Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6143–6147, June 1993
Developmental Biology

Production of calves by transfer of nuclei from cultured inner cell mass cells

(bovine embryonic stem cells/nuclear transfer/totipotency)

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ABSTRACT **We report here the isolation and *in vitro* culture of bovine inner cell mass (ICM) cells and the use of ICM cells in nuclear transfer to produce totipotent blastocysts that resulted in calves born. Of 15 cell lines represented in this study, 13 were derived from immunosurgically isolated ICM of 3 *in vitro* produced day 9–10 bovine blastocysts, while 2 lines were derived from single blastocysts. Approximately 70% of attempted cell lines became established cell lines when started from 3 ICMs. The ability to establish cell lines was dependent on the number of ICMs starting the line. Sire differences were noted in the ability of ICMs to establish cell lines and to form blastocysts. The cell lines were cultured as a low cell density suspension in the medium CR1aa plus selenium, insulin, and transferrin (SIT) and 5% fetal calf serum (FCS) for 6–101 days before use in nuclear transfer, at which time some had multiplied to more than 2000 cells. If allowed to aggregate, cells of established cell lines formed embryoid bodies. A total of 659 nuclear transfer clones were made by fusing the ES cells into enucleated oocytes with polyethylene glycol; 460 of these fused, based on cleavage (70%). After culture of the clones for 7 days *in vitro* in CR1aa/SIT/5% FCS, 109 (24%) of those fused became blastocysts. Thirty-four blastocysts were transferred into uteri of 27 cows, and 13 cows (49%) became pregnant. Four of the 13 cows gave birth to 4 normal calves. DNA typing showed the calves to be derived from the respective sires of the cell lines. The calves were derived from cultures of less than 28 days.**

The isolation and multiplication in culture of totipotent embryonic stem (ES) cells have value in providing a large population of identical cells for use by nuclear transfer to produce clonal offspring (1). ES cells also provide a mechanism for gene transfer by transfection, infection, or injection of genes into the cells (2–6). After insertion of a selectable marker, the transgenic cells can be separated and used either by chimerization into a blastocyst or through use as donor cells in nuclear transfer to produce transgenic offspring (5–7). In addition, homologous recombination techniques can be used with cultured ES cells to add or delete genes at specific sites in the genome (8–11).

All of the above have been accomplished only with ES cells of mice (6, 12). In mice, no offspring from presumed totipotent ES cells have been produced by conventional nuclear transfer (12, 13), although offspring were produced when mouse ES cells were chimerized with tetraploid mouse embryos (14).

For domestic animals, morphological identification of putative ES cells has been published (15–18). Pluripotency has been demonstrated for ES cells of swine (17–19), cattle (17, 20, 21), and sheep (19). Injection of newly isolated blastocyst inner cell mass (ICM) cells into other blastocysts has produced chimeric offspring in sheep (22) and cattle (23). Noncultured ICM cells appear to be totipotent as evidenced by blastocyst formation, pregnancies, and offspring after transfer into enucleated oocytes in rabbits (24), sheep (25), and cattle (26).

Cultured cells with ES cell characteristics have been transferred into bovine oocytes initially with the resulting 5-day cultured embryos surviving only to the 8-cell stage (27). More recently, bovine cell lines derived from ICM (20) or morulae (21) have produced pregnancies by nuclear transfer, which fail in the first trimester. Calves have been born from chimeric embryos but the ES cell contribution is as yet unknown. One chimeric fetus was ES cell positive (20). There are no published reports in domestic species that cultured ICM or putative ES cells are totipotent, as evidenced by offspring derived totally from these cells (6, 12, 17, 19–21, 27).

Most attempts to isolate and culture ICM cells have been based on or adapted from the original methods of Evans and coworkers for mice (2, 17). In general, these methods involve separation of blastocyst ICM from trophoblast trophectoderm cells by immunosurgery followed by isolation of cells with stem cell morphological characteristics from ICM cells as they plate down on a fibroblast feeder layer. The putative stem cells are then maintained as a colony on a monolayer of fibroblast cells with differentiation-inhibiting activity, leukemia inhibitory factor, buffalo rat liver (BRL) cells, or BRL conditioned medium added to inhibit differentiation. This system has allowed culture of pluripotent cells that can become embryoid bodies. Aggregated sheets of cells develop cellular beating heart activity. However, only in mice has it allowed demonstration of or maintenance of totipotency of the cultured cells [reviewed by Stewart (6) and Anderson (12)]. It has been suggested that these mouse-derived differentiation-inhibiting agents do not adequately prevent differentiation of stem cells in species other than rodents (12).

We report here the isolation and short-term *in vitro* culture of bovine ICM cells by using a different approach to prevent differentiation. These cells were used in nuclear transfer to produce blastocysts that resulted in the birth of normal calves. This result provides evidence of totipotency of cultured ICM cells in mammalian species other than mouse.

MATERIALS AND METHODS

All embryos used in this experiment were *in vitro* derived from slaughterhouse ovaries and frozen semen by the methods described by Sirard *et al.* (28), Parrish *et al.* (29), and Rosenkrans and First (30). Oocyte maturation was in TC199 containing 10% fetal calf serum (FCS) and 0.5 μg of NIH ovine luteinizing hormone (NIADDK-OLH-25) per ml. Oocytes were fertilized with sperm from any one of five different bulls

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Abbreviations: ES cells, embryonic stem cells; SIT, selenium, insulin, and transferrin; FCS, fetal calf serum; ICM, inner cell mass.

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with sperm concentration and heparin dose adjusted for each bull. At 40–48 hr postfertilization, embryos were manually stripped of all cumulus cells and extraneous sperm by repeated pipetting through a 190-μm fire-polished pipette. Subsequent embryo culture was carried out in a defined medium called CR1aa (30) for 7–8 days at 39°C in 5% CO_2/95% air with high humidity until the embryos had hatched or were fully expanded, after which they were subjected to immunosurgery. Embryos were first washed in 3 ml of TL Hepes with polyvinylpyrrolidone (Sigma; PVP-40) (1 mg/ml) and polyvinyl alcohol (Sigma; p8136) (1 mg/ml) and then washed through four or five CO_2 equilibrated microdrops (50 μl) of CR1aa, polyvinylpyrrolidone, and polyvinyl alcohol under paraffin oil.

Rabbit anti-bovine antibody (1:10 dilution; Sigma; B8270) was added at a 1:10 dilution for a final concentration of 1:100. Embryos were returned to the 39°C incubator for 30 min. The embryos were removed from the incubator and again washed through four or five fresh microdrops of medium. Then guinea pig complement (Sigma; S-1639) was added to the embryos at a 1:10 dilution from a 1:500 diluted stock for a final dilution of 1:5000. While in the presence of complement, the zonae pellucidae were removed by manual pipetting through a non-fire-polished 150-μm pipette tip. The remaining ICMs were washed and then one to three ICMs per 10-μl drop of the medium CR1aa plus SIT (sodium selenite, insulin, and transferrin; Sigma; I 1884) were placed under paraffin oil. Within 5 days, the ICMs started disassociating from a ball of cells into individual free-floating cells. At this time, the ball of cells was mechanically disaggregated by a micromanipulation needle. The medium was changed every 2–3 days by aspiration and replaced with fresh CO_2-equilibrated CR1aa with SIT. The addition of 5% FCS to the medium was beneficial in reducing the "stickiness" of these cells, allowing easier handling during micromanipulation. The ICM cells were maintained as disassociated cells in suspension culture for periods ranging from 1 week to 2 months, depending on the experimental protocol. The culture conditions were derived through a series of experiments comparing the effects of various growth factors, media, and medium supplements on cell maintenance and growth rates. Cell viability was determined by staining with propidium iodide. All embryos used to make cell lines were derived from embryos cultured in the CR1aa/SIT/5% FCS medium.

The cultured ICM cells were used as nuclear donor cells in nuclear transfer. Recipient oocytes were matured *in vitro* (29) and stripped of cumulus 16–18 hr after initiation of oocyte maturation, using hyaluronidase at a concentration of 2 mg/ml and a fire-polished pipette. Oocytes were selected for the presence of polar bodies and returned to maturation medium for another 2–4 hr. Nuclear transfer was begun ≈20 hr after these metaphase II oocytes were placed into culture. Manipulation was performed with a Nikon Diaphot microscope equipped with Hoffman optics and Narishige micromanipulators. Manipulation was done in culture dishes in which microdrops of medium were arranged with each dish containing both 100-μl drops (TL Hepes with Ca^{2+} and Mg^{2+}) in which the oocytes were placed and 20-μl drops (TL Hepes with Ca^{2+} and Mg^{2+} and 20–50% FCS) to one side containing the cultured ICM cells. This was done to prevent the cells from sticking to the oocytes and to prevent mistaking ICM cells with any remaining cumulus cells. Approximately 10 ICM cells were aspirated into the transfer pipette, and then the tips were moved to the drop containing the oocytes. The cells were drawn higher into the pipette to allow space for enucleation of the oocyte. The oocyte was positioned on a holding pipette so that the polar body was toward the transfer tip. A small amount of cytoplasm from the region directly beneath the polar body and the polar body were removed. The transfer tip was retracted from the zona and the cytoplasm was ejected. The tip was reinserted through the same hole and an ICM cell was deposited beneath the zona. The cell was pressed against the plasma membrane, where it stuck firmly between the zona and plasma membrane. Due to the extreme stickiness of the cells, transfer pipettes were changed frequently. Nuclear (ICM cell) transfer was completed by 24 hr postmaturation, and the unfused units were placed in CR1aa medium overnight. All fusions were done with oocytes 42 hr postfollicular removal.

Fusion proved to be a difficult problem because of the disparate sizes of the cells to be fused. The ICM cells ranged in size from 15 to 25 μm, and the enucleated oocytes were ≈140 μm. Except for Table 4, in which recent experiments (bulls F–I) used electrofusion, fusions were with polyethylene glycol (PEG). The fusion protocol used PEG (M_r 1300–1600; Sigma) 1:0.25 g/ml in Ca^{2+}- and Mg^{2+}-free TL Hepes with polyvinyl alcohol (1 mg/ml) for 45 sec followed by a 1:1 dilution in the same medium for 1 min, another 1:1 dilution for 2 min, and then a final 1:1 dilution for 2–3 min. The most reliable PEG was from Boehringer Mannheim (PEG 1500). A 15-min culture in TL Hepes containing 20% FCS allowed membranes to return to their normal appearance. To activate the ooplasm, the embryos were washed through Ca^{2+}- and Mg^{2+}-free TL Hepes and then exposed to 5 mM ionomycin (Calbiochem) in 1 ml of medium for 45 sec. This was followed by another 15-min culture in TL Hepes containing 20% FCS, after which embryos were returned to CR1aa medium for further maturation.

RESULTS

To prevent differentiation, ICM cells were cultured in suspension at a concentration sufficiently low (1000–1500 cells per 10-μl drop) so that cell aggregation and differentiation did not occur. Several differentiation-inhibiting and mitotic factors were tested in various media combinations for their ability to promote prolonged mitotic activity of ICM cells cultured in loose suspension. Only media consisting of CR1aa plus SIT and either glucose, rifampicin, laminin, or 5% FCS supported mitosis through 2 weeks of culture. Of these, only CR1aa plus SIT plus 5% FCS allowed mitosis and continued proliferation of ICM cells for 4 weeks. ICM cells from day 9 and 10 bovine blastocysts multiplied in culture when cultured in CR1aa plus SIT and 5% FCS with some lines reaching 2000 cells after 2 weeks of culture. These cells have the appearance of mouse ES cells, being small cells with large nuclei, little cytoplasm, and prominent nucleoli (Fig. 1). When

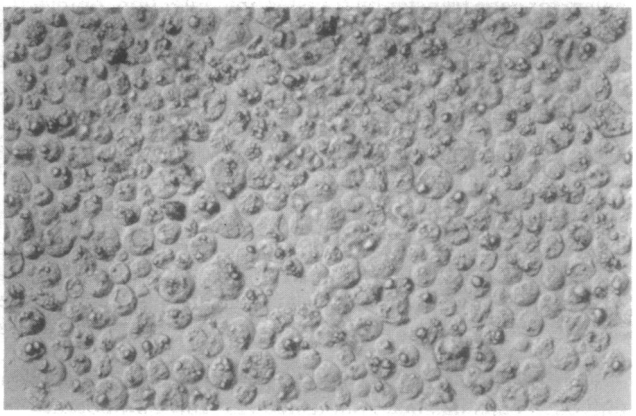

FIG. 1. Bovine ES cells. Cell population in 10-μl microdrops varied from 200 to 2000 cells within 2 weeks of culture. Individual lines were subcultured at 1000–1500 cells per microdrop because embryoid bodies formed when cell population densities exceeded 1000 cells. Note that the nucleus constitutes most of the volume of each cell, the presence of two or three nucleoli per cell, and the large round cells that will soon divide.

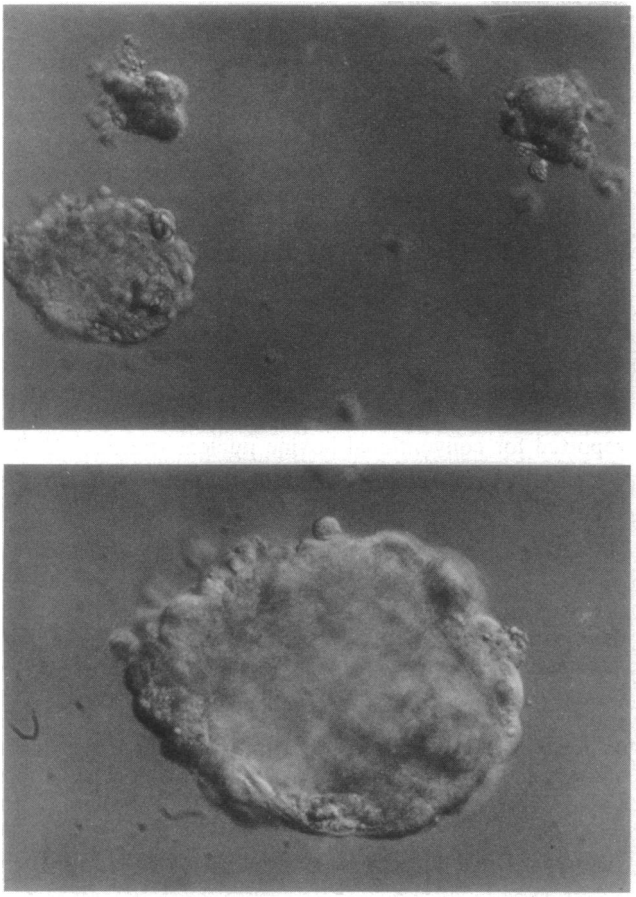

FIG. 2. Embryoid bodies resulting from high cell population density. (*Upper*) Bovine embryoid bodies, two simple and one complex. (*Lower*) Bovine complex embryoid body.

removed from nondifferentiating conditions and allowed to aggregate, the cultured cells formed embryoid bodies (Fig. 2).

Table 1. Effect of number of ICM cells starting a culture on ICM cell survival

No. of ICMs starting cell line	No. of cell lines	
	Started	Surviving at 1 month
1	159	0
3	241	170 (70.54%)

These embryoid bodies do not appear to differ morphologically from mouse embryoid bodies.

The ability of ICM cells cultured in loose suspension microdrops to establish and maintain a proliferating population of ICM cells appears to be dependent on the number of ICMs used to establish the culture (Table 1). ICM cultures derived from three ICMs from two sires established cultures that proliferated, whereas none of the cultures started from one ICM survived to 10 days and even the surviving cultures had a low proliferation rate, with most dying by 1 month. Occasionally, a single ICM initiated a cell culture as the two shown in Table 2. While one cell line, line six, was maintained for 101 days, most ICM lines derived from pooled embryos lost life and nuclear staining after 3 weeks of culture. Live/dead staining at 3 weeks with calcein AM (live) or ethidium homodimer (dead) showed ≈80% live cells (green) and 20% dead (red) and dying (orange-yellow) cells, whereas, at 5 weeks, nearly 80% of the cells were dead or dying.

One way of accurately determining totipotency of embryonic cells is to fuse the cell in question into an enucleated metaphase II oocyte. We report in Table 2 results from derivation and use of cells from 15 bovine ICM cell lines in nuclear transfer. The cell lines ranged from 6 to 101 days of culture at the time of nuclear transfer. A total of 659 embryos (clones) were made by nuclear transfer. After culture for 7 days *in vitro* in CR1aa and SIT plus 5% FCS, 109 became blastocysts (16.6%); of those cleaving, 25% became blastocysts. Each cell line was derived from the ICM of 3 blastocysts except cell lines 14 and 15, which were each derived from the ICM of a single blastocyst. The efficiency of establishing stem cell cultures from ICM cells and the ability of the oocyte ICM cell fusion product to become a blastocyst

Table 2. Use of loose suspension cultured ICM cells as donors of nuclei in nuclear transfer to produce blastocyts

Cell line	Days PIS to nuclear transfer*	Nuclear transfer clones made†	Cleavage (%)	Blastocysts, *n*‡	Blastocysts, % of clones§	Blastocysts, % of cleavage
1	35	24	14/24 (58)	6	25	43
2	42	32	20/32 (63)	4	12.5	20
3		33	21/33 (64)	4	12	19
4	17	92	71/92 (77)	19	21	27
5	71	22	18/22 (82)	4	18	22
6	101	22	15/22 (61)	4	18	27
7	6	44	36/44 (82)	9	20	25
7	6	33	2/33 (6)	0	0	0
8	13	57	43/57 (75)	11	19	26
9	20	42	20/42 (48)	6	14	30
10	27	74	61/74 (82)	12	16	20
11	14	23	17/23 (74)	4	17	24
11	14	5	4/5 (80)	2	40	50
12	21	47	40/47 (85)	4	8.5	10
13		28	21/28 (75)	6	21	29
14¶	54	39	21/39 (54)	6	15	29
15¶	61	42	36/42 (86)	8	19	22
	Total	659	460/659 (70)	109	15%	24%

*PIS, postimmunosurgery. Trophoblast cells were removed and culture of ICM cells was initiated.
†Each clone is the product of attempted fusion of an ICM cell with an enucleated oocyte.
‡Number of blastocysts after *in vitro* culture of the clones for 7 days.
§Frequency of clones becoming blastocysts after 7 days of culture.
¶All cell lines were derived from the pooled ICM of 3 blastocysts, except lines 14 and 15, each of which was derived from the ICM of a single blastocyst.

Table 3. Effect of sire on efficiency of stem cell line production and frequency of blastocysts derived from fusion of stem cells into enucleated oocytes [nuclear transfer (NT)]

	Sire breed	NT clones made, *n*	NT clones becoming blastocysts	
			n	%
A	Angus	89*	14	15.7
B	Holstein	114*	23	20.2
C	Holstein	272*	42	15.4
D	Brahman	184*	30	16.3
E	Brahman	46†	0	0
F	Longhorn	93†	14	15
G	Holstein	102†	22	22
H	Holstein	60†	6	10
I	Holstein	88†	19	22

*Stem cells were fused into enucleated oocytes by using PEG.
†Stem cells were fused into enucleated oocytes by electrofusion.

appears to be partially dependent on the genetics of the embryo as indicated by differences among sires in the frequency of stem cell line formation and blastocyst formation (Table 3).

Totipotency of cultured ICM cells from five cultured cell lines was determined by transfer into cows of blastocysts derived from ICM cell nuclear transfer (Table 4).

Thirty-four of 42 blastocysts derived from cell lines cultured for 6, 13, 20, 27, or 101 days were transferred into uteri of 27 cows. Thirteen of the cows (49%) became pregnant. At 180 days of gestation, 5 (19%) were still carrying 5 (15%) fetuses with heart beats clearly imaged with ultrasonography. Four of the cows delivered normal calves derived from the cultured ICM cells after gestations of normal length. The birth weights of the calves were 75, 80, and 85 pounds for 3 female calves and 86 pounds for a male calf. The gestations were 279, 280, 280, and 279 days, respectively.

The cell cultures producing offspring were cultures 7, 9, and 10. Cell culture 7 was derived from embryos sired by Holstein bull 9805 and the calf born from this culture was Holstein. Cell culture 9 was derived from embryos sired by Brahman bull 9813 and the two calves born were half Brahman. Cell culture 10 was derived from embryos sired by Longhorn bull 12199 and the calf was half Longhorn. DNA typing by Marijo Kent (31) established that each calf was sired by the sire producing the ICM cells from which the calf was derived. The calves were karyotyped and two half-sister Brangus calves from cell line 9 showed tetraploidy of <10% in some lymphocytes at birth but lost the tetraploid lineage by 1 year of age. Karyotypes of integument fibroblasts were normal.

DISCUSSION

The ICM cell culture system reported here prevents differentiation by culturing cells as a loose suspension with <1500 cells per 10-μl drop. Without cell–cell contact, neither cell aggregation nor monolayer formation occurred.

The results presented in Table 3 show that at least some of the ICM cells retain totipotency after culture. The efficiency of blastocyst production from use of the cultured ICM cells in nuclear transfer (15% or 25% of cleaved) is similar to the efficiency of using morulae cells as the donated nucleus in conventional nuclear transfer (18%; ref. 32).

The frequencies of pregnancies (49%) and 180-day maintained pregnancies (19%) after transfer into cows of embryos derived from cultured ICM cells were also similar to the frequency of pregnancies (30%) or maintained pregnancies reported for conventional bovine nuclear transfer (32, 33). The frequency of transferred blastocysts resulting in born offspring was also similar to bovine nuclear transfer and the calves were from three different cell lines.

Pluripotency has been demonstrated previously for cultured cattle ICM cells (12, 18, 20, 21, 27). Our present research was reported as an abstract in 1993 (34). This work demonstrates totipotency from cultured ICM cells of domestic animals, as evidenced by offspring, and the successful use of cultured cells for nuclear transfer (for review of other species, see ref. 12). The methods presented here allowed establishment of ICM cell lines from ≈70% of the blastocysts attempted, when the line was formed from a pool of 3 ICMs. This is approximately equal to the best efficiencies in the production of mouse ES cell lines (6). In mice, some ES cell lines have been shown to be of abnormal karyotype, particularly after several passages. Whether the tetraploidy of calves from line 9 was due to nuclear transfer or ICM cell culture is unknown. While the efficiency of fusion was acceptable in this study (68%), manufacturers' lots of PEG vary considerably in fusogenic activity. A modified electrofusion procedure was used with success for production of blastocysts from cells derived from sires E, F, G, H, and I in Table 3.

The greatest need will be for cell culture systems that promote much greater mitotic activity than the present system while inhibiting differentiation. The CR1aa/SIT/5% FCS culture medium used for this study is adequate only for short-term culture and represents a mere beginning in identification of an optimal culture system for bovine ICM cells.

With the development of culture systems allowing a high rate of cell multiplication, bovine ES cells derived from ICMs or earlier embryo stages should prove useful in propagation and genetic modification of cattle. The use of ES cells in gene transfer could provide more efficient gene transfer with opportunities to select cells for gene integration or expression before offspring are made and opportunities through homol-

Table 4. Production of calves from blastocysts derived from fusion of cultured ICM cells into enucleated bovine oocytes

Cell line*	Days PIS to NT†	NT clones made, *n*	Blastocysts from NT,‡ *n*	Blastocysts (*n*) transferred into cows (*n*)	Cows pregnant at 42 days gestation, *n*	Blastocysts surviving as fetuses *in utero* at gestation day				Calves born
						56	70	150	180	
7	6	44	9	9 into 6	4	5	4	2	2	1
8	13	57	11	6 into 4	0	—	—	—	—	—
9	20	42	6	6 into 4	3	4	4	4	2	2
10	27	74	12	9 into 9	3	1	1	1	1	1
6	101	22	4	4 into 4	3	0	—	—	—	—
			Total	34 into 27	13 of 27 (49%)	10	9 (27%)	7 (21%)	5 (15%)	4 (12%)

*Each of these cell lines was established from the pooled ICMs of 3 blastocysts.
†PIS, postimmunosurgery and start of ICM cell cultures.
‡Number of blastocysts developed per number of clones made after 7 days of culture.

ogous DNA recombination to be site specific in gene transfer or deletion. Bovine ES cells when used as nuclear donors in nuclear transfer could allow the production of large numbers of clonal offspring from one valuable embryo or from the genetically modified ES cells of one valuable embryo.

This manuscript is dedicated to the memory of Julie Busby. We wish to thank George Rueckert, Sue Ann Hubanks, and Julie Busby for typing this manuscript; Brad Haley for obtaining slaughterhouse material; Valerie Schutzkus for maintaining sterile media stocks and supplies; Dr. Marijo Kent for DNA typing and karyotyping the calves; and Drs. Charlie Rosenkrans and George McNamara for their advice and help. This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and W. R. Grace & Co., Connecticut.

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