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Cell cycle synchronization of canine ear fibroblasts for somatic cell nuclear transfer

Ok Jae Koo[2], *Mohammad Shamim Hossein*[2], *So Gun Hong*[2], *Jose A. Martinez-Conejero*[3] *and Byeong Chun Lee*[1]

Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, Korea and Fundacion IVI, Instituto Universitario IVI, Valencia University, Valencia, Spain

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Summary

Cycle synchronization of donor cells in the G_0/G_1 stage is a crucial step for successful somatic cell nuclear transfer. In the present report, we evaluated the effects of contact inhibition, serum starvation and the reagents – dimethyl sulphoxide (DMSO), roscovitine and cycloheximide (CHX) – on synchronization of canine fibroblasts at the G_0/G_1 stage. Ear fibroblast cells were collected from a beagle dog, placed into culture and used for analysis at passages three to eight. The population doubling time was 36.5 h. The proportion of G_0/G_1 cells was significantly increased by contact inhibition (77.1%) as compared with cycling cells (70.1%); however, extending the duration of culture did not induce further synchronization. After 24 h of serum starvation, cells were effectively synchronized at G_0/G_1 (77.1%). Although synchronization was further increased gradually after 24 h and even showed significant difference after 72 h (82.8%) of starvation, the proportion of dead cells also significantly increased after 24 h. The percentage of cells at the G_0/G_1 phase was increased (as compared with controls) after 72 h treatment with DMSO (76.1%) and after 48 h treatment with CHX (73.0%) or roscovitine (72.5%). However, the rate of cell death was increased after 24 and 72 h of treatment with DMSO and CHX, respectively. Thus, we recommend the use of roscovitine for cell cycle synchronization of canine ear fibroblasts as a preparatory step for SCNT.

Keywords: Canine, Cell cycle synchronization, Ear fibroblast, Roscovitine, Somatic cell nuclear transfer

Introduction

Somatic cell nuclear transfer (SCNT) is generally considered to be more difficult in canids due to some unique species-specific reproductive characteristics. Although efforts to clone dogs began in 1997 with a US$2.3 million funded project (Pennisi, 2000), the first cloned dog was not produced until 2005, when our group reported the birth of 'Snuppy' (Lee *et al*., 2005a). However, efficiency of dog cloning, measured as development of transferred embryos to term, was only 0.2%. Two years later, we reported the birth of a litter of three cloned female puppies (Jang *et al*., 2007) that incorporated optimized timing of recovering *in vivo* oocytes from the oviduct (Hossein *et al*., 2007) and improvements in our SCNT technique (Jang *et al*., 2008b). Despite the improvements, overall efficiency of dog cloning was still extremely low (1.8%) and further optimization was needed for practical use.

Synchronization of the cell cycle is regarded as one of the key factors that determines the success of SCNT (Campbell *et al*., 1996). Since 'Dolly', the first cloned mammal using SCNT, was born (Wilmut *et al*., 2007), it is generally accepted that efficiency is improved when donor cells in the G_1 or G_0 phase are used for SCNT (Cibelli *et al*., 1998; Wakayama *et al*., 1998; Onishi *et al*., 2000).

[1]All correspondence to: Byeong Chun Lee. Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151–742, Korea. Tel: +822 880 1269. Fax: +822 873 1269. e-mail: bclee@snu.ac.kr
[2]Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, Korea.
[3]Fundacion IVI, Instituto Universitario IVI, Valencia University, Valencia, Spain.

Several methods have been used for synchronization of donor cells at the G_1 or G_0 phase. Generally, either serum starvation (Cho *et al*., 2002; Lee *et al*., 2005b; Wilmut *et al*., 2007) and contact inhibition (Boquest *et al*., 1999; Holker *et al*., 2005) are the most frequently used methods. However, recent reports have shown that some chemicals, including cycloheximide (CHX) (Goissis *et al*., 2007), dimethyl sulphoxide (DMSO) (Hashem *et al*., 2006) and roscovitine (Sun *et al*., 2008) also can synchronize cells at the G_1 or G_0 phase of the cycle. Interestingly, the use of roscovitine for cell cycle synchronization enhanced survival of cloned embryos as compared with serum starvation in cattle (Gibbons *et al*., 2002).

Only the contact inhibition method has been used for synchronization of donor cell cycles in dog cloning. Possibly, cloning efficiency would be improved if the method of cycle synchronization were to be optimized. However, only limited information about cycle synchronization of canine cells is available. Therefore, the present study was performed to evaluate various methods of cell cycle synchronization, towards the general goal of improving the efficiency of cloning in dogs.

Materials and methods

Chemicals and animals

All chemicals were obtained from Sigma–Aldrich Corp. unless otherwise stated. All animal procedures were performed in accordance with recommendations described in *The Guide for the Care and Use of Laboratory Animals* published by Institutional Animal Care and Use Committee of Seoul National University.

Isolation and culture of canine ear fibroblasts

Ear tissue was obtained from a 4-year-old female beagle dog. The tissue was washed three times in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) and minced with a surgical blade. The minced tissues were dissociated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen) for 1 h at 37°C. Trypsinized cells were washed once in Ca^{2+}- and Mg^{2+}-free DPBS by centrifugation at 300 *g* for 2 min, and seeded onto 100 mm plastic culture dishes (Becton Dickinson). Subsequently, cells were cultured for 8–10 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$, and 1% (v/v) minimal essential medium (MEM) non-essential amino acid solution (Invitrogen) at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured to confluence. The cells were subcultured (at intervals of 4–6 days) by trypsinization (0.1% trypsin and 0.02% EDTA) for 1 min. Trypsinized cells were allocated to three new dishes for further passaging, or stored in liquid nitrogen at –196°C. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) DMSO and 10% (v/v) FBS. Cells at passages three to eight were used for analysis.

Calculation of population doubling time

For each well of a 6-well plate (Becton Dickinson), 2 ml of cells were seeded in culture medium at a density of 5×10^4 cells/ml (Ci: initial concentration). After 24 h of culture, plates were removed from the incubator, cells from two wells (Cf: final concentration) were counted using a haemocytometer (Marienfeld GmbH) and the plate was returned to the incubator. Cell counts were repeated at 48 and 72 h of culture. Population doubling time (PDT) was calculated using the following equation:

$$\text{PDT} = \text{Time (h)} \times \ln 2 / \ln(\text{Cf}) - \ln(\text{Ci})$$

Finally, the mean values of PDT from the 24, 48 and 72 h groups were calculated.

Cell treatment and experimental design

Prior to analysis, cells were thawed and cultured in DMEM supplemented with 10% FBS until 70% confluency. Experiment 1 was conducted to evaluate the effect of contact inhibition on the cell cycle of canine fibroblasts. Cells were harvested by trypsinization upon reaching 70% (control) and 100% (0 h) confluency and at 24 and 48 h after reaching 100% confluency. In Experiment 2, we evaluated the effects of serum starvation on cell cycle synchronization. Cells were cultured in DMEM supplemented with 0.5% FBS for 24, 48 or 72 h after reaching 70% confluency. Cells in the control group were treated as described in Experiment 1. In Experiments 3, 4 and 5, we evaluated the effects of duration (24, 48 or 72 h) of DMSO (0.5%), CHX (10 μg/ml) or roscovitine (15 μM), respectively, on cell cycle synchronization. The concentrations of DMSO, CHX and roscovitine that were used were based on earlier reports performed in other species (Hashem *et al*., 2006, 2007). Preliminary trials were done to confirm that severe cytotoxic effects were not induced using the reagents at the selected concentration (data not shown). After each treatment, cultured cells were harvested for analysis by trypsination.

Cell cycle analysis

Harvested cells were resuspended in PBS and centrifuged at 1200 rpm at 4 °C for 5 min. The supernatant

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was decanted and the cells gently resuspended in PBS. Cells were fixed by adding 0.7 ml cold ethanol (70%) dropwise to 0.3 ml of cell suspension in PBS while vortexing gently. Fixed cells were stored at 4°C. For analysis, fixed cells were centrifuged as above, washed with cold PBS and re-centrifuged. Then, cells were resuspended in 0.25 ml PBS containing 5 μl of 10 mg/ml RNase and incubated at 37°C. After 1 h of incubation, cells were stained by adding 10 μl of 1 mg/ml propidium iodide. Fixed stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson). For each sample, 10 000 events were recorded and histograms of red fluorescence versus counts were generated to evaluate percentages of cells in each phase of the cell cycle. The proportion of cells in each phase was calculated using WinMDI software (Version 2.5, Joseph Trotter). Dead cells were determined by a sub-G_0/G_1 peak in the histograms. Further analysis, by gating only on G_0/G_1, S and G_2/M phases, was done using Excel software (2007 version, Microsoft). All experiments were replicated three times. The same control data (cycling cells) were used for all experiments in the study.

Statistical analysis

All data were subjected to one-way ANOVA followed by Tukey's test using Prism software (Version 4.0, GraphPad Software) to determine differences among experimental groups. Statistical significance was determined when the P value was less than 0.05.

Results

Effect of contact inhibition (Experiment 1)

As shown in Table 1, 70.1, 8.1 and 21.8% of cycling cells (controls) were in the G_0/G_1, S and G_2/M phases, respectively. The proportion of dead cells was 3.1% and the mean PDT was 36.5 h. The proportion of cells at $G_0/G_1$was significantly increased by contact inhibition for 0 to 48 h as compared with cycling cells (76.4% to 81.9% versus 70.1%, respectively). However, extending the duration of culture did not induce a higher frequency of cell cycle synchronization. The proportion of dead cells was significantly higher in the contact inhibition group as compared with the control group (5.3 to 6.8 % versus 3.1%, respectively).

Table 1 Effect of contact inhibition on cell cycle of canine ear fibroblasts.

Duration (h)	Dead cells (sub-G, %)	Cell cycle phase (gated, %)		
		G_0/G_1	S	G_2/M
Cycling cells	3.1 ± 0.4^{a}	70.1 ± 0.8^{a}	8.1 ± 0.4	21.8 ± 1.1^{a}
0	6.0 ± 0.5^{b}	77.1 ± 0.0^{b}	6.9 ± 0.4	16.0 ± 0.4^{b}
24	6.8 ± 0.1^{b}	81.9 ± 0.3^{b}	5.9 ± 0.0	12.2 ± 0.3^{c}
48	5.3 ± 0.5^{b}	76.4 ± 2.5^{b}	9.1 ± 0.6	14.5 ± 0.9^{b}

[a–c]Values with different superscripts in the same column represent significant differences ($P < 0.05$).

Table 2 Effect of serum starvation on cell cycle of canine ear fibroblasts.

Duration (h)	Dead cells (sub-G, %)	Cell cycle phase (gated, %)		
		G_0/G_1	S	G_2/M
Cycling cells	3.1 ± 0.4^{a}	70.1 ± 0.8^{a}	8.1 ± 0.4^{a}	21.8 ± 1.1^{a}
24	7.7 ± 0.0^{b}	77.1 ± 0.3^{b}	4.5 ± 0.0^{b}	18.3 ± 0.3^{a}
48	9.5 ± 0.5^{b}	77.9 ± 0.4^{b}	6.1 ± 0.1^{c}	$16.0 \pm 0.3^{a,b}$
72	18.3 ± 0.9^{c}	82.8 ± 1.2^{c}	3.4 ± 0.1^{d}	$13.8 \pm 1.3^{b,c}$

[a–d]Values with different superscripts in the same column represent significant differences ($P < 0.05$).

Effect of serum starvation (Experiment 2)

As shown in Table 2, after 24 h of serum starvation, the proportion of G_0/G_1 stage cells was significantly higher than cycling cells (77.1% versus 70.1%, respectively). Synchronization rate was further increased gradually after 24 h and shows significant difference in 72 h (82.8%). However, the proportion of dead cells also increased significantly after 24 h of starvation as compared with cycling cells (7.7% versus 3.1%, respectively) and continued to increase over time.

Effect of DMSO (Experiment 3)

After 24 h treatment with DMSO, the proportion of cells at the G_0/G_1 stage was significantly decreased as compared with the control group (67.6% versus 70.1%, respectively). However, the percentage of cells at the G_0/G_1 stage following DMSO treatment for 48 h (70.5%) was similar to that of controls and was significantly higher than controls at 72 h (76.1%). The proportion of dead cells that was increased after treatment of DMSO was increased at all time points as compared with that of controls (Table 3).

Table 3 Effect of DMSO on cell cycle of canine ear fibroblasts.

Duration (h)	Dead cells (sub-G, %)	Cell cycle phase (gated, %)		
		G_0/G_1	S	G_2/M
Cycling cells	3.1 ± 0.4^{a}	70.1 ± 0.8^{a}	$8.1 \pm 0.4^{a,b}$	$21.8 \pm 1.1^{a,b}$
24	7.2 ± 0.4^{b}	67.6 ± 0.1^{b}	$8.0 \pm 0.3^{a,b}$	24.4 ± 0.4^{a}
48	$3.9 \pm 0.0^{a,c}$	$70.5 \pm 0.1^{a,d}$	9.4 ± 0.3^{a}	20.1 ± 0.2^{b}
72	4.6 ± 0.2^{c}	$76.1 \pm 0.4^{c,d}$	7.7 ± 0.2^{b}	16.2 ± 0.6^{b}

[a–d]Values with different superscripts in the same column represent significant differences ($P < 0.05$).

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Table 4 Effect of cycloheximide on cell cycle of canine ear fibroblasts.

Duration (h)	Dead cells (sub-G, %)	Cell cycle phase (gated, %)		
		G_0/G_1	S	G_2/M
Cycling cells	3.1 ± 0.4^a	70.1 ± 0.8^a	8.1 ± 0.4^a	21.8 ± 1.1
24	3.1 ± 0.1^a	68.7 ± 0.6^a	8.2 ± 0.1^a	23.1 ± 0.2
48	6.3 ± 0.8^a	73.0 ± 0.1^b	4.6 ± 0.2^b	22.3 ± 0.3
72	13.8 ± 2.7^b	74.2 ± 0.0^b	4.2 ± 0.3^b	21.7 ± 0.3

[a–b]Values with different superscripts in the same column represent significant differences ($P < 0.05$).

Effect of cycloheximide (Experiment 4)

As shown in Table 4, there were no significant differences between cycling cells and 24 h treatment of CHX. However, 48 h treatment of CHX did induce a significantly higher incidence of cells synchronized at the of G_0/G_1 stage. Treatment with CHX for 72 h did not induce a higher frequency of G_0/G_1 cells, but it did cause a significant increase in the proportion of dead cells.

Effect of roscovitine (Experiment 5)

At 24 h of culture, the percentages of cells at G_0/G_1 cells and G_2/M were significantly lower than in the control group, while the proportion of cells at the S phase was significantly increased. However, synchronization of cells at the G_0/G_1 phase was significantly higher after 48 h of roscovitine treatment and showed a further increase after 72 h treatment (Table 5). The proportion of dead cells was not different from that of controls after 24 h of roscovitine treatment and was significantly lower than controls in the 48 and 72 h groups.

Discussion

In present study, we analysed various cell cycle synchronization methods in canine ear fibroblasts. Cell cycle coordination between nuclear donor and recipient is considered to be a crucial factor for successful cloning in order to maintain correct ploidy of embryos at the end of the first cell cycle (Campbell *et al.*, 1996). Donor nuclei must be in G_0 or G_1 stage when transferred to non-activated oocytes arrested at the metaphase (M)II stage. We have used *in vivo* matured oocytes arrested at the MII stage for dog cloning (Lee *et al.*, 2005a; Jang *et al.*, 2007, 2008a); therefore, donor cells should be synchronized at the G_0 or G_1 stage.

Contact inhibition was the method used for cell cycle synchronization in previous canine cloning studies. Cultured cells exit from the cell cycle and synchronize at the G_0 phase, a non-dividing state, once they come into contact with each other (Suzuki *et al.*, 2000). Although little is known about the molecular mechanisms involved in this phenomenon, it is a well known and frequently used method for cell cycle synchronization. As expected, a significantly higher proportion of G_0/G_1 cells was obtained from contact inhibition treatment (Table 1), but further synchronization did not occur until after an additional 24 h or 48 h of treatment.

Serum starvation has been perhaps the most frequently used method for cell cycle synchronization in SCNT research since the birth of 'Dolly' the sheep (Wilmut *et al.*, 2007). Deprivation of serum from culture medium reduces or removes many growth factors used for cell cycle progression. As a result, cells undergo a rapid exit from the cell cycle and into the G_0 phase, which is characterized by low metabolic activities (Iyer *et al.*, 1999; Kues *et al.*, 2000). In the present study, serum starvation was the most effective method for cell cycle synchronization to the G_0/G_1 phase (Table 2). The highest proportion of synchronized cells obtained in our experiments was produced at 72 h of treatment by this method. Moreover, even after only 24 h of serum starvation, the proportion of cells synchronized at G_0/G_1 was similar to that obtained by the other methods after 72 h of treatment.

Another approach for cell cycle synchronization is the use of chemicals that control check points of cell cycle progression. Cell cycle progression is regulated by serine/threonine kinases, termed cyclin-dependent kinases (CDKs), the activities of which oscillate during the cell cycle. CDKs are associated with the positive co-activators, cyclins, and the negative regulators, CDK inhibitors (Pines, 1995; Sherr & Roberts, 1995). In mammalian cells, cyclin D–CDK4/CDK6, cyclin E–CDK2, cyclin A–CDK2 and cyclin B–CDK2 are the primary cyclin–CDK complexes that regulate the progression of G_0/G_1 to S, mid G_1 to late G_1/S, S phase entry and G_2 to M phases, respectively (Johnson & Walker, 1999; Suzuki *et al.*, 2000). In the present study, we examined the effect of several chemicals (DMSO, CHX and roscovitine), known as cell cycle regulators, on the cell cycle of canine ear fibroblasts.

Table 5 Effect of roscovitine on cell cycle of canine ear fibroblasts.

Duration (h)	Dead cells (sub-G, %)	Cell cycle phase (gated, %)		
		G_0/G_1	S	G_2/M
Cycling cells	3.1 ± 0.4^a	70.1 ± 0.8^a	8.1 ± 0.4^a	21.8 ± 1.1^a
24	$2.4 \pm 0.2^{a,b}$	64.0 ± 0.3^b	21.6 ± 1.3^b	14.1 ± 1.4^b
48	1.5 ± 0.1^b	72.5 ± 0.6^c	18.2 ± 0.5^c	9.3 ± 1.1^c
72	$2.5 \pm 0.3^{a,b}$	78.2 ± 0.2^d	14.7 ± 0.4^d	7.2 ± 0.2^c

[a–d]Values with different superscripts in the same column represent significant differences ($P < 0.05$).

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DMSO reduces expression of cyclin D and induces the overexpression of cyclins A, B and E (Jiang *et al*., 1994; Ponzio *et al*., 1998). Here, treatment with DMSO decreased the proportion of G_0/G_1 cells at 24 h of treatment (Table 3), possibly due to overexpression of cyclins A, B and E. Expression of cyclins A, B and E enhances progression from late G_1 to the G_2/M phase, therefore the proportion of G_0/G_1 cells was reduced at 24 h of DMSO treatment. However, after 72 h of treatment, cells were arrested at early G_1 stage due to absence of cyclin D.

Cycloheximide is a protein synthesis inhibitor that is capable of inhibiting mitosis (Verbin & Farber, 1967) and inducing elongated cell phases (Okuda & Kimura, 1988; Goissis *et al*., 2007). Because protein synthesis was restricted, the effects of CHX are similar to those of serum starvation. In particular, CHX reduces cyclin D and synchronizes porcine fibroblast cells at the G_0/G_1 stage (Goissis *et al*., 2007). In the present study, canine ear fibroblasts were also synchronized at the G_0/G_1 stage after 48 h treatment with CHX (Table 4).

Roscovitine is a CDK2 inhibitor that reduces the effects of cyclins E and A. As a result, roscovitine treatment has been shown to effectively induce cell cycle synchronization at the G_1/G_0 stage in various species (Gibbons *et al*., 2002, 2003; Hinrichs *et al*., 2006). In canine ear fibroblasts, we found that roscovitine induces synchronization at the G_0/G_1 stage after 48 h of treatment. A recent study reported that treatment with various concentrations (5 to 45 μM) of roscovitine did not induce G_0/G_1 synchronization in canine dermal fibroblasts (Khammanit *et al*., 2008); however, the roscovitine treatment interval was for 24 h only. In our opinion, as roscovitine is a CDK2 inhibitor, for proper induction of cell cycle synchronization the treatment period should exceed the PDT. In our study and that of Khammanit *et al*. (2008), the PDT was 36.5 h and 46 h, respectively. Because the PDT was longer than 24 h in both studies, treatment with roscovitine for 24 h would have had no effect even with the use of high concentrations. Thus, we suggest that to determine the proper duration of treatment for chemicals to be used in the induction of cell cycle synchronization, the PDT should first be established.

In present study, all five methods we examined were able to induce cell cycle synchronization at the G_0/G_1 stage. We had two primary consideration in attempting to optimize our SCNT procedure. The first concern was the proportion of dead cells in each treatment group. It is well known that serum starvation induces DNA fragmentation and apoptosis (Kues *et al*., 2000) and this damage may be a cause of embryo/fetal loss and abortion in cloned embryos (Kato *et al*., 1998; Vignon *et al*., 1998; Hill *et al*., 1999). A high portion of cell death may be consistent with DNA damage. If the damage was not repaired properly during the reprogramming of the SCNT embryos, efficiency of fetal development to term will be decreased (Gibbons *et al*., 2002). In present study, all methods except roscovitine treatment induced significantly higher proportions of dead cells as compared with cycling cells (controls). We used 15 μM of roscovitine and the proportion of dead cells was not increased, even after 72 h of treatment. However, Khammanit *et al*. (2008) reported that higher concentrations of roscovitine (30 and 45 μM) did induce cell death in canine dermal fibroblasts. Thus, we recommend that, for cell cycle synchronization, roscovitine be used at a low concentration (i.e. about 15 μM).

The other factor of primary importance was to find a method that would induce synchronization at the G_1 phase, rather than the G_0 phase. Several reports have shown that developmental competence of cloned embryos derived from donor cells at the G_1 stage was higher than that of embryos derived from cells at the G_0 stage (Kasinathan *et al*., 2001; Wells *et al*., 2003). Two of the chemical methods, DMSO and roscovitine, theoretically induce cells into the G_1 stage because they inhibit activity of cyclins, rather than inducing a resting, non-dividing state. From both perspectives, we recommend treatment with roscovitine for cell cycle synchronization in order to improve efficiency of the SCNT procedure.

In conclusion, we have shown that canine fibroblast cells can be synchronized at the G_0/G_1 stage using contact inhibition, serum starvation and treatment with DMSO, CHX and roscovitine. We recommend the use of roscovitine treatment, because it exhibited less toxicity and enabled synchronization at the G_1 stage. Previous reports on SCNT in the bovine (Gibbons *et al*., 2002) and equine (Hinrichs *et al*., 2007) species demonstrated that cell cycle synchronization using roscovitine reduced embryonic and fetal loss, thus we expect that its application to canine cells will improve the efficiency of canine SCNT embryo production. Therefore, in our future efforts to produce cloned dogs we intend to use roscovitine-treated donor cells.

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