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Cell-cycle synchronization of fibroblasts derived from transgenic cloned cattle ear skin: effects of serum starvation, roscovitine and contact inhibition

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Summary

The purpose of the present study was to evaluate the effects of serum-starvation, contact-inhibition and roscovitine treatments on cell-cycle synchronization at the G_0/G_1 stage of ear skin fibroblasts isolated from transgenic cloned cattle. The developmental competence of re-cloned embryos was also examined. Our results showed that the proportion of G_0/G_1 cells from the serum-starved group at 3, 4 or 5 days was significantly higher compared with 1 or 2 days only (91.5, 91.7 and 93.5% versus 90.1 and 88.8%, respectively, $p < 0.05$); whilst there was no statistical difference among cells at 3, 4 or 5 days. For roscovitine-treated cells, the proportion of G_0/G_1 cells at 2, 3, 4 or 5 days was significantly higher than those treated for 1 day only (91.1, 90.1, 89.4 and 91.3% versus 86.51%, respectively, $p < 0.05$). The proportion of contact-inhibited G_0/G_1 cells rose significantly with treatment time, but was similar at 3, 4 and 5 days (89.4, 90.4, 91.4, 91.6 and 92.1%, respectively, $p < 0.05$). The efficiency of obtaining G_0/G_1 phase cells was lower when roscovitine treatment was employed to synchronize the cell cycle compared with the serum-starvation and contact-inhibition methods (89.7 versus 91.1% and 91.0%, $p < 0.05$). Moreover, obvious differences were observed in the rate of fused couplets and blastocysts (89.88 ± 2.70 versus 87.40 ± 5.13; 44.10 ± 8.62 versus 58.38 ± 13.28, respectively, $p < 0.05$), when nuclear transfer embryos were reconstructed using donors cells that had been serum starved or contact inhibited for 3 days. Our data indicate that 3 day treatment is feasible for harvesting sufficient G_0/G_1 cells to produce re-cloned transgenic bovine embryos, regardless of whether serum-starvation, contact-inhibition or roscovitine treatments are used as the synchronization methods.

Keywords: Cell-cycle synchronization, Flow cytometry Somatic cell nuclear transfer, Transgenic

Introduction

Somatic cell nuclear transfer (SCNT) in combination with transfection of somatic cells provides a more efficient approach to produce transgenic animal than the traditional pronuclear DNA microinjection method, which has been the major way of transgenic animal production since it was first applied (Churchill *et al*., 2004). SCNT has shown its tremendous wide-ranging application in high-quality domestic animal reproduction, for the mammary gland bioreactor, for stem-cell techniques and for many other applications. Transgenically cloned large domestic animals have many potential applications, such as the production of therapeutic and nutritional proteins, xenotransplantation and basic research. To date, scientists have successfully produced cloned mammals including sheep (Wilmut *et al*., 1997), mice (Wakayama *et al*., 1998), cattle (Cibelli *et al*., 1998; Kato *et al*., 1998; Wells *et al*., 1999), goats (Baguisi *et al*., 1999),

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pigs (Polejaeva *et al*., 2000), cats (Shin *et al*., 2002), rabbits (Chesne *et al*., 2002), mules (Holden, 2003), horses (Galli *et al*., 2003) and rats (Zhou *et al*., 2001) by SCNT using mammary gland epithelial cells (Wilmut *et al*., 1997), cumulus oophorus (cumulus ovaricus) cells (Wakayama *et al*., 1998), uterine tubal epithelium cells (Kato *et al*., 1998), fetus fibroblast (Baguisi *et al*., 1999), muscle cells (Qi *et al*., 2004) and fetal germ cells (Zakhartchenko *et al*., 1999) at different developmental stages. Nonetheless, the low efficiency of SCNT is a major obstacle to the extensive use of this technology (Renard *et al*., 2002).

The development of reconstructed embryos following nuclear transfer appears to be dependent on a variety of factors. The most important factor identified thus far is cell-cycle synchrony of donor nuclei with recipient enucleated oocytes (Campbell *et al*., 1996; Prather *et al*., 1999). For example, Wilmut *et al.* (1997) believe strongly that their attempts would not succeed without first inducing donor cells into the quiescent stage. Unfortunately, there is currently no system that provides 100% synchronization of cells at a particular stage of the cell cycle (Baguisi *et al*., 1999). The preparation of cell-cycle stage donor cells for SCNT is still a subject for debate (Cibelli *et al*., 1998). Lai *et al.* (2002) and Zhou *et al.* (2001) stated that serum starvation is not necessary for the success of SCNT. Interestingly, although there are several ways of preparing somatic cells for SCNT, many researchers prefer to use serum starvation to synchronize donor cells when they first attempt to clone a new species (Baguisi *et al*., 1999; Chesne *et al*., 2002).

The cell-cycle stage of the donor cells is one of the most important considerations. The importance of the stage of the donor cell-cycle has been noted since the first SCNT mammal – Dolly – was produced (Wilmut *et al*., 1997; Kato *et al*., 1998; Baguisi *et al*., 1999). The use of donor cells arrested at the G_0 stage of the cell cycle always was the first choice in SCNT, although cycling cells have also been used as donor nuclei and in these cases SCNT embryos have developed to full term (Cibelli *et al*., 1998; Kasinathan *et al*., 2001; Urakawa *et al*., 2004).

Yin *et al.* (2006) reviewed methods of arresting cells in the G_0/G_1 phase of the cell cycle (Yin *et al*., 2007). However, to date, studies on cell-cycle synchronization of cells from transgenic cloned cattle, especially when preparing somatic cells for the reproduction of precious cloned animals by using a re-cloning method, are very limited. Therefore, this study was designed to examine the effects of cell-cycle synchronization protocols, such as confluent inhibition (CI), roscovitine treatment (R) and serum starvation (SS) in ear fibroblast cells from transgenic cloned cattle, in order to seek a more reliable and more efficient method to prepare donor cells for the production of re-cloned embryos from transgenic cattle. Here we provide data on cell-cycle synchronization for the re-cloning of transgenic cloned cattle.

Materials and methods

Ear tissue was cut from transgenic cloned cattle aseptically and taken to the laboratory. After removal of the cartilage tissue using iris scissors and forceps, the remaining tissues were rinsed twice in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (DPBS; GIBCO) and cut into 1 mm^3 pieces. The pieces were seeded into T-25 cell culture flask and cultured in an incubator at 37.5 °C, less than 5% CO_2 in air and 100% humidity, for 4–6 h until tissue explants were attached. Then, fresh culture medium, [Dulbecco's Modified Essential Medium (DMEM; GIBCO), 10% fetal bovine serum (FBS; GIBCO) and 1% non-essential amino acids plus penicillin and streptomycin], was added. After the cells grew to 70–80% confluency, the spent culture medium was discarded and DPBS was used to wash the cells three times, followed by addition of 0.25% trypsin and 0.02% EDTA to harvest the cells. After centrifugation at 1500 rpm for 5 min, supernatants were removed and one group of pellets was resuspended in fresh cell-culture medium and seeded into new T-25 flasks. A second group of pellets was resuspended in cell cryopreservation medium [DMEM, 10% dimethylsulfoxide (DMSO) and 20% FBS] and aliquoted into cryovials. After freezing at −70 °C overnight, pellets were then stored at −196 °C in liquid nitrogen.

Flow cytometry analysis

DNA content of fetal cells was analysed by ethanol fixation and staining with propidium iodide. Trypsinized cells were resuspended in DMEM with 10% FBS and dispensed into 15 ml tubes, at 5×10^5 cells per tube. Cells were pelleted by centrifugation at 500 *g* for 10 min and pelleted cells were washed in DPBS. Ethanol (3 ml at 4 °C) was added slowly to each tube with vortexing. After ethanol fixation (at least 12 h at 4 °C), cells were pelleted (1000 *g* for 5 min), then washed once with PBS + 10% FBS (1000 *g* for 5 min). Pelleted cells were washed with 2 ml Triton X-100 (Sigma), and the pellet was stained with 1 ml PBS containing 30 mg/ml propidium iodide (Sigma) and 0.3 mg/ml RNase A (Sigma). Staining was carried out at room temperature for a minimum of 1 h. Stained cells were filtered through a 30 mm nylon mesh (Spectrum) immediately prior to flow cytometric analysis.

Preparation of recipient oocytes

Ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermos flask

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filled with physiological saline at 25–35 °C. Cumulus–oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) and selected based on their morphology. Complexes were washed twice in maturation medium, which was comprised of tissue culture medium (TCM-199; Life Technologies) supplemented with 10% FBS, 0.01 U/ml FSH, 0.01 U/ml LH, 1 mg/ml 17β-estradiol and 1% (v/v) penicillin/streptomycin (Life Technologies). Approximately 50–60 COCs were transferred into 0.5 ml of maturation medium in 4-well dishes, overlaid with paraffin oil and cultured at 38.5 °C in 5% CO_2 in air for 18–20 h. After maturation, cumulus cells were removed by vortex in 0.1% (w/v) hyaluronidase for 2–3 min.

Nuclear transfer

Enucleation was achieved by piercing the zona pellucida with a glass needle, followed by removal of the polar body and surrounding cytoplasm. Successful enucleation was confirmed by Hoechst 33342 staining of pushed-out karyoplasts. Donor cells were then transferred individually into the perivitelline space of enucleated recipient oocytes. Reconstructed embryos were fused electrically, 24 h after maturation, in a chamber filled with Zimmerman cell fusion medium, by two stainless steel electrodes. Cytoplast–cell complexes were aligned manually with a fine glass needle, so that the contact surface between cytoplast and donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 2.5 kV/cm for 10 μs, each at 1 s apart, and delivered by a BTX2001 Electro Cell Manipulator (BTX). Activation was induced by incubation in 10 μg/ml cycloheximide and 2.5 μg/ml cytochalasin-D in CR1aa medium supplemented with 0.1% (w/v) bovine serum albumin (BSA) for 1 h and then cycloheximide (10 mg/ml) alone for a further 4 h. After activation, embryos were cultured further in CR1aa medium supplemented with 0.1% (w/v) BSA for 48 h in an atmosphere consisting of 5% O_2, 5% CO_2 and 90% N_2. Cleaved embryos were then selected and cultured for an additional 5 days in CR1aa medium supplemented with 5% (v/v) FBS on cumulus cell monolayers in an atmosphere of 5% CO_2 in air. The medium was changed every 2 days throughout the culture period.

Experimental design

In Experiment 1, the cell-cycle stages of ear fibroblasts from transgenic cloned cattle were evaluated. Three experimental groups were designed and cells were passaged four times and cultured near to confluency: (i) cells were cultured either in DMEM + 10% FBS until confluency [confluent inhibition (CI) group]; (ii) in DMEM + 10% FBS + 15 μM roscovitine [roscovitine (R) group]; or (iii) in DMEM + 0.5% FBS [serum starvation (SS) group] for 5 days. Every group sample was evaluated at 24 h, 48 h, 72 h, 96 h and 120 h after treatment.

In Experiment 2, nuclear transferred embryos (NTEs) were reconstructed using the serum-starvation and contact-inhibition treatment for 3 days and ear fibroblasts from transgenic cloned cattle as donors.

Statistical analysis

The data were analysed using the chi-squared test. A value of chi-squared corresponding to $p < 0.05$ was considered significant.

Results

In the cycling fibroblasts (50–60% confluent), 59.29% cells were allocated in the G_0/G_1 phases. The efficiency of obtaining G_0/G_1 phase cells was lower when R was employed to synchronize the cell cycle than for the SS and CI methods (89.7 versus 91.1% and 91.0%, respectively, $p < 0.05$) (Table 1).

For the SS group, the proportion of G_0/G_1 cells was significantly higher when treatment lasted for 3, 4 and 5 days rather than treatment for 1 or 2 days only (91.5, 91.7 and 93.5% versus 90.1 and 88.8%, respectively, $p < 0.05$). No statistical difference was observed between values for cells treated for 3, 4 or 5 days (Table 2).

For the CI group, as expected, an increasing number of G_0/G_1 stage cells was found following 3 days of CI (from 89.4% at day 1 and 90.4% at day 2 to 91.4% at day 3, $p < 0.05$), the latter being similar to values for day 4 (91.6%) and day 5 of CI treatment (92.1%) (Table 2).

For the R group, the number of cells that had developed to the G_0/G_1 stage was significantly lower after 1 day of treatment than that after 2 to 5 days (86.51 versus 91.1, 90.1, 89.4 and 91.3%, respectively, $p < 0.05$), for which similar numbers of cells in G_0/G_1 phase were observed (Table 2).

Nuclear transferred embryos were reconstructed using SS- or CI-treated ear fibroblasts from transgenic cloned cattle cells as donors. Obvious difference were found in fused couplets and blastocysts (89.88 ± 2.70

Table 1 Cell-cycle synchronization of transgenic cloned cattle fibroblasts cultured in different conditions

	Group (culture conditions)		
	SS group	CI group	R group
% G_0/G_1 cell cycle (mean ± SEM)	91.11 ± 1.77^a	90.97 ± 1.18^a	89.70 ± 2.23^b

[a,b]Mean statistical differences $p < 0.05$.

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Table 2 Distribution of cell cycle stages with the treatment time

	Serum starvation, contact inhibition and roscovitine treatment(s)				
Treatment	1 day	2 days	3 days	4 days	5 days
SS	88.78 ± 0.68^{d}	$90.10 \pm 1.07^{c,d}$	91.47 ± 0.40^{b}	91.70 ± 0.56^{b}	93.52 ± 0.87^{a}
CI	89.36 ± 0.52^{c}	$90.37 \pm 0.23^{b,c}$	$91.43 \pm 1.09^{a,b}$	$91.60 \pm 0.56^{a,b}$	92.11 ± 0.82^{a}
R	86.51 ± 3.08^{a}	91.10 ± 1.05^{b}	90.05 ± 0.19^{b}	89.54 ± 1.38^{b}	91.26 ± 0.40^{b}

$^{a-d}$Mean statistical differences, $p < 0.05$.

Table 3 *In vitro* developmental competence of cloned embryos

	In vitro developmental competence of cloned embryos			
Treatment	Reconstructed	Couplets fused (%)	Embryos cleaved (%)	Blastocysts (%)
SS	1015	89.88 ± 2.70^{a}	70.20 ± 5.30^{a}	44.10 ± 8.62^{b}
CI	968	87.40 ± 5.13^{b}	69.74 ± 6.30^{a}	58.38 ± 7.28^{a}

Embryos were examined using serum starvation and contact inhibition treatment transgenic cloned cattle ear fibroblasts cells as donors.
$^{a-d}$Mean statistical differences, $p < 0.05$.

versus 87.40 ± 5.13; 44.10 ± 8.62 versus 58.38 ± 13.28, respectively, $p < 0.05$) (Table 3) while no significant difference was found between cleaved embryos.

Discussion

Data from our present study indicate clearly that ear fibroblasts from transgenic cloned cattle could be synchronized effectively at G_0/G_1 stages using all the three different treatments: CI, R and SS. Most of the cells (>80%) in all groups were arrested at the G_0/G_1 stage, while in the cycling fibroblasts (50–60% confluent), 59.29 % cells allocated in the G_0/G_1.

Serum starvation has long been known to withdraw growth factors and other related components from cultured cells (Chesne *et al*., 2002). In other studies, effective synchronization of donor cells in presumptive G_0/G_1 has been achieved by SS treatment for 4 days of sheep cells (Wilmut *et al*., 1997), 5–10 days in rabbits cells (Zhou *et al*., 2006) and 5 days in cattle cells (Kubota *et al*., 2000). It takes 5 days of continuous starvation to reach the plateau (93%) found in this study and no statistical difference was observed following 3, 4 or 5 days of treatment. Cho *et al*. (2005) reported that they achieved 82.9% cattle fetal fibroblasts cells in G_0/G_1 phase with SS treatment (Cho *et al*., 2005). Differences in results may be attributed to the influence of individuals, cell types and treatment methods.

Culture to 100% confluency is another strategy used to move cells into G_0 in response to overcrowding (Zetterberg and Auer, 1970). Boquest *et al*. (1999) reported that a larger portion (85%) of confluent porcine foetal fibroblasts cells was in G_0/G_1 phase when compared with those in cycling cultures. Cho *et al.* (2005) reported that 86.9% of confluent cattle fetal fibroblasts cells were in G_0/G_1 phase. Differences between results might be attributed to the influence of cell types and passage. However, these results show that monitoring the cell-cycle stage distribution for each cell line and for each treatment before their use as donors is of great value.

In this study no significant different was observed in the G_0/G_1 cells that underwent the SS and CI treatments (91.11 ± 1.77 and 90.97 ± 1.18, $p > 0.05$), which is similar to results found for rabbit fibroblast (Liu *et al*., 2004) and porcine mammary cells (Prather *et al*., 1999). We also tested the response of transgenic cloned cattle fibroblasts to the cell cycle inhibitor roscovitine, which has been shown to arrest cultured fetal fibroblast cells from cattle in G_0/G_1 (Cho *et al*., 2005). Roscovitine, a potent inhibitor of specific Cdk_2 and maturation promoting factor (MPF) effectively arrested human fibroblasts in the G_0/G_1 phase of the cell cycle and, following its removal, cells arrested in G_0/G_1 resumed cycling and entered the S phase as expected (Alessi *et al*., 1998). Gibbons *et al*. (2002) reported that roscovitine-treated adult bovine granulosa cells were more efficiently synchronized in G_0/G_1 phase of the cell cycle than serum-starved cells (Gibbons *et al*., 2002). Our results showed that the efficiency of SS and CI treatment was higher than that of R treatment in synchronized cells in G_0/G_1 in ear fibroblast cells from transgenic cloned cattle. Cho *et al.* (2005) reported that 82.8% fetal fibroblasts cells in R were synchronized in G_0/G_1 phase (Cho *et al*., 2005). Furthermore, Gibbons (2002) showed that roscovitine culture could synchronize the donor cells and can increase cloning efficiency. Unfortunately, these finding

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have not been confirmed by other studies to date (Gibbons *et al*., 2002).

In our current research, the efficiency of SS and CI treatment was higher than that of R treatment, so that nuclear-transferred embryos were reconstructed using ear fibroblasts cells from transgenic cloned cattle and SS or CI treatment as donors. Although several researchers have confirmed that normal non-transgenic offspring could be obtained using SCNT with SS- or CI-treated cells as donors (Wilmut *et al*., 1997; Cibelli *et al*., 1998; Wells *et al*., 1999), information on effects of different cell cycle synchronization methods and on the subsequent development of re-cloned embryos is very scarce. Here in the present study, we observed obvious differences in the rate of fused couplets and blastocysts (89.88 ± 2.70 versus 87.40 ± 5.13; 44.10 ± 8.62 versus 58.38 ± 13.28, respectively, $p < 0.05$), while there was no difference in the number of cleaved embryos.

Three different treatments (confluent, roscovitine and serum starvation) could be synchronized effectively at the G_0/G_1 stages. Cho *et al*. (2005) analysed chromosomes from all of the three groups and showed that approximately 70% of cells had normal chromosome sets (58 autosomes and two sex chromosomes) (Cho *et al*., 2005). These results suggested that these donor treatments could be used for SCNT.

In conclusion, a detailed analysis of cell-cycle stages for ear fibroblasts from transgenic cloned cattle was presented in this study. To our knowledge, few studies have been carried out on the cell-cycle stage of ear fibroblast cells from transgenic cloned cattle. These cells could be synchronized effectively at the G_0/G_1 stages by all three treatments, confluent, roscovitine and serum starvation. Our data indicate that to harvest adequate G_0/G_1 stage cells for re-cloning transgenic cattle from fibroblasts and established from ear skin from newborn transgenic cloned calves, 3 days of treatment is sufficient regardless of method. Further research is needed to evaluate the developmental competence of cloned embryos derived from nuclear donors prepared by these treatments. A more efficient synchronization or different control regimes of the cell cycle stages needs to be addressed for ear fibroblasts from transgenic cloned cattle, which would be informative for future SCNT in transgenic cloned cattle re-cloning studies.

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