Nuclear Transfer in the Bovine Using Microinjected Donor Embryos: Assessment of Development and Deoxyribonucleic Acid Detection Frequency

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ABSTRACT

Bovine embryos that had been microinjected with DNA were examined for their potential use as donor embryos in nuclear transfer. Donor embryos were obtained from oocytes collected by transvaginal oocyte aspiration, matured and fertilized in vitro, microinjected with a murine whey acidic protein-human protein C genomic DNA construct, and cultured in vitro on liver cells of buffalo rat (Rattus norvegicus). Blastomeres from these embryos were transferred into enucleated bovine oocytes received from an abattoir by electrofusion at 40 h postmaturation. Following 7 d of culture, the developmental stage was recorded, and resulting embryos were prepared for analysis by polymerase chain reaction. Embryos that were derived from microinjected donor embryos did not differ from control donor embryos (11 vs. 8.6%) in development to the morula and blastocyst stage. Of the biopsies from 20 microinjected donor embryos, 19 were positive for the injected DNA. Of 37 embryos developing normally, only 12 (32.4%) were positive for the injected DNA. These results indicate that microinjected embryos can be successfully used in a nuclear transfer program to produce additional viable embryos and that these embryos may be reliably screened for the transgene before transfer.

(Key words: transgenic, cloning, polymerase chain reaction)

Abbreviation key: BRL = buffalo rat liver, FCS = fetal calf serum, hPC = human protein C, PCR = polymerase chain reaction, TL HEPES = modified Tyrode’s medium with BSA.

INTRODUCTION

Transgenic cattle have great value in the production of human pharmaceutical proteins and the improvement of animal performance. The low efficiency of DNA integration into the embryonic genome and the high cost of maintaining recipients carrying nontransgenic offspring are major limitations to this technology. The polymerase chain reaction [PCR; (15)] is one technique that has been used to screen preimplantation embryos (12). Only embryos containing the injected DNA are transferred to recipients. However, detection frequency of DNA by PCR is higher than expected based on live transgenic birth rates (4, 12). Unintegrated DNA may be responsible for these high detection frequencies. To date, no other reliable method has been developed to screen embryos before transfer.

For transgenic technology to be feasible in cattle, a method is needed to produce a greater number of viable preimplantation embryos that can be reliably screened before transfer. Nuclear transfer, or cloning, of microinjected embryos offers this possibility. A single embryo that develops successfully to the early morula stage can be multiplied by transferring each blastomere into an enucleated oocyte, fusing the two with an electric pulse and allowing the resulting embryo clones to develop (5, 14). The additional culture period may provide time for unintegrated DNA to be degraded by the embryo. At this point, PCR could be successfully used to screen embryos before transfer. This experiment was designed to investigate the effect of using microinjected embryos as donor

1995 J Dairy Sci 78:1282-1288
embryos for nuclear transfer and to test the effectiveness of PCR as a screening method on the resulting embryo clones.

MATERIALS AND METHODS

Donor Embryo Production

Donor embryos were produced entirely in vitro. Oocytes were obtained by transvaginal follicular aspiration, guided by ultrasound. Aspiration was performed twice per week on one Jersey and eight Holstein cows for 12 wk. Aspirations began on d 3 of the cycle (estrus = d 0). The ovaries of each cow were visualized using an ultrasound machine (Aloka 500V; Corimetrics, Wallingford, CT). The follicles were aspirated with the aid of a 5-MHz sector scanner (Corimetrics) attached to a vaginal probe. The oocytes were collected through 17-gauge, 50-cm needles affixed to the dorsal surface of the vaginal probe by a 16-gauge needle guide. Fluid was aspirated directly into an embryo filter (Professional Embryo Transfer Supply, Inc., Canton, TX). The oocytes were rinsed away from the filter with PBS (Gibco, Long Island, NY) supplemented with 10% (v/v) newborn calf serum (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), and 25 U/ml of heparin (Sigma Chemical Company, St. Louis, MO). After the oocytes were recovered, they were washed in modified Tyrode's medium containing 0.3% (w/v) BSA fraction V (TL HEPES) medium and held at 39°C. Oocytes were then placed into 0.5 ml of gassed maturation medium and transported to the laboratory in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). Maturation medium consisted of TC199 (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS; HyClone, Logan, UT), bovine follicle-stimulating hormone and LH (0.1 U/ml each: NOBL Labs, Sioux Center, LA), and 1% (v/v) penicillin-streptomycin (Gibco). Oocytes were matured for 24 h and washed three times in TL HEPES medium; 50 oocytes were then placed into 500 μl of fertilization medium (3).

A Percoll separation procedure was used to prepare frozen-thawed Holstein semen for in vitro fertilization. Frozen 5-ml straws were thawed at 35°C for 1 min. Semen was layered on top of a Percoll (Sigma Chemical Company) density gradient (90%-45%) in a 15-ml centrifuge tube and centrifuged for 30 min at 700 × g. After centrifugation, sperm concentration was determined. Sperm were added to fertilization wells with the washed oocytes to give a final concentration of 1 × 10⁹ sperm/ml. Heparin (5 mg/ml), penicillamine (20 mM), hypotaurine (10 mM), and epinephrine (1 mM) were included (2).

Embryos were removed from the fertilization medium at 14 to 16 h after fertilization. Approximately 15% of zygotes were placed directly into cultures as controls. The remaining zygotes were vortexed to remove cumulus cells and centrifuged at 12,000 × g for 6 min to permit visualization of pronuclei. Microinjections were performed in TL HEPES medium on a heated stage.

The construct used for microinjection was a 12-kb fragment consisting of genomic human protein C (hPC) under the control of the murine whey acidic protein promoter. The murine whey acidic protein and hPC fusion gene was designed by Henryk Lubon and cloned in the Holland Laboratory of The American Red Cross (Rockville, MD). The genomic construct was purified by digestion with the restriction endonuclease NotI (Stratagene Cloning Systems, La Jolla, CA), followed by electrophoresis on a 1% low electroendoosmosis agarose gel with 0.5 mg/ml of ethidium bromide. Agarose containing the transgene was excised, and the DNA was purified (Prep-a-Gene kit; Bio-Rad Laboratories, Hercules, CA). The construct was then resuspended in buffer [10 mM Tris-HCl and 0.25 mM EDTA; pH 7.4 (6)] and filtered through a 0.45-μm filter. Embryos were microinjected with 1 to 3 μl of the DNA solution (1.5 mg/ml of DNA at 100 copies per pl).

After microinjection, embryos were co-cultured in wells that were prepared with buffalo rat (Rattus norvegicus) liver (BRL) cells as described by Voelkel and Hu (18). The medium used for coculture consisted of TCM199, 10% FCS, 1% BSA, and 1% penicillin-streptomycin. Embryos were cultured 25 per well for 5 d in an atmosphere of 5% CO₂ and 95% air at 39°C. On the 5th d of culture, embryos at the early morula stage were removed for nuclear transfer. The remain-
ing embryos were moved to fresh BRL wells and cultured for an additional 2 d.

Recipient Oocytes

Oocytes used as cytoplasm donors were collected from ovaries obtained from an abattoir. Bovine oocytes were purchased (Utah State University, Logan), shipped overnight in 1 ml of gassed maturation medium as described, and held in a temperature-controlled (39°C) portable incubator (Minitube of America). Oocytes were matured for 22 to 24 h and selected for the presence of the first polar body. Selected oocytes were placed in aging medium consisting of TCM199 supplemented with 10% (vol/vol) of FCS and 1% (vol/vol) penicillin-streptomycin for an additional 16 to 18 h (1). Aged oocytes were enucleated in PBS supplemented with 5 μg/ml of cytochalasin B (Sigma Chemical Co.), using a beveled micropipette to puncture the zona pellucida and aspirate a small amount of cytoplasm adjacent to the first polar body (9).

Nuclear Transfer

Embryos that had been selected as donors for nuclear transfer were placed into Ca-free, Mg-free PBS (Gibco) for 30 min before manipulations. Donor embryos and enucleated oocytes were then placed into a 100-μl drop of cytochalasin B-PBS for micromanipulation. A beveled micropipette was used to puncture the zona pellucida of the donor embryo. Cells were aspirated from the embryo and placed into an enucleated oocyte, one blastomere per oocyte (21). Two cells from each donor embryo were left in the zona pellucida and used for PCR analysis.

Blastomere-oocyte units (fusion units) were allowed to equilibrate in cell fusion medium (21) for 15 min before membrane fusion. Fusion units were placed into fusion medium in a chamber consisting of two wire electrodes mounted on a plexiglass slide. The fusion units were manually aligned so that the two membranes to be fused were parallel to the two electrodes. Electrofusion was accomplished with the use of a BTX 200-cell fusion machine (110 V; 15-μs single pulse; BTX, San Diego, CA). Immediately after fusion, embryos were transferred to BRL culture wells and cultured for 7 d as described. Embryos were moved to fresh culture wells on d 4 of culture.

PCR

The PCR (16) was used to determine the presence of the transgene in cells of 20 donor embryos and 288 cloned embryos of various developmental stages. Uninjected embryos were analyzed as negative controls. After examination for development, each embryo was transferred in 1 μl of medium into 4 μl of embryo-lysis buffer (20 mM Tris (pH 8.0), 0.9% Tween 20, 0.9% Nonidet (Sigma Chemical Co.), and 0.4 mg/ml of proteinase K (Amresco, Solon, OH)) in 0.5-ml microcentrifuge tube. Embryonic cells were handled in the same manner. Samples were then covered with 25 μl of paraffin oil and frozen at −80°C until analysis. Initial digestion of the embryos was performed at 55°C for 30 min, followed by a 15-min denaturation period at 98°C, and held at 85°C until 20 μl of the PCR reaction mixture was added (water, 2.5 μl of Taq buffer, 2 mM each 2'-deoxyadenosine, 5'-triphosphate, 2'-deoxycytidine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate, and 2'-deoxy guanosine 3'-triphosphate, 1.5 mM MgCl2, 5 mM of each primer, and 0.025 units/ml of Taq polymerase) for a final volume of 25 μl. The primers used to amplify a 559-bp target sequence in the transgene were human ProC 5' (hPC-specific sense, 5'TGGGAGAAGTGGAGCTGGACCTG) and ProC A9 (hPC-specific antisense, 5'CAGCTCTTCTGGGGGGTCCTTG). This procedure was followed by 45 cycles of annealing (55°C for 60 s), elongation (75°C for 75 s), and denaturation (96°C for 15 s). A dilution series of plasmids digested by the restriction enzyme was used as a positive control, and control bovine DNA was used as a negative control. Biopsies from control embryos were evaluated with PCR using primers to the endogenous bovine trophoblastic protein-1 gene to verify the sensitivity of the PCR procedure with only two cells. The primers used to amplify a 390-bp target sequence in the bovine trophoblastic protein-1 gene were 5'CCAGCACGCAACCCACATCTTTCCCATGAGCTG (sense) and 5'CCCTGACCTTGCGAAGCCGCCGGTGCACTACCCTC (antisense). After the initial digestion, the procedure was followed by 35
Viable embryos are morulae and blastocysts on d 7 of culture.

<table>
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<td>%</td>
<td>8.6</td>
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\(^1\)Viable embryos are morulae and blastocysts on d 7 of culture.

Developmental and PCR data were analyzed by chi-square test.

RESULTS

Development of cloned embryos from control and microinjected donor embryos is presented in Table 1. Five control donor embryos yielded 81 clones (16.2 per donor). Twenty microinjected donor embryos yielded 337 clones (16.9 per donor). Microinjected donor embryos (37 of 337; 11.0%) did not differ in development of resulting cloned embryos from control donor embryos (7 of 81; 8.6%; \(P > .05\)). For each control embryo cloned, 1.4 embryos were produced. For each microinjected embryo cloned, 1.9 embryos were produced.

Developmental data also were examined based upon the number of fusions per donor embryo. Nuclear transfer embryos derived from donors producing at least 15 cloned embryos (n = 12) had greater development to the morula and blastocyst stages (36 of 274; 13.1%) than did embryos from donors producing 14 or fewer clones (n = 8; 1 of 63, 1.6%; \(P < .05\)).

Data from PCR analysis are presented in Figure 1. Of 20 biopsies of donor embryos that were analyzed by PCR, 19 (95%) were positive for the presence of the transgene. All 5 biopsies of control donor embryos were positive for the endogenous bovine trophoblastic protein-1 gene. Of 288 cloned embryos at all developmental stages analyzed by PCR, 176 (61.1%) were positive for the transgene. Of the 37 morulae or blastocysts produced by nuclear transfer, 12 (32.4%) were positive for the transgene. The number of embryos that were positive for PCR decreased as cell number increased, and the number of negative embryos increased as cell number increased. In other words, embryos testing positive for the presence of the transgene had more arrested one-cell embryos and fewer morulae and blastocysts.
blastocysts than did those embryos testing negative ($P < .05$).

The 12 positive cloned morulae and blastocysts produced were derived from 7 different positive donor embryos. The distribution of morulae and blastocysts and of nonviable, PCR-positive and negative nuclear transfer embryos for each positive microinjected donor embryo is in Figure 2. Five of these 7 donor embryos also produced morulae and blastocysts that tested negative for the presence of the transgene. Only 2 positive donor embryos produced exclusively positive morulae and blastocyst clones, although both of those donor embryos produced nonviable embryos that tested negative. Three positive donor embryos produced no positive embryos of 13 morulae and blastocysts in total. Nine positive donor embryos produced no positive viable clones. Six of these 9 donors produced 14 or more clones. All positive donor embryos produced cloned embryos (viable and nonviable), testing both positive and negative for the transgene. Figure 3 depicts the PCR analysis of embryos resulting from nuclear transfer of 2 microinjected, cloned embryos that tested positive for the transgene. Embryo 01 produced 5 morulae and blastocysts, 2 of which tested positive for the transgene. Embryo 02 produced three morulae and blastocysts, 1 of which was positive for the transgene.

**DISCUSSION**

The production of transgenic cattle is hampered by problems that make the procedures labor intensive and extremely expensive. In vitro maturation and in vitro fertilization procedures have been used successfully to produce transfer quality embryos and a transgenic calf (4, 11, 17). However, many zygotes must be injected to produce a few transferable embryos. Finally, no good method currently exists to screen the embryos for the transgene before transfer to a recipient. Analysis by PCR produced a high number of positive results, possibly because PCR detected unintegrated DNA as well as integrated transgenes (4, 12). Injected DNA might form large concatamers (7) that may be difficult for the embryo to degrade. Microinjection of DNA was reported (10) to be associated with embryo mortality. Positive PCR results declined over a culture period of 21 d, possibly reflecting degradation of the DNA (13). For d-7 embryos, the PCR might detect this unintegrated DNA as well as integrated transgenes. Nuclear transfer might help to circumvent these problems.

This experiment demonstrated that microinjected embryos could be used successfully in a nuclear transfer program. Injected donor embryos were no less viable than control donor embryos when used for the cloning procedure. Overall, for each microinjected embryo cloned, 1.9 morulae and blastocysts were produced. However, donor embryos (n = 12) having at least 17 blastomeres produced 3 embryos (n = 37) per donor embryo cloned. Thus, in this experiment the number of embryos available for transfer was tripled.

Another advantage of cloning microinjected embryos for the production of transgenic cattle was that PCR became a more reliable test. The additional 5 d of culture may allow the embryos additional time to degrade unintegrated DNA. Analysis of biopsied d-5 microinjected
NUCLEAR TRANSFER WITH MICROINJECTION

Embryos revealed that 95% (19 of 20) of these embryos were positive for the transgene by PCR. After nuclear transfer, only 12 of 37 (32.4%) clones produced from positive donor embryos were still positive by PCR analysis. By transferring only clones that tested positive for the transgene, the number of needed recipients could be reduced (11), and the efficiency of producing a transgenic calf would probably be increased. Even though fewer embryos are ultimately transferred, the possibility of producing a transgenic calf is increased. The PCR assay used in this study does not permit discrimination between integrated and unintegrated DNA. Only transfer of positive cloned embryos to produce transgenic calves will clarify the reliability of PCR analysis of cloned embryos.

In this study, the number of positive embryos decreased as cell number increased in cloned embryos. This result suggested that embryos that were positive for the transgene were less viable than embryos that were negative. In pronuclear-injected embryos that have not been cloned, this effect was difficult to distinguish from the mechanical damage from the injection process. The loss of embryo viability because of integrated DNA might be an alternative hypothesis for the disparity between DNA detection frequency at d 7 and reported transgenic birth rates (11). The PCR might detect integrated transgenes of d 7, but cells containing the gene might die later in fetal development or be allocated to extra-embryonic tissues. Alternatively, the degradation of unintegrated DNA over time might be reflected in the increase of PCR-negative embryos as development proceeds.

Another facet of transgenic animal production was brought to light by this experiment. Because each nuclear transfer embryo was derived from a single blastomere, the transgenic composition of the donor embryo could be examined by cell. This study indicated that microinjected bovine embryos appeared to be highly mosaic, having integration of the transgene in only one or a few blastomeres. From 30 to 60% of microinjected murine embryos have been found to be mosaic (8, 19, 20). All positive donor embryos produced cloned em-

![Figure 3. Polymerase chain reaction analysis of viable nuclear transfer embryos from two positive, microinjected donor embryos (control, bovine liver genome copy number of 1000 and 100; blast, blastocyst; and Genomic PC, protein C standards of 1000, 100, 10, and 1 copy). Embryo number is not the same as that shown in Figure 2.](image-url)
bryos that were both positive and negative for the presence of the transgene. Therefore, simply because a cloned embryo was derived from a donor embryo that was positive for PCR did not mean that the cloned embryo would be positive as well. In fact, twice as many negative (n = 25) transferable embryos were produced by cloning PCR-positive embryos than positive embryo clones (n = 12). Nuclear transfer has the possibility to reduce mosaicism because the donor blastomeres had 5 d either to degrade or to incorporate the microinjected DNA, although the DNA might still be unintegrated at cloning, which was another advantage of using nuclear transfer technology in a bovine transgenic system.

CONCLUSIONS

Microinjected embryos might be used successfully to produce cloned embryos in a nuclear transfer program. Embryos produced by nuclear transfer may be more accurately screened by PCR, a simple and expedient technique, than microinjected embryos that were not cloned.

ACKNOWLEDGMENTS

The authors express their appreciation to R. L. Page, A. S. Larsen, S. K. Carlin, and W. H. Eyestone for technical support and Transpharm and Center for Innovative Technology of Virginia for financial support.

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