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Full Length Research Paper

Cell cycle regulation of human foreskin fibroblasts

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Therapeutic cloning has broad application prospects in the medical field. However, interspecies cloning efficiency is very low. It is generally believed that G0/G1 stages of cell cycle are more beneficial to cell reprogramming of cloning. The purpose of this study was to evaluate the effects of serum starvation, contact inhibition and 2-methoxyestradiol (2-ME) treatment on cell-cycle synchronization in the G0/G1 stage in human foreskin fibroblasts. Our results show that the proportion of G0/G1 cells from the serum-starved group at 3 and 4 days was significantly higher when compared with 2 days and cells not subjected to serum starvation (97.3% and 97.99% vs. 93.8% and 81.59%, respectively; $p < 0.01$). No significant difference was observed among cells with 3 and 4 days of starvation. The proportion of contact-inhibited G0/G1 cells significantly increased when compared with cells with 70 to 80% confluence (84.89 vs. 81.59%; $p < 0.05$) and decreased significantly when compared with cells subjected to serum starvation for 3 and 4 days. In cells treated with 2-ME (0.1 to 10 μM) combined with vibration, the proportion of G0/G1 cells was not significantly different (74.32, 77.75, 78.65, 76.96, 80.39 and 81.4%; $p > 0.05$). After the recovery of cells that were frozen for 4 to 5 months, the proportion of cells in the G0/G1 phase was significantly lower when compared with normal cells (75.14 vs. 81.59%; $p < 0.05$). Our results show that serum-starvation for 3 days is the most effective method for synchronizing human foreskin fibroblasts in the G0/G1 phase, which is may more suitable as donor cells for cloning.

Key words: Cell cycle synchronization, 2-methoxyestradiol, serum starvation, culture to confluence, human foreskin fibroblast, induced pluripotent stem cells.

INTRODUCTION

The emergence of induced pluripotent stem cells (iPSCs) opens a new field of stem cell research and provides a new research method for organ transplantation and gene repair (Amabile and Meissner, 2009). The latest reports show that iPSCs cells have the same developmental potential as embryonic stem cells but with significant differences in their gene expression and methylation (Stadtfeld et al., 2010; Lister et al., 2011). Zhao et al. (2011) found that when compared with embryonic stem cells, iPSCs still caused immune rejection when there is auto transplantation. Furthermore, because of the low efficiency of reprogramming, carcinogenicity and immune rejection of iPSCs, their use is far from ideal when dispensable, and discovering embryonic stem cell compared with embryonic stem cells (Pappas and Yang, 2008). Embryonic stem cell research continues to be sources for patient remains a difficult problem. In addition to fertilization *in vitro*, nuclear transfer is a good way to generate human blastocysts of the same genetic background (Hwang et al., 2005). To generate a potentially unlimited autologous source of totipotent human embryonic stem cells for transplantation medicine, research has utilized human skin fibroblast cells as nuclear donor cells and enucleated oocytes from humans and other species as recipient cytoplasts to reconstruct nuclear-transferred embryos (Zhang and Zhou 2008; Yamanaka, 2007).

One of the most important factors for determining the success of the development of cloned embryos is the cell cycle stage of nuclear donor cells (Hashem et al., 2007; Sun et al., 2008). The G0/G1 stages of the cell cycle may provide optimal results in terms of proper ploidy and the development of reconstructed embryos (Hashem, 2006). Several methods can be employed to control the cell

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Abbreviation: 2-ME, 2-Methoxyestradioin

cycle, such as serum deprivation, contact inhibition and chemical inhibitors. Chemical inhibitors can be divided into the following types: (i) Protease inhibitors, such as 6-dimethylaminopurine (6-DMAP), cycloheximide and cytochalasin B (Hashem, 2006), (ii) antioxidants, such as β-mercaptoethanol, cysteine and glutathione (Hashem, 2006), (iii) cyclin-dependent kinase 2 (CDK2) inhibitors, such as roscovitine (Choresca et al., 2009) and butyrolactone I (Kues et al., 2000), (iv) polymerization of microtubules inhibitors, such as colchicines (Khammanit et al., 2008) and demicoline (Liu et al., 2004), (v) the DNA polymerase inhibitor aphidicolin (Liu et al., 2004) and (vi) the topoisomerase inhibitor Hoechst 33342 (Kühholzer and Prather, 2001). More experiments are needed to elucidate the mechanism of action of some inhibitors, such as 2-methoxyestradiol (2-ME).

We chose foreskin fibroblast cells as the material, because the cells have better activity *in vitro*, such as, high proliferative ability and good morphology as well (Hovatta et al., 2003). To optimize donor nuclei treatment prior to nuclear transfer, this study examined the cell cycle characteristics of human foreskin fibroblasts under serum deprivation for 2, 3 and 4 days, contact inhibition, and 2-ME treatment at different concentrations. 2-Methoxyestradiol is an endogenous metabolite of estradiol and is one of the most promising chemotherapeutic agents (Qadan et al., 2001). The toxic effects of 2-ME are not mediated by conventional estrogen receptors (Maran et al., 2002). It can inhibit tumor growth and angiogenesis, but it does not harm quiescent or proliferating normal cells (Perez-Stable, 2006). This study sought to synchronize human foreskin fibroblast cells in the G0/G1 phase using 2-ME combined with vibration. The data were analyzed using a BD FACS Calibur cytometer flow cytometer at 488 nm.

MATERIALS AND METHODS

Establishment of foreskin fibroblast cell lines

Foreskin tissue was taken from a 9-year-old boy at an orphanage after circumcision surgery with the guardian's consent (Inner Mongolia Medical College Hospital Centre for Reproduction provided the material). The foreskin tissue was washed three times in calcium and magnesium chloride-free phosphate-buffered saline (PBS) supplemented with 200 U penicillin-streptomycin (PS), immersed in Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS) and 100 U PS, and returned to the laboratory within 2 h. The tissue was disinfected for 1 min with 75% alcohol and washed three times in PBS again. After the final wash, the foreskin tissue was cut, minced and affixed in a 25 ml culture flask. Four hours later, DMEM/F12 supplemented with 100 U PS and 10% FBS (v/v) was added to the tissue. The culture dishes were placed in an incubator at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was changed for 3 to 4 days until the cells had reached 70 to 80% confluence. The cells were then digested by 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) at 37°C for 1 min and dispersed pipetting. After digestion was terminated by DMEM/F12 containing 10% FBS, the trypsinized cells were reseeded in a new 25 ml culture flask for subculture. In this process, the attached epithelial cells were discarded. The cells were frozen in DMEM/F12 containing 10% dimethyl sulfoxide (DMSO) and 20% FBS in liquid nitrogen. In Experiments 1 and 2, we used passage 6 of the culture. However, in Experiment 2, the cells were frozen in passage 4 for 4 to 5 months, then thawed and generated to passage 6.

Experimental treatments

Experimental 1 evaluated the effects of culture to confluence and serum starvation on cell cycle synchronization in the G0/G1 phase. Normally, growing fibroblasts that reached 70 to 80% confluence were used as a control. To assay the effect of confluence, when the cells reached 95 to 100% confluence, they were fixed and analyzed. To assay the effect of serum starvation, cells were grown to 70 to 80% confluence, subjected to serum starvation for 2, 3, and 4 days by reducing FBS to 0.5%, harvested, and fixed for analysis. The experiments were replicated three times.

Experimental 2 was essentially conducted according to a previous study, and cell cycle synchronization was attained by using 2-ME (Sigma) at 0.1, 0.5, 1, 2, 5 and 10 μM for 30 min followed by shaking. After shaking the plate for 1 min, DMEM/F12+FBS with the same concentration of 2-ME were added to the same plate, and the culture was continued for 30 min. We then digested and collected the cells and fixed them with 75% alcohol for analysis.

The cell number before plating was normalized by a hemocytometer, and each treatment was performed in triplicate with 1 x 10^5 cells per plate. The experiments were replicated twice.

Cell fixation, staining and cell cycle analysis

Cultured cells were harvested using 0.25% trypsine-EDTA (Sigma) and resuspended in DMEM/F12 at 1 x 10^7 cells/tube. The cells were resuspended in PBS and centrifuged at 1500 rpm at 4°C for 5 min. The supernatant was decanted, and the cells were gently resuspended in PBS. Cells were fixed by adding 0.7 ml cold ethanol (70%) drop-wise in a tube containing 0.3 ml of cell suspension in PBS while gently vortexing. Fixed cells were left undisturbed at 4°C for 48 h before further analysis. The fixed cells were centrifuged as earlier mentioned, washed once with cold PBS, and recentrifuged. Centrifuged cells were resuspended in 0.25 ml PBS containing 5 μl of 10 mg/ml RNase (Sigma) and incubated at 37°C for 1 h. Incubated cells were stained by adding 10 μl of 1 mg/ml propidium iodide (Sigma). The cells were then analyzed by FACS-CALIBUR at 488 nm. Histogram plots were created using Cell Quest software (Beckton Dickinson, San Jose, CA). The percentages of cells within the various phases of the cell cycle were calculated using the modfit program.

Statistical analysis

Differences among groups were analyzed using one-way analysis of variance (ANOVA) and the protected least significant different (LSD) test using general linear models in the SAS software program. When statistically significant effects were found for each experimental parameter, data were compared using the least squares method. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Experiment 1

The effects of different cell culture conditions, control,

Table 1. Effect of fully confluent cells on different cell cycle stages.

Cell culture condition	Percentage of different cell cycle stages (mean ± SD)		
	G0/G1	S	G2/M
70 to 80% confluence	81.59 ± 0.49[a]	7.79 ± 0.31[b]	10.92 ± 0.45[b]
Complete confluence	84.89 ± 0.78[b]	6.63 ± 0.36[a]	8.48 ± 0.51[a]

[a, b]Within columns, values with different superscripts are significantly different ($p < 0.05$).

Table 2. Effect of serum starvation on different cell cycle stages.

Cell culture condition	Percentage of different cell cycle stages (mean ± SD)		
	G0/G1	S	G2/M
Serum starvation (2 days)	93.81 ± 0.82[b]	1.28 ± 0.15[b]	4.92 ± 0.82[b]
Serum starvation (3 days)	97.36 ± 0.43[c]	2.26 ± 0.63[b]	0.39 ± 0.31[c]
Serum starvation (4 days)	97.99 ± 0.51[c]	1.63 ± 0.21[b]	0.38 ± 0.3[c]

[b,c]Within columns, values with different superscripts are significantly different ($p < 0.01$).

complete confluence, and serum-starvation on the different cell cycle phases are shown in Tables 1 to 3. Table 1 shows significant differences in the G0/G1 ($p < 0.01$), S and G2/M ($p < 0.05$) phases of the cell cycle in control and fully confluent cells. Additionally, as shown in Table 1, human foreskin fibroblasts without any treatment and with only 70 to 80% confluence displayed a relatively high proportion of cells in the G0/G1 phase (81.59%). Table 2 shows that with 2 (93.81%), 3 (97.36%) and 4 days (97.99%) of serum starvation, the proportion of cells in the G0/G1 phase was significantly higher than the untreated group (81.59%; $p < 0.01$). However, with 3 days of starvation, the proportion of G0/G1 phase cells was not significantly different when compared with 4 days. When compared with the controls, the fraction of S phase cells in the serum starvation group was significantly different. No difference in the proportion of S phase cells was observed between the groups subjected to starvation for 3 and 4 days. The percentage of G2/M phase cells was significantly decreased ($p < 0.01$) as the days of serum starvation increased. We then compared the cell cycle distribution of fully confluent and serum-starved cells (3 days). The culture in serum starvation medium (97.36%) yielded a significantly higher percentage of cells arrested in the G0/G1 phase ($p < 0.01$) when compared with cells cultured to confluence (84.89%). For the human foreskin fibroblasts, the 3 to 4 days of serum starvation synchronized cells very effectively in the G0/G1 phase.

Experiment 2

The effects of different concentrations of 2-ME followed by shaking on the synchronization of cell fibroblasts are shown in Table 4.The statistical analysis indicates that this method cannot significantly increase or decrease the G0/G1 phase proportion in human foreskin fibroblasts ($p > 0.05$). Additionally, the proportion of S and G2/M phase cells did not significantly change ($p > 0.05$). Human foreskin fibroblast cells were grown to 70 to 80% confluence, and the cell cycle distribution of cells subjected to 4 to 5 months of freezing and control cells were compared. Table 5 shows that the proportion of G0/G1 phase cells in the frozen cells was significantly lower when compared with cells that were not frozen ($p < 0.01$), and the percentage of S and G2/M phase cells significantly increased ($p < 0.05$). The results suggest that cryopreservation can significantly affect the cell cycle state.

DISCUSSION

In this study, we examined the effect of fully confluent, serum-starved cells and treatment with different concentrations of 2-ME on cell cycle synchronization in the G0/G1 phase and the proportion of normal cells in the G0/G1 phase when compared with the cells that were frozen for 4 to 5 months. We found that in human foreskin fibroblasts without any processing, the cells were still in the G0/G1 phase at a relatively higher proportion (81.59%) than other cells reported before (Francesca et al.,1998), similar results of human foreskin fibroblasts were also reported by Li et al. (2009), this maybe the characteristics of these cells. Complete confluence and serum starvation can effectively synchronize the cell cycle in the G0/G1 phase, but serum starvation had more marked effects in our experiment. The proportion of cells in the G0/G1 phase were not significantly different in cells subjected to serum starvation for 3 and 4 days, indicating that serum starvation for 3 days is sufficient to synchronize the cell cycle. As the time of serum starvation increased, the proportion of cells in the G0/G1

Table 3. Effect of complete confluence and serum starvation on different cell cycle stages.

Cell culture condition	Percentage of different cell cycle stages (mean ± SD)		
	G0/G1	S	G2/M
Complete confluence	84.89 ± 0.78[a]	6.63 ± 0.36[a]	8.48 ± 0.51[a]
Serum starvation (3 days)	97.36 ± 0.43[b]	2.26 ± 0.63[b]	0.39 ± 0.31[b]

[a,b]Within columns, values with different superscripts are significantly different ($p < 0.01$).

Table 4. Effects of different concentrations of 2-ME on the synchronization of cell fibroblasts.

2-Methoxyestradiol concentration (µM)	Percentage of different cell cycle stages (mean ± SD)		
	G0/G1	S	G2/M
0	75.14 ± 0.62[a]	9.09 ± 0.66[a]	15.77 ± 0.04[a]
0.1	74.32 ± 4.45[a]	7.85 ± 0.88[a]	17.84 ± 3.56[a]
0.5	77.75 ± 1.22[a]	5.86 ± 0.57[a]	16.4 ± 1.79[a]
1	78.65 ± 4.33[a]	7.08 ± 3.03[a]	14.27 ± 1.3[a]
2	76.96 ± 7.52[a]	8.07 ± 3.74[a]	14.98 ± 3.78[a]
5	80.39 ± 5.45[a]	4.74 ± 1.09[a]	14.88 ± 4.36[a]
10	81.4 ± 3.26[a]	4.57 ± 0.87[b]	14.04 ± 2.39[a]

[a,b]Within columns, values with different superscripts are significantly different ($p < 0.05$). Cells with 70 to 80% confluence before treated.

Table 5. Effect of freezing on different cell cycle stages.

Cell culture condition	Percentage of different cell cycle stages (mean ± SD)		
	G0/G1	S	G2/M
70 to 80% confluence	81.59 ± 0.49[a]	7.79 ± 0.31[b]	10.92 ± 0.45[b]
70 to 80% confluence (after freezing)	75.14 ± 0.62[b]	9.09 ± 0.66[a]	15.77 ± 0.04[a]

[a,b]Within columns, values with different superscripts are significantly different ($p < 0.05$).

phase increased, and the proportion of cells in the S and G2/M phases decreased. However, the proportion of G2/M phase cells continuously decreased. The proportion of S phase cells somewhat decreased but not continuously. We speculate that the number of cells in metaphase have a threshold in a cell population. The existence of this threshold may be attributable to the cell type and the different culture conditions. Our experiment also found that cells frozen for 4 to 5 months and normal cultured cells, although, in the same passage, had different proportions of cell cycles. This shows that freezing significantly affects the cell state, and the effect may last for a period of time after thawing. The freezing process may play a role in determining the cell cycle (Lima-Neto et al., 2010). Cryopreservation in DMSO may have also affected the cell cycle.

This study essentially followed previous methods (Urakawa et al., 2004) to obtain a higher percentage of cells arrested in the G0/G1 phase. Different concentrations of 2-ME combined with vibration did not significantly affect cell cycle synchronization in human foreskin fibroblasts. Urakawa et al. (2004) used 1 µm 2-ME combined with vibration for 30 min, repeated the treatment, and then identified cells with the appropriate diameter (20 µm) from the cell population. The number of cells synchronized in the G0/G1 phase significantly improved the rate of nuclear transfer blastocysts after this treatment. However, this method did not significantly increase the rate of cells in the G0/G1 phase. Two possibilities may have led to these results. First, the different types of cells may have yielded disparate results. Second, the selection of a suitable cell diameter (20 µm) in this experiment may have played a major role. Because cell diameter generally correlates with DNA content (Zhou et al., 2001), most foreskin fibroblast cells with a diameter of 20 µm are in the normal M-phase. Further experiments are needed to address this issue. However, with increasing concentrations of 2-ME in this study, we observed a trend in the proportion of cells in the G0/G1 phase, which was similar to human prostate cancer cell cycle changes observed in a previous study that incubated cells for a prolonged period of time (Perez-Stable, 2006). We may conclude that repeated 2-ME treatment combined with shock for 1 min may have

affected the present results, but not appreciably. To achieve synchronization, a longer drug treatment or more appropriate cell diameter may be required.

2-Methoxyestradiol is an endogenous metabolite of estradiol with promise for cancer chemotherapy. 2-Methoxyestradiol does not harm quiescent or proliferating normal cells and does not exert significant estrogenic effects on estrogen receptor binding (Maran et al., 2002). 2-Methoxyestradiol appears to block mitosis by inhibiting microtubule assembly and interfering with mitotic spindle dynamics (Bu et al., 2002). Low doses of 2-ME resulted in G1 cell cycle arrest in a p53 wildtype LNCaP cell line, but with minimal induction of apoptosis. Similar doses of 2-ME did not cause G1 accumulation in the other cells, possibly because their G1 cell cycle checkpoints are defective (Perez-Stable, 2006). Treatment of cells with a high concentration of 2-ME increased the proportion of cells in the G2/M phase, with a concomitant decrease in G1 (Perez-Stable, 2006). Previous studies have shown very different results with 2-ME depending on the cell type. Different cells may have different mechanisms of action. The heterogeneity of these results may also be attributable to different drug concentrations.

In conclusion, our results show that the human foreskin fibroblasts stay at G0/G1 phase at a higher proportion (81.59%) than other cells without any processing. Complete confluence and serum starvation can effectively synchronize the cell cycle in the G0/G1 phase (84.89 and 97.99%), but serum starvation had more marked effects in our experiment. These results suggest that human skin fibroblast cells may be a good source of donor cells to reconstruct nuclear transfer embryos.

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