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Effects of Gene Methylation Reprogramming in Cloned Calves Derived from In Vitro-Transfected Somatic Cells

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Abstract

In vitro transfection of cultured cells combined with nuclear transfer currently is the most effective procedure to produce transgenic livestock. In the present study, bovine primary fetal fibroblasts were transfected with a green fluorescent protein (GFP) reporter transgene and used as nuclear donor cells in oocyte reconstructions. To examine the role of host cytoplasm on transgene expression and developmental outcome, GFP-expressing fibroblasts were fused to oocytes reconstructed either metaphase or telophase activation, and PCR technology was also employed. The results showed that GFP became detectable at the 8- to 16-cell stage, approximately 80 h after reconstruction, and remained positive at all later stages. Embryonic development to the blastocyst stage was not significantly different among metaphase and telophase groups. Therefore, GFP transgene technology can be used to select embryoes derived from transgenic animals.

Keywords: Transgenic animal; Cloned calves; Embryo; In vitro fertilization; Gene methylation

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1. Introduction

Improvements in technologies to produce transgenic farm animals are highly desirable, because the economic savings would benefit both biotechnology and basic research. The main barrier for transgenic animal production remains the identification of more efficient systems of transgene delivery and better mechanisms to optimize regulation of transgene expression levels. Although pronuclear microinjection has been used for more than two decades to produce transgenic mice, rabbits, pigs, sheep, and cattle [1-3], variable transgene expression patterns and uncertain transmission through the germ line preclude widespread application of this technology. The production of somatic cell clones derived from different tissue types of cultured cells opens new horizons for transgeneic technologies [4]. Indeed, results obtained from various species, including bovine [5], ovine [6], goats [7], and pigs [8], have indicated that, a high proportion of offspring derived by nuclear transfer are transgenic. Nonetheless, the general efficiency of somatic cloning (i.e., production of viable offspring) remains very low [9].

Successful cloning by nuclear transfer using somatic cells depends on the appropriate combination of cell-cycle stages between the donor and host cell. It has been proposed that better results are obtained with donor cells arrested at the G_0/G_1 phase following serum starvation and host cytoplasts arrested at metaphase II [10]. However, our preliminary data have shown that transgene transfection and selection procedures require prolonged culture periods, leading to slower and fewer cycling cells, which interferes with proper G_0/G_1 synchromization of donor cells for nuclear transfer. Thus, cycling cells are often erroneously chosen and fused to metaphase-arrested enucleated oocytes, leading to chromatin condensation, pulverization, and abnormal spindle assembly [11], with dramatic effects on embryonic ploidy and developmental outcome [12]. On the other hand, preactivated cytoplasts have been successfully used in nuclei transfer experiments with interphase blastomere nuclei [13], suggesting that activated host oocytes could provide an alternative for somatic cell cloning when donor nuclei synchronization cannot be efficiently attained.

In the present study, our prime objective was to determine the development potential of transgenic embryos produced by nuclear transfer using nonsynchronous donor cells with oocytes at the telophase stage, and to screen transgenic cloned embryos before embryo transfer and the resulting offspring for somatic and germ line transmission.

2. Materials and Methods

2.1. Establishment and transfection of fibroblast cell lines

Fibroblast cell cultures were established from a slaughterhouse-derived male bovine fetus at approximately 50 days of gestation. Fetal tissues were minced and digested with 0.25% trypsin and 0.02% EDTA at 37 ℃ for 10 min. Isolated cells were washed with Dulbecco modified eagle medium supplemented with 10% fetal bovine serum (FBS) and 0.5% antibiotics (penicillin, 10 000 U/mL; streptomycin, 10 mg/mL) at 37 ℃ in 5% CO_2. Once confluent, cells were frozen in 10% dimethyl sulfoxide in DMEM supplemented with 10% FBS.

Frozen aliquots were thawed and transfected with the CEEGFP plasmid containing the enhanced, humanized version of the GFP-reporter gene driven by the human elongation factor promotor and cytomegalovirus enhancer and the neomycin-resistance cassette under the control of SV-40 promoter. Cells were transfected according to the manufacturer' instructions. A total of 14 GFP-expression clones were generated and the clones were expanded and frozen at the concentration of 0.5-1 $\times$ 10^6 mL for subsequent use as donor unclei. Two clonal lines, which had been selected by visual assessment, were used for nuclear transfer (Fig. 1). Normal chromosomal number was determined before use of cells for

nuclear transfer. Briefly, fetal fibroblasts were cultured with colcemid to increase the number of cells in mitotic metaphase, spread onto slides, stained with Giemsa, and assessed for chromosome number and sex.

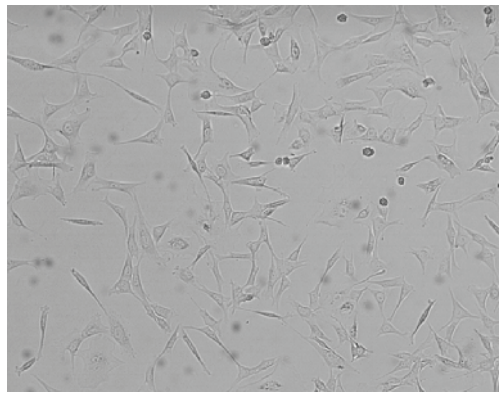

Fig. 1. GFP-transfected fetal fibroblast nuclear donor cells shown immediately after trypsin treatment in preparation for ooctye reconstruction protocol

2.2. Production of host cytoplasts

Ovaries were collected from a local abattoir and transported in saline at 30-35 ℃ to the laboratory within 2 h of slaughter. Follicles with diameters between 2 and 8 mm were punctured with a 19-gauge needle, and cumulus-oocyte complexes with several layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium supplemented with 10% of FBS. Groups of 20 COCs were placed in 100 μL of bicarbonate-buffered TCM-199 supplemented with 10% FBS, 50 μg/mL of LH, 0.5 μg/mL of FSH, 1 μg/mL of estradiol-17β, 22 μg/mL of pyruvate, and 50 μg/mL of gentamicin. After 20-22 h of vitro maturation, host oocytes were vigorously shaken in a 0.2% hyaluronidase solution to remove the cumulus cells, selected for the presence of the first polar body, and used for nuclear transfer.

2.3. Reconstruction of embryos by nuclear transfer

Transfected fibroblasts were thawed at 37 ℃, washed with DMEM containing 10% FBS, and seeded into a 60-mm Petri dish using the same medium. After 4-6 h in culture, the dish was rinsed with PBS to remove floating, unattached cells, and trypsin was added to isolate the attached fibroblasts, which were used as donor cells for nuclear transfer. Two protocols of oocyte enucleation were used to determine the role of the cell-cycle stage of the host cytoplasm on developmental outcome.

In the first protocol (metaphase enucleation), cumulus-denuded oocytes were placed in PBS containing 7.5 μg/mL of cytochalasin B, and approximately 30% of the cytoplasm adjacent to the first polar body was removed. After microsurgery, oocytes were placed in medium containing 5 μg/mL of Hoechst 33342 for 15 min and then subjected briefly to ultraviolet irradiation to confirm the removal of chromatin. A GFP-positive donor cell was introduced into the perivitelline space of the enucleated oocyte, and the resulting couplet was placed in a 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and subjected to a 1.5-kV electric pulse lasting 70 μsec. Previous experiments have indicated that exposure to an electric pulse at 24-26 h of IVM causes low rates of activation. After electrical stimulation, couplets were washed and cultured in 50 μL drops of Menezo B2 medium supplemented with 20% FBS

in the presence of bovine oviduct epithelial cells under equilibrated mineral oil at 39 ℃ in a humidified atmosphere of 5% CO_2 in air. After 1-2 h in culture, couplets were examined to determine fusion and then exposed to 5 M ionomycin for 4 min to induce parthenogenetic activation. Reconstructed oocytes were cultured in MB2 and BOEC for 7 days.

In the second protocol (telophase enucleation), denuded and selected oocytes were cultured for an additional 4 h (from 24 to 28 h) in IVM drops, activated with ionomycin, and placed for 2 h in the incubator to allow for extrusion of the second polar body. During or immediately after polar body extrusion, telophase II-stage oocytes were exposed for 15 min to medium containing cytochalasin B, and approximately one-tenth of the cytoplasm adjacent to the second polar body was removed. A GFP-positive fibroblast cell was injected into the perivitelline space and fused to the host cytoplast at approximately 2.5 h after activation. Electrofusion parameters and culture conditions of reconstructed embryos were equal to those described above.

2.4. Synchronization of recipient heifers and embryo transfer

Heat synchronization of the recipient heifers was induced by injecting 500 μg of the prostaglandin analogue cloprostenol. Six to seven days after the standing heat, one or two fresh blastocysts were transferred to the uterine horn ipsilaterally to the presence of the ovary with a corpus luteun. Embryos were washed with TCM-199 Hepes-buffered. After embryo transfer, heifers were monitored daily for heat behavior and examined by ultrasound after 30 days from embryo transfer to confirm the pregnancy.

2.5. GFP presence and expression in embryos and calves

During the period of in vitro culture, part of the reconstructed embryos were briefly exposed to blue light to determine expression of the GFP at different stages of development. At day 7 after construction, embryos that attained the blastocyst stage were examined to certify the expression of GFP. Some embryos were fixed with 10% formalin for 10 min, placed onto slides in a mounting solution containing 5 μg/mL of Hoechst 33342, and examined by epifluorescence to determine the number of nuclei perblastocyst.

Skin biopsies were performed on two cloned calves at 48 h after birth to establish fibroblast cell cultures. After a few passages, fibroblasts were treated with trypsin, fixed in 2% paraformaldehyde, and analyzed by fluorescence-activated cell sorting to determine the percentage of cells expression GFP. Some GFP-positive and –negative fibroblast cells were used in nuclear transfer. Cell cultures were also established from the placental tissue, and expression of GFP in these cells was determined by exposure to blue light. Different tissues were recovered after slaughter, snap-frozen at -70 ℃, and examined. Tissues recovered included brain, hypophysis, heart, lung, liver, kidney, pancreas, spleen, bladder, intestine, testis, muscle, tongue, and skin. Tissues were cryosectioned, mounted onto slides, and analyzed under blue light to determine the expression of GFP.

Somen samples were obtained from one of the cloned calves at 15 mo of age by means of an artificial vagina and were frozen in liquid nitrogen using a sodium citrate, egg yolk, and glycerol extender. Slaughterhouse-derived oocytes were in vitro fertilized and cultured as described previously [14]. Cleaved embryos at different stages of development were briefly exposed to blue light to determine both the proportion and the stage when GFP was first expressed. At Day 9 of culture, DNA was extracted from GFP-positive and – negative embryos using the Dneasy Tissue Kit. Then, PCR reactions from each embryo were made to verify the presence of GFP sequences, using the primers described above.

3. Results and Analysis

3.1. Metaphase and telophase nuclear transfers

The cell-cycle stage of donor cells was characterized by flow cytometry at 5 h after thaw (i.e., the expected time of embryo reconstruction). Results demonstrated that donor cells were not synchronized at any specific stage of the cell cycle. Although a fair proportion of the cells (76%) were at the G_0/G_1 stage, the remaining cells were equally distributed at the S (12.5%) and G_2/M (11.5%) stages, a typical cell-phase pattern found in cycling fibroblasts. Moreover, as determined by 5-bromodeoxyuridine incorporation, approximately two-thirds of the cells were undergoing DNA synthesis during a 2-h period preceding oocyte reconstruction. To verify whether actively cycling cells require a specific host cytoplasm stage to complete nuclear reprogramming and support development, donor cells were electrofused with host cytoplasts either at 1-2 h before (metaphase) or at 2.5-3 h after (telophase) activation with ionomycin. The rate of development to the blastocyst stage after 7 days of in vitro culture and the quality of the blastocysts produced, as evidenced by morphological evaluation and the number of nuclei, were not significantly different between metaphase and telophase host oocytes, demonstrating that both cytoplasts have similar potential to produce transgenic blastocysts (Table 1).

Table 1. Development outcome of embryos reconstructed by nuclear transfer from GFP-transfected fetal fibroblasts

GFP embryos	Metaphase	Telophase
Reconstructed oocytes	182	168
Day 7 blastocysts (%)	24 (13.2)	25 (14.9)
Expressing blastocyst (%)	24 (100)	25 (100)
Nuclei per blastocyst	112±12	123±12
Embryos transferred	15	11
Recipients	8	6
Pregnant at Day 60 (%)	4 (50)	1 (17)
Pregnant at Day 280 (%)	4 (50)	1 (17)
Live of offspring	3	1
Alive embryos transferred (%)	20	9

Some of the embryos that developed to the blastocyst stage at Day 7 were transferred into surrogate heifers synchronized with prostaglandin. A total of 15 metaphase- and 11 telophase-derived embryos were transferred to eight and six heifers, respectively, detected by ultrasound on Day 55 after embryo transfer. All five recipients were pregnant at 280 days of gestation, and four produced live offspring after cesarean section.

3.2. Transgene expression during preimplantation stage

To verify whether the pattern of control of GFP expression was affected by the cytoplast stage, embryos reconstructed with metaphase and telophase host cytoplasts were analyzed during preimplantation development. In both cytoplast groups, GFP disappeared immediately after embryo reconstruction and remained undetected up to the 8-cell stage at 60 h. Independently of the cytoplast stage, GFP was observed in nuclear transfer embryonic genome activation in bovine embryos [15]. After 80 h,

embryos that continued development to the blastocyst stage at Day 7 and beyond were consistently GFP positive in both metaphse- and telophase-reconstructed groups, indicating a similar pattern of remodeling of the somatic chromatin activity after nuclear transfer both before and after oocyte activation.

3.3. Transgene identification and expression

The presence and activity of the GFP transgene in cloned calves was confirmed by PCR, FISH, and GFP expression in tissues and primary cell cultures derived from offspring and their placental membranes. The FISH analysis of fibroblast cultures established from a metaphase- and the telophase-derived cloned calves revealed approximately 50 % GFP-positive cells. The PCR identification results are presented in Fig. 2.

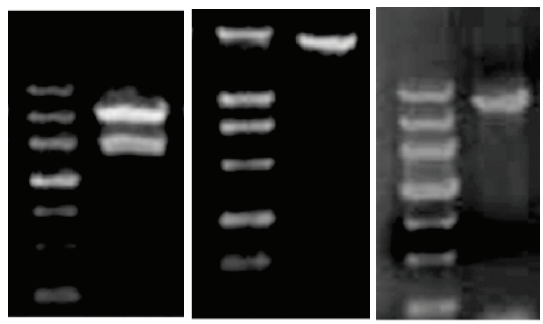

Fig. 2. GFP-transfected fetal fibroblast nuclear donor cells shown immediately after trypsin treatment in preparation for ooctye reconstruction protocol

However, although the patterns of PCR were normally distributed in the metaphase-derived clone, fibroblast samples from the telophase-derived clone contained a bimodal distribution, with more GFP expression.

To determine whether unclear transfer could reprogram somatic cells in which GFP expression was silenced, GFP-negative and -positive skin-derived fibroblasts obtained from cloned offspring were fused to metaphase-enucleated oocytes and examined at Days 1, 5, and 8 of development in vitro. As reported above with GFP-positive fetal-derived fibroblasts, reconstructed embryos derived from GFP-positive skin fibroblasts were negative at Day 1 but became positive at Days 5 and 8 after nuclear transfer, indicating that the oocytes' cytoplasm was unable to reprogram or activate the silenced transgene after nuclear transfer.

4. Discussion

In the present study, we demonstrate the expression and germ line transmission of a GFP transgene in bovine clones generated by somatic cell nuclear transfer obtained with embryos reconstructed from transfected cycling fibroblasts fused to host oocytes enucleated before (metaphase) or after (telophase) activation. Embryonic development to the blastocyst stage was not significantly different among metaphase and telophase groups, and transgenic cloned calves were produced with both protocols of oocyte reconstruction. These results show that the production of transgenic calves from genetically manipulated an in vitro-cultured somatic cells does not require a stringent synchronization protocol for donor nuclei or necessitate the use of metaphase-arrested host ooplasts for nuclear transfer.

Recent reports have shown that animals generated by nuclear transfer from genetically transformed cells are mostly also transgenic, which greatly improves the low efficiency levels obtained with

traditional pronuclear micro-injection-based protocols. Nonetheless, improvements are needed both in more accurately selecting nuclear donor cells that adequately express the transgene and in obtaining more consistent, full-term gestations and liveborn offspring after nuclear transfer. Indeed, some offspring derived by nuclear transfer from transfected donor cells are not transgenic because of difficulties in selecting a pure clonal population of transfected somatic cells. Identified in jellyfish, GFP is a single peptide of 238 amino acids that absorb blue light and emits green light without any substrate or cofactor. Expression of GFP has been shown in several species, and it has been widely used as an expression marker to study mechanisms of gene regulation. The GFP-reporter vectors have been inserted in somatic cells and embryos and offspring have been produced after nuclear transfer. In the present study, we demonstrate that GFP gene can be used as a selection marker in bovine transgenesis, not only for the nuclear donor cells before nuclear transfer and for selecting reconstructed embryos but also for selecting germ line transmission by the identification of transgene-expressing embryos generated from founder transgenic sires.

The FACS analysis of fibroblast cultures established from metaphase- and telophase-reconstructed clones, as well as the in situ assessment of several tissues from cloned offspring, demonstrated that GFP was not expressed in all of the cells. It is possible that the transgene is affected by epigenetic modification during development and/or culture in vitro. This epigenetic silencing of GFP expression seems to be fairly stable, because the transfer of nuclei from GFP-negative cells did not lead to reprogramming of the silenced transgene. Moreover, PCR amplification analysis performed in embryos produced using sperm from a transgenic cloned bull suggest that one of two of the inserted transgenes identified were silenced after passage through the germ line.

In summary, we demonstrate in the present study that GFP transgenic calves can be obtained from transfected somatic cell nuclei transplanted into either metaphase- or telophase-enucleated oocytes. Moreover, the GFP transgene is transmitted in the germ line and can therefore be use to select embryoes derived from transgenic animals.

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