Asian-Aust. J. Anim. Sci.
Vol. 19, No. 11 : 1574 - 1579
November 2006
www.ajas.info

Development of Bovine Nuclear Transfer Embryos Using Life-span Extended Donor Cells Transfected with Foreign Gene

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ABSTRACT : This study was performed to determine the developmental potentials of nuclear transfer (NT) embryos using life-span extended cells transfected with a foreign gene as donor cells. A life-span extended bovine embryonic fibroblast cell line was transfected with an expression vector in which the human type II collagen (BOMAR) and ear fibroblasts were used as a donor cell. Cytogenetic analysis was performed to analyze the chromosomal abnormality of donor cells. The fusion rate of 1.8 kV/cm for 15 μsec given twice was significantly higher than that of other groups ($p<0.05$) and the embryos lysed were significantly higher after 1.8 kV/cm for 20 μsec given once compared to other groups ($p<0.01$). The blastocyst development in the ear cell group was statistically significant compared to both BOMAR groups ($p<0.01$). Both BOMAR groups cultured more than 40 passages (>40 passages) had a lower number of chromosomes; however, fresh granulosa cell (GC) and BOMAR groups cultured less than 20 passages had normal chromosome numbers. Both >40 passages BOMAR groups had numerous obscure debris in metaphase spreads. The transfected foreign gene was expressed in all BOMAR groups, but not in the GC group. Based on these results, the lower developmental potential of NT embryos using life-span extended donor cells transfected with a foreign gene might be a cause of chromosomal abnormality in donor cells. (**Key Words :** Chromosomal Abnormality, Foreign Gene Transfection, Life-span Extended Donor Cell, Somatic Cell Nuclear Transfer)

INTRODUCTION

Cloned animals using somatic cell nuclear transfer (SCNT) have been successfully generated in sheep (Campbell et al., 1996), mice (Wakayama et al., 1998), cattle (Kato et al., 1998), pigs (Onishi et al., 2000; Polejaeva et al., 2000), cats (Shin et al., 2002), horses (Galli et al., 2003), and dog (Lee et al., 2005). SCNT using transfected donor cells provides a new strategy for the production of transgenic animals (Schnieke et al., 1997; Cibelli et al., 1998). However, problems are still remained inefficiencies in the cloning techniques, high rates of embryo and fetal loss, and a low percentage of cloned embryos developing to term. The causes of the high incidences of embryo and fetal death and abnormalities are not known. One of the reasons of these phenomenons has been suggested chromosome abnormalities (Collas et al., 1992; Campbell et al., 1993; Hanada et al., 2005).

The overall proliferate life-span of the cells is one of the major limitations of gene targeting in somatic cells. To increase the efficiencies of selection and expansion of targeted clones, long-term culture is required. Methods to extend life-span of donor cells, however, have tremendous implications for the genetic engineering of somatic cells. To reduce the implications, spontaneously immortalized cell line was used as nuclear donors (Cui et al., 2003; You et al., 2004).

In the present study, we performed to examine the productive efficiency of nuclear transfer (NT) embryos using transfected donor cell and to analyze chromosomal abnormality.

MATERIALS AND METHODS

Oocyte collection and maturation

Bovine ovaries were collected from an abattoir and

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Received December 26, 2005; Accepted April 5, 2006

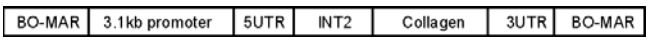

Figure 1. An explanatory diagram of the expression vector was as follows: CMV promoter was removed from pcDNA3.0 used as backbone. Bovine MAR/bovine β-casein promoter/Human type II collagen 5'UTR/human type II collagen 2nd intron/human type II collagen cDNA/human type II collagen 3'UTR/Bovine MAR.

transferred in 0.9% (w/v) saline to the laboratory within 2 h of collection. Ovaries were rinsed in pre-warmed saline (39°C) prior to follicular aspiration. Aspirations were conducted using a custom-fabricated modified vacuum system. Follicles ranging from 2 to 6 mm were aspirated using an 18-gauge needle to collect cumulus-oocyte complexes (COCs). COCs were selected on the basis of homogeneous cytoplasm and at least three complete layers of surrounding cumulus cells. Selected COCs were matured in TCM-199 medium supplemented with 10% fetal bovine serum (FBS, GIBCO BRL., Carlsbad, CA, USA), 10 mg/ml follicle stimulating hormone (FSH, Sigma Aldrich Chemicals, St. Louis, USA), 0.1 IU/ml luteinizing hormone (LH, Chorulon; Intervet Pty. Ltd., Australia), and 1 mg/ml estradiol-17β (E2, Sigma Aldrich Chemicals, St. Louis, USA) for approximately 20 h at 39°C in a humidified incubator (REVCO, Thermo Electron Corp., MA, USA) containing 5% CO_2 in air.

Gene cloning

Human type II collagen cDNA was cloned by RT-PCR using mRNAs extracted from the primary cultured human chondrocytes. For amplification of correct sequence, cDNA fragment was divided into three, then were amplified by three pairs of primers individually and they were simultaneously religated into the pGEM-T easy vector (Promega, Fitchburg, WI, USA) for complete type II collagen cDNA. We prepared 3.1 kb bovine β-casein promoter by ligating the newly cloned 1.4 kb 5'-flanking region of bovine β-casein promoter to the already known 1.7 kb bovine β-casein promoter. Two promoters respectively amplified by PCR primers were digested by endonuclease StuI and religated. We designed 5'-end primer of 1.4 kb new region using goat β-casein promoter sequence similar to bovine because there is the only 1.7 kb 5'-flanking sequence of the bovine β-casein gene on the Genbank. 5' and 3' UTR and 2nd intron of human type II collagen were amplified with primer pairs containing specific restriction enzyme sites using human genomic DNA, as PCR template, isolated from Hep3B, human hepatoma cell line. Bovine matrix attachment region derived from lactating mammary gland was also amplified with primers designed from bovine genomic DNA. We cloned all of the PCR products using pGEM-T easy vector and then confirmed each sequence by ABI3700 DNA analyzer (PE Applied Biosystems, Foster, CA, USA).

Construction of expression vectors

CMV promoter was removed from pcDNA3.0 used as backbone prior to construction of overexpression vector. The newly cloned 1.4 kb promoter and already known 1.7 kb promoter and type II collagen cDNA were digested with the specific restriction enzyme and ligated into the site between BamHI and XbaI of the pcDNA3.0 (ΔCMV) simultaneously. Immediately, intron was inserted into between 3.1 kb promoter and type II collagen cDNA. The insertion of 5' UTR into between 3.1 kb promoter and intron was performed with insertion of 3' UTR into 3'-end XbaI site of type II collgen cDNA. Finally, BoMARs were inserted into the front of 3.1 kb promoter and the back of 3' UTR (Figure 1).

Preparation of donor cells for nuclear transfer

The life-span expanded bovine embryonic fibroblast cells were transfected with an expression vector in which the human type II collagen is placed under the control of the newly cloned promoter (BOMAR). The BOMAR cells were donated from Dr. Seungkwon You (Korea University, Korea). The ear tissue samples were collected from Hanwoo cow (Korean native beef cattle). They were minced and incubated in PBS containing 0.25% (w/v) Trypsin (GIBCO BRL., Carlsbad, CA, USA) and 0.04% (w/v) ethylenediaminetetraacetic acid (EDTA, GIBCO BRL., Carlsbad, CA, USA) for 30 min. Digested tissue was transferred into 0.1% (w/v) gelatin coated flasks containing tissue culture medium (Alpha MEM; JRH Biosciences, Victoria, Australia) plus 1.5 mM L-glutamine, 50 IU/ml penicillin and streptomycin solution, supplemented with 20% (v/v) FBS. Primary culture cells were lifted and passaged upon reaching 70% confluence. Ear fibroblasts were cultured in an actively proliferating state and were obtained between passages 4-6 for all nuclear transfer experiments. To prepare donor cells, the cells were detached using Trypsin/EDTA, collected by centrifugation (1,500 rpm, 5 min), and re-suspended in tissue culture medium without serum.

NT procedures

After maturation, the cumulus cells were removed by vortexing in TCM-199 medium containing 0.2% (w/v) hyaluronidase (Sigma Aldrich Chemicals, St. Louis, USA). Oocytes with the first polar bodies (Pb1) were cultured in TCM-199 supplemented with 10% (v/v) FBS. After enucleation, single donor cells were individually transferred to the perivitelline space of recipient cytoplasts. Fusions were done by two direct current pulse of 1.5 kV/cm for 20

Table 1. Fusion rate of reconstructed embryos on electric pulse conditions

Electric pulse	No. of NT embryos (%)		
	Examined	Fused	Lysed
1.5 kV/cm 20 μsec×1	76	47 (67.1±5.7) [a]	6 (7.9±3.2)
1.8 kV/cm 15 μsec×2	120	91 (75.3±7.4) [b]	17 (13.6±2.9)
1.8 kV/cm 20 μsec×1	66	41 (63.0±4.9) [a]	20 (30.3±10.2)*

The results were expressed as mean±SD.
[a, b] $p<0.05$; * $p<0.01$.

Table 2. Developmental rate of NT embryos on donor cell types

Donor cell	No. of embryos (%)		
	Reconstructed	Cleaved	Blastocyst
BOMAR I[1]	108	77 (75.5±8.5)	0
BOMAR II[1]	108	79 (71.2±15.3)	3 (3.6±3.5)
Ear fibroblast	60	50 (80.7±8.1)	19 (39.3±3.5)*

The results were expressed as mean±SD.
Asterisk indicates significant difference ($p<0.01$) in same column.
[1] Cultured more than 40 passages.

μsec 1 time, 1.8 kV/cm for 15 μsec 2 times, and 1.8 kV/cm for 20 μsec 1 time using an Electro Cell Manipulator 2001 (BTX, Holliston, MA, USA) in 0.26 M mannitol containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.05% (w/v) BSA. Fused couplets were activated at IVM 24-25 h with 5 mM ionomycin for 4 min, followed by treatment with 1.9 mM 6-dimethyl-aminopurine (6-DMAP; Sigma Aldrich Chemicals, St. Louis, USA) in TCM-199 supplemented 10% (v/v) FBS for 4 h at 39°C in 5% CO_2 in air. After activation, fused couplets were cultured in CR1aa supplemented with 0.3% (w/v) BSA at 39°C in 5% CO_2 in air for three days. After that the embryos were transferred to CR2aa medium containing 5% (v/v) FBS with a monolayer of cumulus cells until embryo transfer.

Polymerase chain reaction (PCR) amplification

Genomic DNA was isolated from each cell line using a DNA purification kit (Promega, Fitchburg, WI, USA) and analyzed by polymerase chain reaction for the presence of human type II collagen gene. Primers used to amplify the 451-base pair fragment were forward; 5'-CTG AGT GGA AGA GTG GAG ACT AC-3' and reverse; 5'-TGT TTC GTG CAG CCA TCC TTC AG-3'. After Denaturation at 94°C for 1 min, 30 cycles of PCR amplification were performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR products were applied to a 1.0% (w/v) agarose gel, and then visualized under UV light.

Chromosome analysis

The ploidy of the transfected cells was examined for their cytogenetic composition. Granulosa cells were also analyzed as controls. The procedures were as follows: The cells were incubated in 0.05 mg/ml colcemid (Gibco BRL., Carlsbad, Ca, USA) in culture medium for 5 h and then treated with 1% (w/v) trisodium citrate for 10-15 min, and transferred individually onto a clean glass slide and fixed. Slides were stained with 1% (v/v) Giemsa for 10 min. Images were captured by digital camera with PIXERA Viewfinder Program (Pixera Corporation, Los Gatos, CA, USA) under Zeiss microscope (Zeiss, Göttingen, Germany). The cells that did not show an interpretable metaphase spread because of gross overspreading or clumped chromosomes were not classified. The numbers of chromosomes were counted under a light microscope at 1,000×magnification. At least 50 metaphase spreads/lines were examined.

Table 3. Effects of cell passages on chromosomal abnormality

Donor types	No. of			Abnormality (%)
	Passages	Spreads	Chromosomes	
Granulosa cell	Fresh	50	59.7±0.7	13.2
BOMAR	<20	52	59.6±0.7	22.5
BOMAR I	>40	55	56.4±1.6	100
BOMAR II	>40	50	55.4±1.9	100

Statistical analysis

Differences among groups were analyzed by one-way analysis of variance (ANOVA) procedure of SAS program (Release 9.1, Cary, NC, USA: Inst. Inc.; 2002). A probability of $p<0.05$ was considered statistically significant.

RESULTS

To determine an appropriate fusion conditions for BOMAR donor cell, we examined several electric pulse conditions (Table 1). The fusion rate in 1.8 kV/cm for 15 μsec 2 times was statistically higher compared to that of other groups ($p<0.05$). The number of lysed NT embryos was significantly higher in 1.8 kV/cm for 20 μsec 1 time than other groups ($p<0.01$). The developmental rate of the reconstructed NT embryos was shown in Table 2. The cleavage rate was similar among three experimental groups. However, the NT embryos developed to blastocyst (BL) were significantly higher in ear cell group than in both BOMAR groups ($p<0.01$). To evaluate the reason why both BOMAR groups did not develop to BL, we performed chromosome analysis to donor cells (Table 3). The number of chromosomes in granulosa cell (GC; as control) and BOMAR cultured less than 20 passages was shown normal ranges. However, BOMAR I, II (>40 passages) groups were shown abnormal chromosome number ranged from 55 to 56. The abnormality of the BOMAR groups cultured more than 40 passages counted were 100% compared to GC (13.2%) and BOMAR (<20 passages; 22.5%).

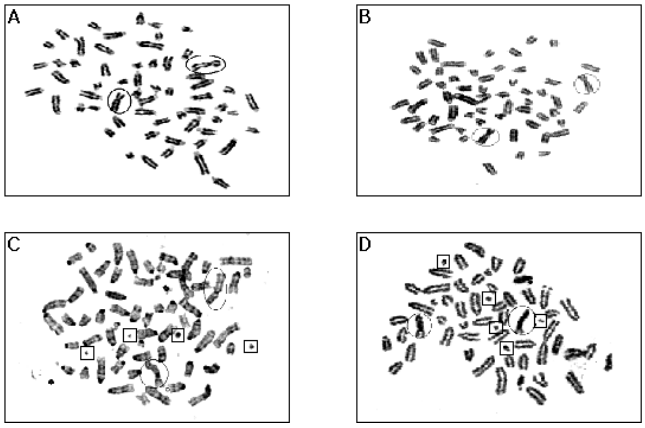

Figure 2. Cytogenetic analysis of the donor cells: (A) Granulosa cells (fresh); (B) BOMAR (<20 passages); (C) BOMAR I (>40 passages); and (D) BOMAR II (>40 passages). There were numerical obscure particles (square) in metaphase spreads in (C) and (D). The sex chromosomes were in circles.

As shown in Figure 2, GC (A) and BOMAR less than 20 passages (B) had normal shape of chromosomes. But, BOMAR I (C), II (D) spreads showed many small chromosome debris (square). They were not appeared in GC and BOMAR (<20 passages). The circles were sex chromosomes. In spite of these abnormalities, all BOMAR groups expressed the foreign gene (Figure 3).

DISCUSSION

The results of the present study indicate that the low development rate of NT embryos developed to BL might be caused by the chromosomal abnormalities of donor cells cultured excessively to select and expand of targeted clons. To reconstruct NT embryos, an establishment of fusion conditions, such as appropriate voltage, duration, frequency, and donor cell type were critically important. The fusion rate has varied according to research groups. The rate was ranged from 30% to >90% (Zakhartchenko et al., 2001; Chen et al., 2002). In this study, we examined several fusion conditions for the transfected donor cells (BOMAR) to increase the fusion rate. Among treatment groups, 1.8 kV/cm for 15 μsec 2 times group showed significantly higher fusion rate and moderate lysis rate. Low fusion rate in this study might be a cause of the donor cells. They were sensitive to the duration of electric pulse. So to increase the fusion and decrease the lysis rate, an appropriate fusion conditions have to be examined according to donor cell types.

The NT embryos developed to BL was significantly different between transfected and normal somatic cells. We failed to obtain BL embryos from BOMAR I and obtained low developmental rate of BL in BOMAR II group, however, the ear cell group showed significantly higher BL

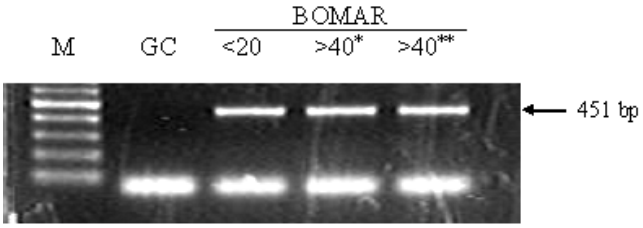

Figure 3. The expression of foreign gene in different cell types: M: marker (100 bp); GC: granulosa cell; <20: BOMAR cultured less than 20 passages; * BOMAR I cultured more than 40 passages; ** BOMAR II cultured more than 40 passages.

formation. The efficiency of the BL formation of NT embryos is extremely variable. The percentage of nuclear transfer embryos developing to blastocyst stage ranges from less than 5% to greater than 65% (Hills et al., 2000; Kubota et al., 2000; Dong et al., 2004; Hong et al., 2005). Recent studies of SCNT-derived embryos have shown that a high proportion of embryos contain cells with abnormal chromosome numbers from 0% to 75% (Mohamed and Takahashi, 2000; Arat et al., 2002; Booth et al., 2003; Slimane-Bureau et al., 2003). King (1990) revealed that the most common of abnormalities involving chromosome number involves irregularities of cell division during culture. Bureau et al. (2003) found that the incidence of cloned embryos with chromosomally abnormal cells was higher in embryos derived from the parent cell cultures with the higher incidence of abnormal cells. They speculated that some cell lines and the embryos derived from them have a higher susceptibility for abnormal cell division. The NT procedures, i.e. removal of oocyte nuclear material, transfer of a differentiated cell into an enucleated cytoplast, fusion with electric pulse, chemical activation, culture conditions, and manipulation of donor cell for transgenic, can all lead to potential cell cycle blockages. All of these artificial manipulations can potentially influence of the chromosomal composition (Li et al., 2004).

To facilitate a genetic modification of somatic cell, researchers have established spontaneously immortalized cell lines (Zakhartchenko et al., 1999; Cui et al., 2003; You et al., 2004). To make life-span extended cell line, the cells were cultured more than 40 or 50 passages. In spite of these excessive culture periods, there were no problems to use the cell lines as donor cell (You et al., 2004). In this study, we analyzed the chromosome abnormalities of the donor cells transfected and life-span extended. Unfortunately, we could not examine the chromosomal abnormalities in ear fibroblasts because of lack of samples. We prepared donor somatic cells each experiment newly. That is the reason why we did not include the chromosome analysis data of ear cell in this experiment.

First, we examined BOMAR I group and GC. The

chromosome numbers in BOMAR I group were less then normal. The BOMAR I group had many obscure particles in most of metaphase spreads. We counted the particles as chromosomes because of an indefinite origin. Despite of this calculation, the numbers of chromosome in BOMAR I group were around 55, however, most of GC was shown 60 chromosomes and they did not have any kind of particles in metaphase spreads. Then, we examined new BOMAR group (<20 passages), BOMAR II (>40 passages), and GC as control. Like previous experiment, the BOMAR II group had numerical and morphological chromosomal abnormalities, but not in BOMAR and GC groups. These results are similar to data from Kubota et al. (2000), who reported that about 20 to 30% of donor cells cultured less than 15 passages showed abnormal chromosome numbers.

Interestingly, in spite of these chromosome abnormalities, the foreign gene transfected was expressed in all BOMAR group and they did not have any morphological abnormalities during culture periods. Therefore, demonstration of genetic instability of cells after prolonged culture is pivotal to combining site-specific genetic manipulations and cloning.

Based on these results, we conclude that the low developmental potential of NT embryos used life-span extended donor cells transfected with foreign gene might be a cause of chromosomal abnormality in donor cells.

ACKNOWLEDGEMENT

This work was supported by a grant from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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