from the recloned group, 38% resulted in fetuses present at Day 60; however, none survived to Day 100 of pregnancy. In the FBA treatment, seven fetuses were lost in the third trimester, including three sets of twin pregnancies. These losses resulted from an excessive accumulation of allantoic fluid. Ten heifer calves were delivered by cesarean section between Days 276 and 281 from eight recipient cows. All 10 cloned calves have survived (Fig. 1d). After the first few hours of life, regular animal health tests showed that the calves were physiologically healthy. The calves were derived from mural granulosa cells at either passage three or five of culture. There was no effect of length of serum deprivation or passage number on embryo survival rates.

Postnatal Characteristics of Cloned Calves

The average birth weights of the four twin calves and the six singles were 30.1 ± 2.1 kg and 44.1 ± 2.1 kg, respectively (range: 26.5–51.0 kg). After cesarean section, the average time for the majority of calves (8 of 10) to stand unaided was 39 min (range 30–50 min). However, two larger calves with birth weights of 49 and 51 kg required 90 and 120 min, respectively. All calves had a strong suckling reflex and, once standing, all drank colostrum from the bottle. In the case of one calf, 1 mg epinephrine and 20 mg doxapram were administered within 5 min of delivery in order to stimulate the cardiac and respiratory systems, respectively. This calf responded well to treatment and was standing and feeding 40 min later. The average rectal temperature within 10 min of delivery was 39.7 ± 0.1°C. However, by 1 h and 8 h postdelivery, rectal temperatures had fallen to 38.9 ± 0.2°C and 38.2 ± 0.1°C, respectively. Plasma bicarbonate was generally normal within the first hour (25.7 ± 0.8 mM; n = 3); however, one calf had metabolic acidosis over this period (16 mM HCO^{3-}). Of the five calves examined, four had normal plasma glucose levels (3.6 ± 0.5 mM); however, one calf was hypoglycemic after 90 min (1.4 mM glucose) and took several hours before it stabilized to normal levels. While all the calves were viable, a number of abnormalities were noted in the placentas of four recipient cows, which included cases of enlarged umbilical vessels, edematous membranes, and greater than usual allantoic fluid volume. None of these abnormalities appeared to compromise fetal development.

Microsatellite Analyses

Microsatellite DNA analyses examining 16 loci confirm that all the calves are genetically identical to the donor cow from which the granulosa cells used for nuclear transfer were obtained. Additionally, the cloned calves are not genetically related to the respective recipient cows (Fig. 2; a–d represent microsatellite markers texan10, bms1789, bm711, and bms941, respectively). In all of the autoradiograms, lane 1 represents the donor cow and lanes 2–11 the adult cloned calves, with lanes 12–19 representing the eight recipient cows. The other microsatellite markers examined were agla232, bmc4214, bms1353, bms2614, cssm38, oarfcb20, rm216, rm327, rm737, tgla122, tgla126, and tgla227 (data not shown).

DISCUSSION

We have shown that mural granulosa cells obtained from a living adult cow can be reprogrammed by nuclear transfer and result in the production of viable cloned calves. Exposure of the quiescent nucleus to cytoplasmic factors present in the MII oocyte for 4–6 h resulted in relatively high

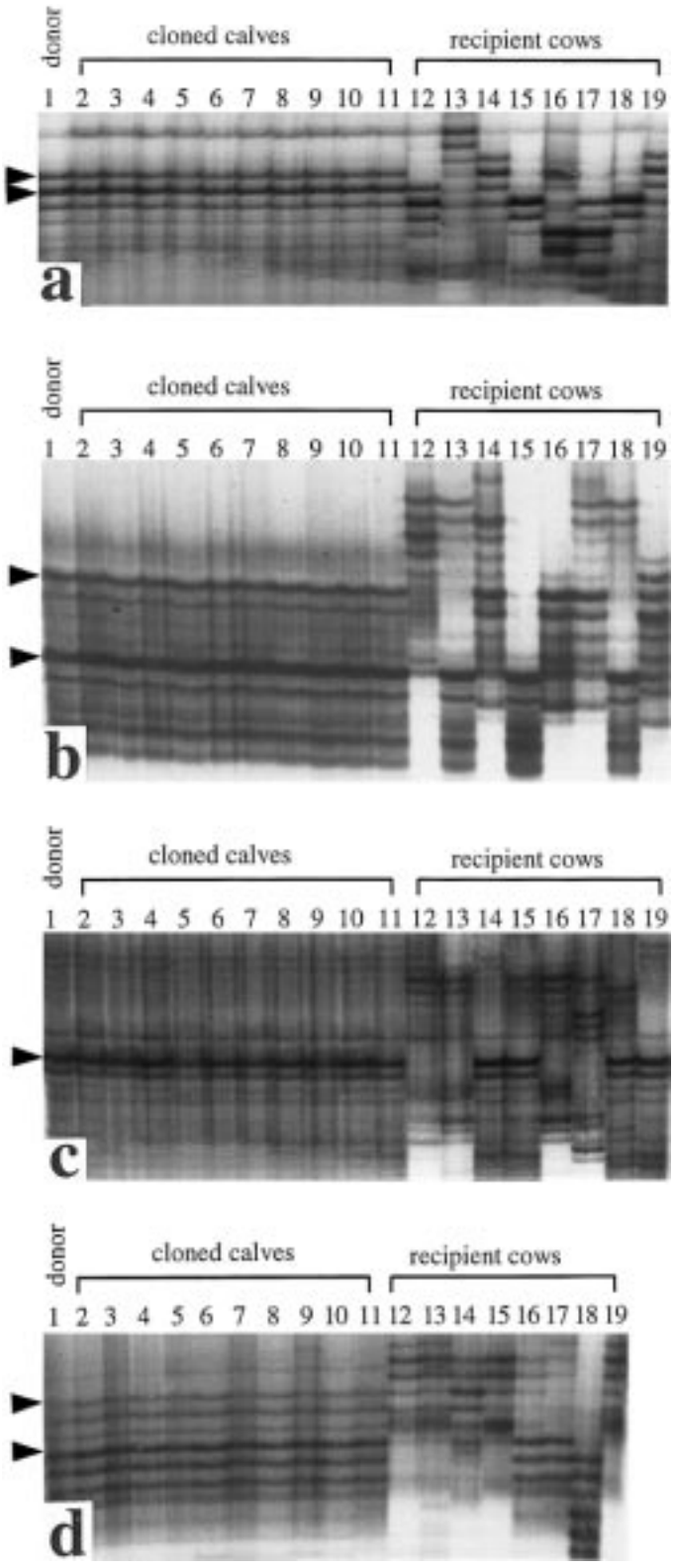

FIG. 2. Autoradiograms demonstrating the genetic origin of the cloned calves using microsatellite DNA markers texan10, bms1789, bm711, and bms941, in **a, b, c,** and **d,** respectively (see text for details).

rates of development to transferable-quality embryos in vitro (28%) and survival to term (10%). Collectively these data suggest improved reprogramming of the somatic cell nucleus. However, the technique remains limited by the substantial fetal loss that occurs throughout gestation and the abnormalities associated particularly with placentation and parturition.

It has not yet been determined which cell types from the adult animal are the most successful for somatic cell nuclear transfer. Earlier reports have shown that quiescent cells from the mammary gland in the sheep [2] and the cumulus cells surrounding the ovulated mouse oocyte [3] are both successful cell types, with overall efficiencies (in terms of live animals from successfully fused or injected cytoplasts) of 0.4% and 0.9%, respectively. In comparison, with the bovine mural granulosa cells used here for nuclear transfer, we report an overall efficiency of 2.8%, from the in vitro development and subsequent transfer of grade 1 and 2 blastocysts only (we have no data on the viability of grade 3 blastocysts). Mural granulosa cells differ in both their function and fate from cumulus granulosa cells [28]. Since the EFC cells were isolated before terminal differentiation into luteal cells, it is probable that the mural granulosa cells used here were less differentiated than expanded and mucified cumulus granulosa cells obtained from ovulated oocytes [4]. In an earlier study, bovine cumulus granulosa cells failed to produce pregnancies after nuclear transfer [29]; however, this cell type has recently resulted in the birth of adult clones in the mouse [3]. We suggest that mural granulosa cells will prove to be a suitable cell type from which to clone genetically elite cows because of the ease of repeated and noninvasive collection of cells using standard ''ovum pick-up'' techniques [19].

It has been shown in amphibians that the efficiency of nuclear transfer, represented as the proportion of clones developing normally, decreases as nuclei from more differentiated cell types, or more advanced developmental stages, are used [30]. Thus it had been postulated that as development proceeds, the totipotency of nuclei becomes restricted. With sheep and cattle in our laboratory, at least, this effect has not been observed. The proportion of fused embryos that develop to blastocysts (grade 1–3) from embryonic [31, 32], fetal [12], and adult (this study) cell types are 26%, 52%, and 51%, respectively. The corresponding production of viable offspring from embryos transferred in each of these categories is 5%, 11%, and 10%, respectively. Interestingly, these efficiencies with cultured somatic cells are not dissimilar to those achieved in some studies after nuclear transfer with embryonic blastomeres [33, 34]. By way of comparison, the efficiency of standard in vitro production (IVP) of bovine embryos in our laboratory is typically 40% development to blastocyst, with 39% of transferred embryos surviving to term [23]. Significantly, the quality of adult cloned embryos as determined by the cell number of grade 1 and 2 blastocysts on Day 7 is not different from that of bovine IVP embryos [23]. Thus the proportion and quality of blastocysts that develop are similar after in vitro fertilization and somatic cell nuclear transfer; however, the development to term of cloned embryos is currently only one-quarter that of IVP.

All ten nuclear transfer-derived calves reported here resulted from the transfer of embryos produced after the fusion of quiescent mural granulosa cells with enucleated MII oocytes, which were then activated to commence development 4–6 h later. We have previously demonstrated that embryo development is significantly increased by fusing quiescent donor cells with metaphase II cytoplasm before activation (FBA treatment), in comparison to simultaneous fusion and activation at either 24 or 30 hpm with either bovine fetal fibroblasts [12] or adult mural granulosa cells [13]. The prolonged exposure of transferred nuclei to oo-

cyte cytoplasmic factors possibly facilitates nuclear remodeling and reprogramming, as suggested previously [9, 35]. Improved embryo development with the FBA treatment, compared to simultaneous fusion and activation, has also been observed with unsynchronized cultures of bovine embryonic cells [18] and with mouse cumulus cells [3], in which the majority of cells were reported to be either in G1 or in a natural G0/G1 stage of the cell cycle, respectively. In addition to the effect of FBA, there may be benefits in synchronizing cells in G0, since blastocyst rates in the bovine species with unsynchronized embryonic [18] or fetal [10] cells have only been 10–12% compared to 51–52% reported with both quiescent adult and fetal cells (the present study and [12]). The lower development from unsynchronized cell populations may have been a consequence of inappropriate cell cycle coordination in approximately 40% of reconstructed embryos, as they would have received cells that were not in G1 [10, 18], and/or a consequence of inadequate nuclear reprogramming from using nonquiescent cells. It is significant to note, however, that the proportion of embryos transferred that resulted in viable calves was not different (around 10%) between MII cytoplasts reconstructed with donor cells presumed to either be in G0 ([12] and this study) and in G1 [10]. Further investigations are needed: first to verify the exact stages of the cell cycle being used in nuclear transfer studies, and then to determine the effects of various cell cycle combinations between cultured somatic cells and cytoplasts on subsequent in vitro and in vivo development.

For the FBA treatment protocol, it is important to ensure that the cytoplasts were not prematurely activated, particularly during electro-fusion. Under the experimental conditions used here, and in agreement with Stice and colleagues [18], a negligible proportion of reconstructed embryos in the FBA group would have been activated (around 1%) by the electrical stimulation employed to achieve fusion at 22–24 hpm,. Despite the presence of calcium and magnesium in the fusion buffer, added to increase fusion rates [12, 13], the young age of the cytoplasts in combination with the electrical fusion parameters used did not result in premature activation of the reconstructed embryos before exposure to ionomycin and 6-DMAP at 30 hpm.

With the introduction of the donor nucleus before activation, it is vital to control the ploidy of the reconstructed embryo after the activating stimulus is applied, in order for normal development to proceed. The relatively high rates of embryo development in the FBA group here may therefore have been in part due to the presence of 6-DMAP in the medium following exposure to ionomycin. This protein kinase inhibitor may have inhibited phosphorylations necessary for the spindle apparatus (as suggested in [36]) and therefore prevented micronuclei formation known to occur when fusion precedes activation [11]. Other researchers have used nocodazole as a microtubule inhibitor to control ploidy, but in their studies in sheep, with a quiescent embryonic cell line [1], there was no apparent benefit of FBA treatment, either in terms of embryo development or embryo survival. The lack of any treatment to prevent micronuclei formation may partly explain the poor embryo and fetal development in an earlier cloning study [29] following the transfer of a small number of embryos derived from bovine cumulus cells, presumably in G0/G1 [4] and apparently exposed to MII cytoplasm for a short period before electrical activation. In the mouse, a polar body is typically extruded after activation of MII cytoplasts reconstructed with donor nuclei [37, 38], quite unlike the situation with sheep and cattle [15]. Cytochalasin B was therefore added to control ploidy when mouse cumulus cells were fused before activation in embryos reconstructed by nuclear transfer [3].

This study also examined the recloning of embryos initially produced after nuclear transfer with quiescent granulosa cells. The aim was to investigate the effect of additional exposure of transferred nuclei to oocyte cytoplasmic factors, in order to allow a longer opportunity for nuclear reprogramming to occur by effectively passing the original differentiated nucleus through two rounds of early embryo development. In amphibians, recloning improved the developmental capacity of terminally differentiated nuclei, resulting in more advanced larval development compared to a single round of nuclear transfer [9]. However, in the experiments here, there was no improvement in terms of either embryo or fetal development compared to first-generation FBA cloned embryos derived directly from granulosa cells. The activation protocol in both the FBA and recloning groups was the same and involved a combination of ionomycin and 6-DMAP, although cytoplasts were activated at different ages: 30 and 24 hpm, respectively. Despite the difference in timing, the efficiency of oocyte activation in control oocytes, as evidenced by pronuclear formation, was the same (both 95%) and similar to that in a previous report [36]. In the experiments here, the blastomeres used for recloning were obtained from compacting morulae in excess of 30 cells. At this stage of development, the transcription of embryonic genes may have commenced in the first-generation cloned embryo [39]. It may be beneficial to reclone embryos before the expected onset of transcription. However, it is difficult at the 8-cell stage to visually identify those cloned embryos that are likely to have the potential for further development. Thus, compacting morulae were recloned, as our experience shows that good-quality morulae reliably develop into good-quality blastocysts. In the experiments here, preactivated (presumptive S-phase) cytoplasts, capable of accepting nuclei at any stage of the cell cycle [25], were fused with unsynchronized blastomeres. It may be necessary to investigate alternative cell cycle coordination options to improve developmental rates.

It is widely acknowledged that nuclear transfer, even with embryonic blastomeres, results in increased rates of abortion throughout pregnancy, high birth weight, perinatal deaths, and poor adaptation to extra-uterine life [40–42]. These effects appear more extreme with somatic cell nuclear transfer [1, 2, 10, 31, 32, 43] and may relate to one deficiency or a combination of deficiencies in either the nuclear transfer procedure itself, leading to incomplete nuclear reprogramming of the cultured donor cells, or in the in vitro maturation and embryo culture systems used. These deficiencies, either collectively or singularly, may lead to inappropriate patterns of gene expression at specific key stages during embryo, fetal, or placental development, contributing to pregnancy loss.

The survival of adult cloned embryos reconstructed in the FBA treatment 60 days after transfer, as indicated by ultrasonography, was relatively high (45%) in this study. This is similar to data from our laboratory with twin embryo transfer of bovine embryos produced after either nuclear transfer with quiescent fetal cells [12] or IVP [44], and is higher than with cloned embryos derived from either nonquiescent fetal cells [10] or embryonic blastomeres [33, 34]. However, the nuclear transfer process in cattle with both cultured embryonic [18] and fetal cells [12] and embryonic blastomeres [33, 34] is currently associated with

high rates of fetal loss throughout gestation. This was exemplified here, with 78% of adult cloned fetuses present at Day 60 not surviving to term, compared to a typical loss of 30% with bovine IVP embryos [23, 45]. It appears that the failure of normal placentation is a problem frequently observed with cloned embryos and also with a proportion of IVP embryos. Approximately 25% of the early embryonic mortality with the IVP embryo appears to be due to an unsuccessful transition from yolk sac to allantoic nutrition, whereby the growth of the allantois is severely retarded, or even nonexistent, and is characterized by a lack of vascularization by Day 34 [46]. It is therefore likely that the IVP cloned embryo will have a similar deficiency during this stage of development. There are reports of high fetal losses during the middle of the first trimester [12, 18]. Despite apparently normal fetal development, part of this loss may be due to a failure of normal placentome development [18]. These losses coincide with the stage at which functioning placentomes are required for the exchange of nutrients and gases [47], and this may in part be due to a deficiency in the underlying allantois (A.J. Peterson, personal communication). The majority of the fetal losses in the third trimester in this study were a consequence of hydrallantois in three twin-bearing recipient cows. This excessive accumulation of allantoic fluid may be a consequence of low numbers of placentomes, leading to placental dysfunction [48]. Reports in the literature with both IVP and cloned conceptuses describe increased incidence of hydrallantois in late gestation, fewer and enlarged placentomes, enlarged umbilical vessels, and edematous placental membranes [10, 42, 49, 50]. These abnormalities of placentation were all observed in some (but not all) recipients here. It is suggested that the embryo survival rate may have been greater had embryos in this study been transferred singularly to minimize pregnancy complications.

With the nuclear transfer pregnancies reported here, it appeared that the appropriate signaling in preparation for birth did not occur normally and there was a lack of ''communication'' between the maturing fetus and the recipient cow in the weeks leading up to expected full term (Day 282). This was characterized by few overt signs of readiness for birth and by inadequate mammary gland development in the recipient cows over this time. This may reflect another abnormality in placental function, as there are both direct and indirect actions of placental hormones on mammogenesis during pregnancy [51]. The deficiencies in parturition and mammogenesis noted here have been experienced by us previously with sheep [31, 32], and have prompted the current decision to deliver cloned offspring by elective cesarean section. Because of the decision to deliver calves between Days 276 and 281, and to not allow a sufficiently long period for them to calve following corticosteroid injection [52], we did not provide the opportunity to observe whether parturition would have been initiated after induction, or whether it might have occurred naturally at some later point in gestation. Offspring generated from both IVP [50, 53, 54] and cloned embryos [40, 42] do tend to have longer gestation lengths. If, however, there is a problem with either the fetal hypothalamic-anterior pituitary-adrenal axis and/or the transduction of the resulting rise in fetal cortisol near the time of birth to the cow [55], it certainly has not compromised the viability of the cloned calves themselves. Normally, the transmission of fetal cortisol to the dam is mediated by the changing activities of steroidogenic enzymes in the placenta [56], leading to an elevation in maternal estrogen, which in turn initiates the cascade of events leading to parturition [55]. Currently, it is not known whether the cloned calves had naturally high levels of cortisol, as the recipient cows each received an injection of corticosteroid 17 h before cesarean section in order to hasten fetal lung maturation [57]. Nevertheless, it is tempting to speculate that the lack of a typical parturition response may have been primarily due to the inability of the placenta to convert progesterone to estrogen.

The embryo culture system used here has no effect on the birth weight of IVP calves [23]. Despite the lack of control data, the birth weights observed here for the singles, but not the twins, tended to be greater than those generally reported in New Zealand for Friesians [58] and following IVP [23]. Although the birth weight of two cloned calves approached 50 kg, these were not as extreme as some reported elsewhere [40, 42]. All but one of the calves delivered were fundamentally viable and only required basic neonatal veterinary assistance. In the other case, the calf did require treatment to alleviate a slow, irregular heartbeat and respiration rate immediately after delivery. The immediate postnatal behavior of the cloned calves was more vigorous than previously reported [41], and all were standing within 30 min to 2 h and had suckled colostrum. It has been suggested that cloned calves exhibit defects in energy metabolism as evidenced by cases of hypothermia, hypoxemia, hypoglycemia, and metabolic acidosis, and that this may be a consequence of abnormal placental function [41]. With the calves reported here, oxygen therapy was provided by us as a standard procedure, and there was evidence of a metabolic imbalance immediately after birth in two of the calves, which was corrected without intervention.

While microsatellite DNA analyses are consistent with the cloned calves' being genetically identical to the Friesian donor cow (Fig. 2), there are differences in the black and white coat color patterns both between the calves and compared to the cow (Fig. 1, c and d). However, the variation is no different from that commonly observed in genetically identical twins that occur either naturally or after embryo bisection (Wells, personal observations). This aspect of piebald patterning appears not to be under absolute genetic control: environmental influences in utero result in a degree of variability in the multiplication and migration of melanoblasts, which form the melanocytes necessary for pigment production in the developing skin of the fetus [59, 60]. Subtle differences in the detail of the pigmentation markings between genetically identical cloned amphibians have also been noted [30].

The development of an efficient method for producing animals from cultured somatic cells after nuclear transfer will have a number of advantages for both agriculture and biomedicine [61, 62]. These particularly relate to new opportunities for introducing precise genetic modifications into livestock species, following homologous recombination in the cultured cells. We have identified a cell population in the adult female that has proven to be relatively successful in generating cloned offspring after nuclear transfer. Although mural granulosa cells do have a specialized function, they are not terminally differentiated, and, when combined with nuclear transfer methods whereby quiescent cells are fused before activation, relatively high rates of embryo development and survival have resulted. Overall, the technology is currently one-quarter the efficiency of IVP in the bovine species. To better enable the commercial application of this technology, however, the efficiency of the procedures needs to be substantially improved. Re-

search is needed to better understand the reprogramming of differentiated nuclei, in order to improve both the establishment and maintenance of healthy conceptuses. Studies should also focus on the development and function of the placenta and on parturition.

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