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Flow cytometric cell-cycle analysis of cultured fibroblasts from the giant panda, *Ailuropoda melanoleuca* L.

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Abstract

In animal cloning, it is generally believed that the inactive diploid G_0 or G_1 stage of the cell cycle is beneficial to initiate cell-cycle coordination and reprogramming following transfer of the donor nucleus. Previous experiments have demonstrated that serum starvation results in quiescent cell stage. Some experiments show that the majority of cells in a fully confluent cell culture are also in an inactive G_1 stage.

In order to provide more G_0/G_1 stage cells for giant panda cloning, we carried out a flow cytometric analysis of the cell cycle of fibroblasts from the abdominal muscle of a giant panda at different passage numbers under different growth conditions, and after different periods of serum starvation. The percentage of G_0+G_1 stage cells differed significantly under different growth conditions. Serum starvation effectively increased the percentage of G_0+G_1 stage cells, and the cell cycle characteristics following serum starvation for varying periods of time differed with this and the initial confluency of the cultures. The data should help in choosing the optimal stage for preparing donor cells as well as increasing the potential cloning efficiency in our study of giant panda cloning.

Keywords: Fibroblast; Giant panda; Cell cycle; Flow cytometry; Preparation for cloning

1. Introduction

Somatic cell cloning has succeeded in sheep (Wilmut et al., 1997), mice (Wakayama and Yanagimachi, 1999; Wakayama et al., 1998), cattle (Hill et al., 2000; Kato et al., 1998; Keefer et al., 2001; Kubota et al., 2000; Wells et al., 1998, 1999; Zakhartchenko et al., 1999a,b), goat (Baguisi et al., 1999) and pigs (Polejaeva et al., 2000). The conditions of nuclear donor cells clearly influences the efficiency of animal cloning, and studies have shown that progeny can be successfully obtained by nuclear transfer of serum-starved fibroblast cells in all the abovementioned references. It is generally believed from this evidence that an inactive, diploid G_0 or G_1 stage of the cell cycle is beneficial to initiate cell-cycle coordination and reprogramming of the donor nucleus. Experiments in sheep demonstrated that the serum starvation resulted in a beneficial, quiescent cell stage, and arrest in G_0 by serum starvation was the key in allowing donor somatic cells to support development of embryos to term (Wilmut et al., 1997). Other research found that the majority of cells in a fully confluent cell culture are also in an inactive G_1 stage and can be used as nuclear donors in animal cloning (Betthauser et al., 2000; Onishi et al., 2000; Zou et al., 2001).

In our interspecies giant panda cloning study, we have successfully reached the blastocyst stage using serum-starved somatic cells of giant panda, and plan to further develop this research (Chen et al., 1999). The giant panda, *Ailuropoda melanoleuca*, is a critically endangered species with a wild population estimated at only 1000 individuals. Thus cells and tissues are difficult to obtain. Indeed, only three opportunities for cell culture have arisen since the research plan for giant

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panda cloning began in 1997, and therefore the preparation of donor cells has become very much more important and indispensable in the interim. Since many G_0/G_1 stage cells are required for cloning, we used flow cytometry to estimate the percentage of G_0/G_1 stage cells at different passage numbers, under different growth conditions and with varying exposures to serum starvation in order to get the most efficient donor cells for giant panda cloning.

2. Materials and methods

2.1. Culture of fibroblasts from giant panda

The abdominal muscle was collected from the youngest of triplets (female, 3 days old, Wolong Nature Reserve, China) as soon as it died. A large sample of abdominal muscle was rinsed three times with sterile saline containing 200 U/ml penicillin and 200 μg/ml streptomycin, and minced finely with crossed scalpels. Small pieces were disaggregated in cold 0.25% trypsin (GIBCO BRL, Life Technologies, Irvine, Scotland) for 4 h at 4 °C, and then transferred to 37 °C for 30 min. The disaggregated tissues and cells were seeded in culture flasks, to which were added Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham)=1:1 (D-MEM/F-12, GIBCO BRL) and 20% fetal bovine serum (FBS, GIBCO). The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Cell-specific marker staining

The cytoskeleton of 132 m cells were analyzed by immunochemical method as described previously (Han et al., 2001). Briefly, the sixth passage '132 m' cells cultured on 8 × 8 mm cover glass were fixed in 3.7% paraformaldehyde for 20 min at room temperature (RT), then washed twice by 0.01 mol/l PBS (pH 7.4) containing 0.01%Triton X-100 (T-PBS). Treated the cover glasses in 0.1% Triton X-100 containing 3 mg/ml BSA for 30 min at 37 °C, then washed with T-PBS three times at RT. Reduced free aldehydes in 0.01 mol/l PBS (pH 7.4) containing 150 mmol/l glycine and 3 mg/ml BSA for 30 min at 37 °C. Vimentin (fibroblast-specific cell marker) were labeled with monoclonal antibody to vimentin (Sigma Chemical Co.) for 0.5 h at 37 °C, then washed with T-PBS three times at RT. Then both were incubated with FITC-anti-mouse IgG (Sigma Chemical Co.) for 0.5 h at 37 °C and washed with T-PBS three times at RT. DNA were labeled with 10 μg/ml propidium iodide (PI, Sigma Co.) for 10 min and washed with T-PBS for 10 min. Then observed under the Laser Confocal Microscope (Leica TCS-4D, Heidelberg, Germany).

2.3. Cell treatment

Cell-cycle comparisons were made among cycling cells, serum starvation cells, and cells cultured to different confluent state. Cells of different passages were seeded in culture flasks and cultured in D-MEM/F-12 with 10% FBS to different confluent state. Cells with different confluent states were washed three times in D-MEM/F-12 with 0.5% FBS and cultured in this low serum medium for different periods.

2.4. Cell-cycle analysis by flow cytometry

Cells were trypsinized and resuspended in D-MEM/F-12 at a concentration of approximately 1×10^6 cells/tube. Cells were pelleted and resuspended twice in D-PBS (136.8 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Cells were fixed with 75% ethanol overnight at 4 °C, washed with D-PBS and pelleted. Treatment for 30 min with RNAase (1 mg/ml) at 37 °C followed, after which the cells were pelleted and resuspended in D-PBS. Cells were stained for 5 min at RT with 100 μg/ml PI containing 0.1% Triton X-100 before flow cytometric analysis. Cells were analyzed on a FACS Calibur (Becton-Dickinson, San Jose, CA, USA). The single-parameter histogram of DNA allowed discrimination of cell populations existing in G_0/G_1 (2C DNA content), S (between 2C and 4C), and G_2/M (4C) phases of the cell cycle. Percentages were calculated based on the gated cells displaying fluorescence correlating to a cell-cycle stage. Cell-cycle data were analyzed by ANOVA using SPSS 10.2 software package.

3. Results

3.1. Characterization of cells from abdominal muscle of giant panda

The primary spindle-shape fibroblasts from abdominal muscle of giant panda were cultured for 3 days and then subcultured in a conventional manner; this was designated '132 m'. Phase-contrast microscopy showed that 132 m cells take on normal fibroblasts morphology characteristics after three subculturings (Fig. 1).

Cell-specific marker staining showed that the cultured cells contain normal vimentin, confirming they were fibroblasts, as seen by laser confocal microscopy (Fig. 2).

3.2. Cell-cycle analysis of 132 m fibroblasts from different passages at 70–85% confluence

We analyzed cultured fibroblasts that were 70–85% confluent by flow cytometry at passages 5, 6, 7, 8, 9, 10, and 13. The results show that 72–79% of the fibroblasts

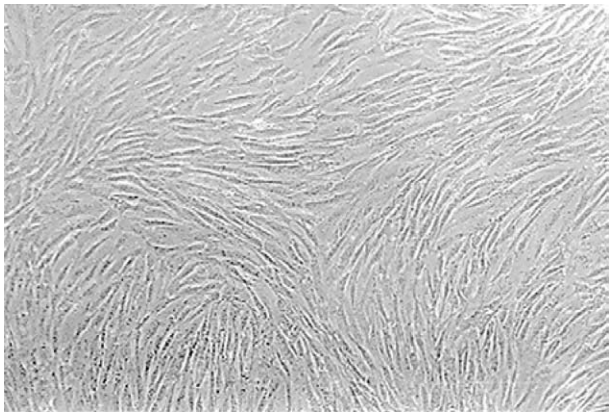

Fig. 1. Phase-contrast micrographs of cultured 132 m cells (100 ×).

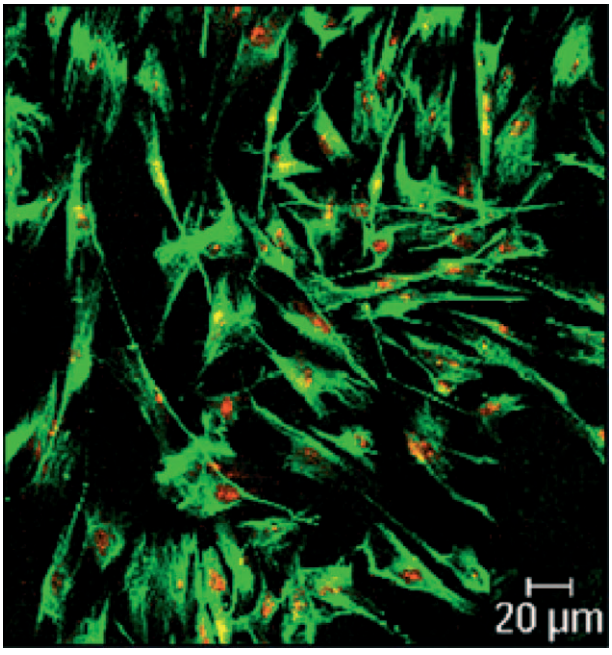

Fig. 2. Confocal micrograph of 132 m cells (stained for vimentin with green fluorescence and nuclei with red).

were in the G_0+G_1 stage, and that there was no significant difference between the different passages (Table 1, Fig. 3).

3.3. Cell-cycle analysis of 132 m fibroblasts at different growth conditions

Based on the analysis results of different passages, we analyzed 132 m fibroblasts at 50–60, 70–85, and 90% confluence and after 2 days of 100% confluence. The results showed that the percentages of G_0+G_1 stage cells differed significantly at each level of confluence (Table 2, Fig. 4).

Table 1
Cell-cycle stages of 132 m fibroblasts from different passages at 70–85% confluency (mean ± SD)

Passages	G_0+G_1*	S*	G_2+M*
Passage 5	77.1 ± 2.6	13.1 ± 9.3	9.8 ± 6.8
Passage 6	79.3 ± 0.5	8.4 ± 1.5	12.4 ± 1.4
Passage 7	78.0 ± 3.4	10.1 ± 3.5	11.9 ± 1.1
Passage 8	77.1 ± 2.6	10.4 ± 2.4	12.5 ± 2.9
Passage 9	72.4 ± 0.9	11.9 ± 5.0	15.5 ± 5.8
Passage 10	74.7 ± 2.6	11.4 ± 5.5	13.9 ± 2.9
Passage 13	75.3 ± 2.6	17.0 ± 1.4	7.7 ± 1.2

*There is no significant difference among different passages (P>0.05).

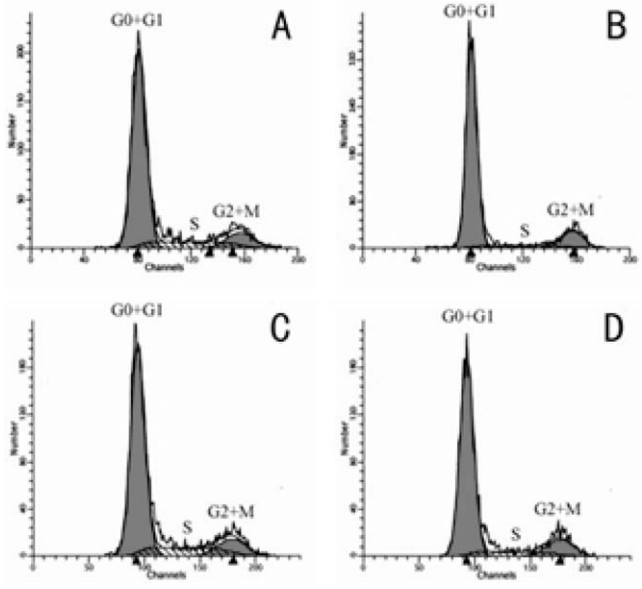

Fig. 3. Representative cell-cycle histograms of 132 m fibroblasts from passages 5 (A), 8 (B),10 (C), and 13 (D) at 70–85% confluence.

Table 2
Cell-cycle stages of 132 m fibroblasts at different growth conditions (mean ± SD)

Growth conditions	G_0+G_1*	S*	G_2+M*
50–60% Confluence	62.5 ± 2.7^{a}	23.9 ± 2.9^{a}	13.6 ± 2.8^{a}
70–85% Confluence	76.8 ± 2.8^{b}	11.1 ± 4.1^{b}	12.1 ± 3.4^{a}
90% Confluence	87.4 ± 1.9^{bc}	4.1 ± 1.4^{bc}	8.5 ± 2.8^{b}
Fully confluence 2 days	92.2 ± 1.1^{bcd}	2.9 ± 0.6^{bc}	4.9 ± 1.3^{b}

*Percentages with different superscripts within column differ significantly (P<0.05).

3.4. Cell-cycle analysis of 132 m fibroblasts with 70–85% confluence after different serum starvation periods

To examine the effect of serum starvation, we analyzed the 132 m fibroblasts with 70–85% confluence after 24, 48, 72 and 120 h serum starvation. The results showed markedly increased percentages of G_0+G_1 stage cells, with their percentages after 72 and 120 h serum starvation being higher than at 24 and 48 h serum

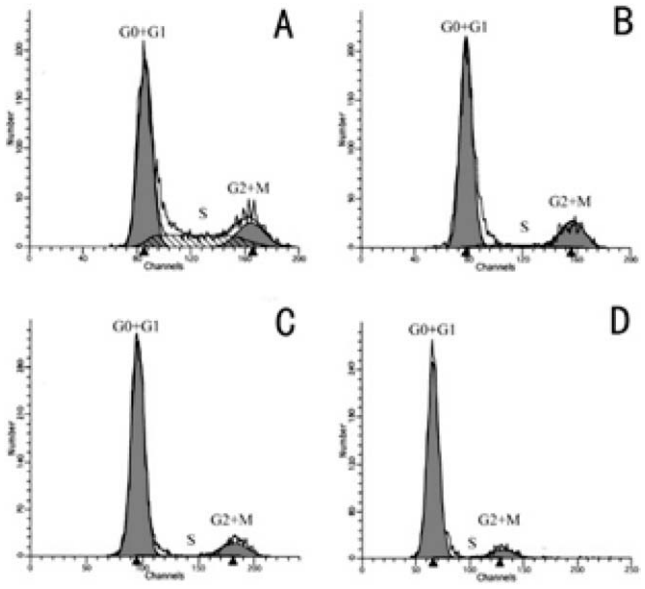

Fig. 4. Representative cell-cycle histograms of 132 m fibroblasts from passage 7 at 50–60% (A), 70–85% (B), 90% confluence (C) and over 2 days after fully confluence (D).

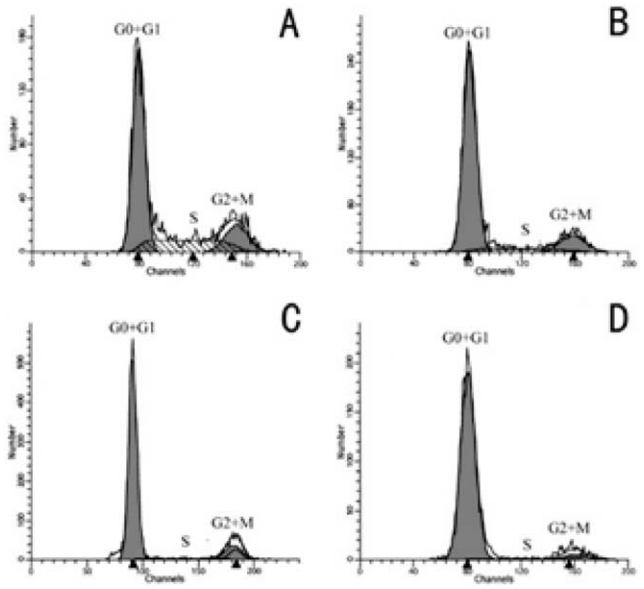

Fig. 5. Representative cell-cycle histograms of 132 m fibroblasts from passage 6 of 70–85% confluence with serum starvation 0 h (A), 24 h (B), 72 h (C) and 120 h (D).

Table 3
Cell-cycle stages of 132 m fibroblasts with 70–85% confluence at different serum starvation periods (mean ± SD)

Serum starvation periods	G_0+G_1*	S*	G_2+M*
70–85% Confluence	76.8 ± 2.8^{a}	11.1 ± 4.1^{a}	12.1 ± 3.4^{a}
24 h	87.8 ± 1.0^{bc}	6.0 ± 1.1^{b}	6.2 ± 1.91^{b}
48 h	88.3 ± 1.8^{bc}	6.2 ± 0.7^{b}	5.5 ± 2.5^{b}
72 h	90.4 ± 0.9^{bd}	5.0 ± 1.80^{b}	4.6350 ± 2.12^{b}
120 h	90.9 ± 0.7^{bd}	2.7 ± 1.2^{b}	6.4 ± 1.9^{b}

*Percentages with different superscripts within column differ significantly ($P<0.05$).

Table 4
Cell-cycle stages of 132 m fibroblasts at different serum starvation periods (mean ± SD)

Serum starvation periods	G_0+G_1*	S*	$G_{2+}M$
50–60% Confluence	62.5 ± 2.7^{a}	23.9 ± 2.9^{a}	13.6 ± 2.8
72 h	81.3 ± 0.5^{b}	5.7 ± 0.5^{b}	13.0 ± 1.0
120 h	83.9 ± 1.1^{b}	4.1 ± 1.5^{b}	12.0 ± 0.4
216 h	82.2 ± 1.5^{b}	1.8 ± 0.3^{b}	16.0 ± 1.8

*Percentages with different superscripts within column differ significantly ($P<0.05$).

starvation. However, there was no significant difference between the 72 and 120 h starvation cultures (Table 3, Fig. 5).

3.5. Cell-cycle analysis of 132 m fibroblasts with 50–60% confluence at different serum starvation periods

Fibroblasts of 132 m with 50–60% confluence analyzed after 72, 120, and 216 h serum starvation showed markedly increased percentages of G_0+G_1 stage cells, but there is no significant difference between the shorter and the longer starvation periods (Table 4, Fig. 6).

4. Discussion

Coordination of the cell cycle of donor nucleus and recipient cytoplasm is very important for successful development in somatic cell cloning. In an embryo reconstructed by nuclear transfer, the donor nucleus is transferred into a cytoplasmic environment with high maturation promoting factor (MPF) activity. Regardless of the cell-cycle stage of the donor nucleus at the time of transfer, this causes nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC). The effects of NEBD and PCC on the donor nucleus depend on the cell-cycle stage at the time of transfer. Following activation MPF levels decline, chromatin decondenses and a nuclear envelope is formed. All nuclei that have undergone NEBD will then undergo DNA synthesis. Hence donor nuclei must be in G_0 or G_1, when transferred to metaphase II recipient oocytes with high levels of MPF in order to condense normally and maintain correct ploidy of reconstructed embryos at the end of the first cell cycle.

In the cell-cycle analysis of different passages of giant panda fibroblasts at 70–85% confluent culture, there was no significant difference. Based on this result, we compared the cell cycle characteristic of different growth conditions and found that the percentages of G_0+G_1 stage cells differed significantly following increase in the confluent state, and >92% of cells are in G_0+G_1 stage when examined 2 days after becoming fully confluent. However, continued culture led to DNA fragmentation and the abnormal detachment of cells.

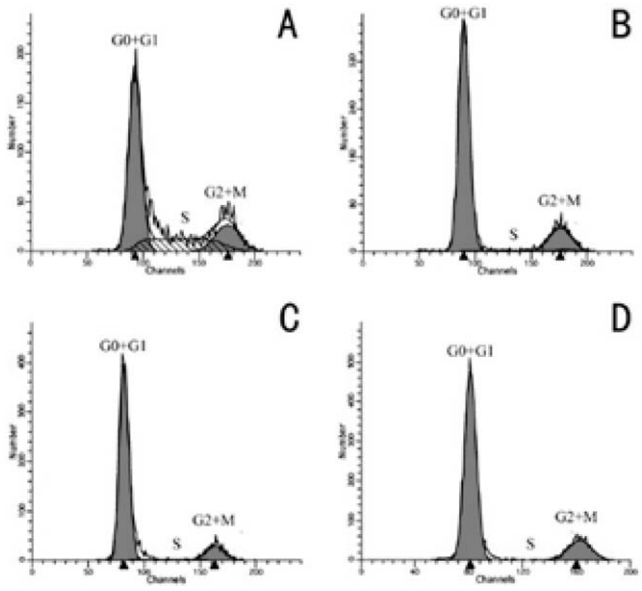

Fig. 6. Representative cell-cycle histograms of 132 m fibroblasts from passage 6 of 50–60% confluence with serum starvation 0 h (A), 72 h (B), 120 h (C) and 216 h (D).

Our results demonstrate that serum starvation has rapid and drastic effects on the cell-cycle state of giant panda fibroblasts. We compared the cell cycle characteristic of different serum starvation periods with different original confluent state and found that its major effect on the cell cycle of 70–85% confluent cells was already evident by 72 h and the percentages of G_0+G_1 stage cells failed to increase with more extended starvation periods, but the effect of serum starvation on the cell cycle of 50–60% confluence had still not achieved this level by 216 h. In fully confluent and contact inhibited cells, serum starvation failed to increase the percentage of G_0+G_1 stage cells and induced abnormal detachment of the cells. In addition, cells with enlarged flattened phenotype were determined to fail to increase cell number in culture, the percentage of G_0+G_1 stage cells is higher than normal phenotype, and serum starvation could not increase the percentage of G_0+G_1 stage cells and will lead to DNA fragmentation and the convergent growth which is a sign of cell aging.

In conclusion, flow cytometric analysis indicated the serum starvation and confluent culture could induce the fibroblasts to G_0 or G_1 by different mechanisms. So the results of this study will direct us to choose the optimum stage for preparing the donor cells in the research of giant panda cloning, which is beneficial in increasing the efficiency.

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