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Cell Cycle Synchronization of Porcine Fetal Fibroblasts: Effects of Serum Deprivation and Reversible Cell Cycle Inhibitors[1]

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ABSTRACT

The success of somatic nuclear transfer critically depends on the cell cycle stage of the donor nucleus and the recipient cytoplast. In this study we tested serum deprivation as well as two reversible cell cycle inhibitors, aphidicolin and butyrolactone I, for their ability to synchronize porcine fetal fibroblasts at either G0 stage or G1/S or G2/M transition. The synchronization efficiency of the various protocols was determined by fluorescence-activated cell sorting (FACS), cell proliferation assays, and semiquantitative multiplex reverse transcription-polymerase chain reaction detection of the cell cycle-regulated porcine Polo-like kinase mRNA (*Plk-p*). FACS measurements revealed that 66.6–73.3% of the porcine fetal fibroblasts were in G0/G1 stage (2C DNA content) in serum-supplemented medium. Short periods of 24–72 h of serum deprivation significantly increased the proportion of cells at G0/G1 phase to 77.9–80.2%, and mitotic activity had already terminated after 48 h. Prolonged culture in serum-deprived medium induced massive DNA fragmentation. Aphidicolin treatment led to an accumulation of 81.9 ± 4.9% of cells at the G1/S transition. Butyrolactone I arrested 81.0 ± 5.8% of the cells at the end of G1 stage and 37.0 ± 6.8% at the G2/M transition. The effects of both chemical inhibitors were fully reversible, and their removal led to a rapid progression in the cell cycle. The measurement of *Plk-p* expression allowed discrimination between the presumptive G0 phase induced by serum deprivation and the G1/S transition arrest achieved by chemical inhibitors. These data indicate that porcine fetal fibroblasts can be effectively synchronized at various cell cycle stages without compromising their proliferation capacity.

INTRODUCTION

The success rate of nuclear transfer using somatic cells is insufficient, and only 1–2% of reconstituted sheep, cow, mouse, and goat embryos develop to term [1–5]. Fetal fibroblasts have been frequently employed as donor cells, and the rate of development of reconstituted embryos is thought to be enhanced when donor nuclei are at the G0 phase of the cell cycle [1,6].

Serum deprivation is a commonly used method to synchronize cell lines in the G0 phase of the cell cycle. Mammalian fibroblasts require mitogens (e.g., growth factors) to progress through the G1 phase of the cell cycle. When cells have passed the checkpoint late in G1 they can enter the S phase and complete the cell cycle without further stimulation by mitogens [7–9]. The absence of mitotic signals, e.g., through serum deprivation in G1 phase, leads to a rapid exit from the cell cycle into a nondividing state, termed G0, characterized by low metabolic activity [10,11]. Apart from serum deprivation, several chemical inhibitors are effective in blocking the cell cycle at various positions. It has been shown that rodent and human fibroblasts can be synchronized in a reversible manner at G1/S transition with either 6 μM aphidicolin [12,13] or 118 μM butyrolactone I [14]. Aphidicolin is a reversible inhibitor of mammalian DNA polymerases and blocks the cell cycle at the transition from G1 to S phase [15]. Butyrolactone I is a specific inhibitor of the cyclin-dependent kinase (Cdk) family. It directly inhibits most Cdks and arrests the cell cycle at the transition from G1 to S stage and at the transition from G2 to M stage [14]. Butyrolactone I maintains the cell cycle in G1 prior to phosphorylation of retinoblastoma protein (pRB), whereas aphidicolin acts after pRB phosphorylation [13,14].

Regulation of the cell cycle is achieved by the well-orchestrated expression of various genes, such as kinases. One of the genes involved in cell cycle regulation is the Polo-like kinase (Plk); Plk belongs to a family of related kinases and has been shown to be essential for cell cycle progression [16,17]. Recent analysis of the Plk promoter has revealed that cell cycle regulation of the Plk activity occurs predominantly at the level of transcription [18]. Expression was lowest at G1 stage, increased in S phase, and peaked at G2/M in rodent and human cells [19–21].

In the present study we have investigated the effectiveness of three different cell cycle synchronization protocols in porcine fetal fibroblasts and analyzed the effects of cell synchronization on cell viability, proliferation, and reentry into the cell cycle. Cell cycle synchronization was achieved either by (1) serum deprivation or with the reversible cell cycle inhibitors (2) aphidicolin and (3) butyrolactone I. In addition, we applied a multiplex reverse transcription-polymerase chain reaction (RT-PCR) of porcine Polo-like kinase (*Plk-p*) mRNA levels to monitor the cell cycle stage of the synchronized porcine fetal fibroblast cultures at the transcriptional level.

MATERIAL AND METHODS

Preparation of Porcine Fetal Fibroblasts and Cell Culture

Primary fibroblasts were prepared from German Landrace pig fetuses 25 days postcoitum. Eight pregnant gilts were slaughtered on 3 different days, and the uteri were immediately transported to the laboratory. A total of 72 fetuses were collected within 15 min after slaughter. The fetuses were washed in PBS (Sigma Chemical Co., Deisen-

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TABLE 1. Used primers and size of amplification products.

Primer	Sequence	Product size
GAPDH[a]	upper: 5′-GTT CCA GTA TGA TTC CAC CCA CGG CAA GTT	763 bp
	lower: 5′-TGC CAG CCC CAG CAT CAA AGG TAG AAG AGT	
Plk-p[b]	upper: 5′-CTC CTG GAG CTG CAC AAG AGG AGG AA	454 bp
	lower: 5′-TCT GTC TGA AGC ATC TTC TGG ATG AG	

[a] GAPDH primers correspond to base pair positions 92–121 and 826–855 of the porcine cDNA (GenBank accession no. U48832).
[b] Plk-p primers have not yet been published.

hofen, Germany); then the heads and inner organs were excised. The remnants were pooled, minced into small pieces, and digested in a 0.05% trypsin/0.02% EDTA solution (Sigma) for 30 min at room temperature. The supernatant containing single cells was decanted and added to Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS, lot No. 14731321; Boehringer-Mannheim, Mannheim, Germany). The cells were separated by centrifugation at 500 × *g* for 10 min, and the cell pellets were resuspended in DMEM containing 10% FCS, 1 mM glutamine, penicillin (100 IU/ml; Sigma), and streptomycin (100 μg/ml; Sigma). Ten square centimeters of culture area was seeded per 2.5×10^6 cells. Culture was performed at 37°C and 5% CO_2 until fibroblast cells reached subconfluency. Primary fibroblasts were expanded for at least two passages. Karyotyping showed that > 95% of passage 2 and passage 3 cells contained the euploid chromosome number of n = 38. Passage 2 cells were trypsinized and stored as frozen aliquots (in culture medium with 10% dimethyl sulfoxide; Sigma). Recent experiments in our laboratory had shown that porcine fetal fibroblasts could be expanded for 15 passages before senescence occurred.

Cell Cycle Synchronization

For the synchronization experiments, cells were seeded at a concentration of 2×10^4 cells/cm^2 either in multiwell dishes (5-bromo-2′deoxy-uridine [BrdU] incorporation; terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling [TUNEL] assay) or in T25 culture flasks (fluorescence-activated cell sorting [FACS] and RNA isolation) and grown to 60–80% confluency to obtain cultures in the logarithmic growth phase [22]. For serum deprivation experiments, cultures were washed 3 times with DMEM and then cultured in DMEM supplemented with 0.2% FCS. For chemical synchronization, cultures were growth arrested by serum deprivation (DMEM with 0.2% FCS) for 48 h followed by incubation in medium supplemented with 10% FCS containing either aphidicolin (6 μM, Sigma) for 14 h [12,13] or butyrolactone I (118 μM; Affiniti, Exeter, UK) for 5 h [14]. Since fetal fibroblasts proliferate asynchronously under standard culture conditions, presynchronization by serum deprivation [13, 14] was chosen to minimize the length of incubation times with aphidicolin or butyrolactone I. To block cells at G2/M transition, cultures were released in medium supplemented with 10% FCS for different time intervals before butyrolactone I was added. All synchronization experiments employing serum deprivation or chemical synchronization schedules as reported in Tables 2, 4, and 5 were performed in parallel to ensure maximum homogeneity of the employed material. Therefore the 0.2% FCS control values are identical for Tables 2, 4, and 5, respectively. Stock solutions of aphidicolin and butyrolactone I were prepared in dimethyl sulfoxide and stored at −20°C.

Identically treated cultures, grown in parallel, were harvested for FACS and mRNA preparations at the indicated time points. Synchronization experiments were repeated at least three times.

Determination of Cell Cycle Stage by FACS

Cellular DNA content was determined by staining cells with propidium iodide and measuring fluorescence in a Becton Dickinson (Rutherford, NJ) FACScan [23]. Fetal fibroblasts were harvested by trypsinization and fixed in cooled 80% methanol (−20°C). The cells were incubated in a solution containing 1 mg/ml RNase, 20 μg/ml propidium iodide, and 0.1% saponin (all reagents from Sigma) for 30 min. Subsequently, the cells were transferred in PBS. For each cell population, 10 000 cells were analyzed in a Becton Dickinson FACScan, and the proportion in G0/G1, S, and G2/M phases was estimated using the Modfit cell cycle analysis program. FACS measurements were performed on at least three independent synchronization experiments.

Semiquantitative Multiplex RT-PCR

Relative changes in the abundance of the *Plk-p* mRNA in cell culture samples were determined by a multiplex RT-PCR assay, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as an internal standard. Total RNA was isolated from pig fetal fibroblasts with the RNAeasy Mini kit (Qiagen, Hilden, Germany) according to the recommendations of the supplier. Purified total RNA (0.5 μg) was used in a random hexamer-primed reverse-transcription reaction in a total volume of 40 μl consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM dNTPs (Amersham, Braunschweig, Germany); 2.5 μM random hexamers (Perkin-Elmer, Vaterstetten, Germany); 10 units RNase inhibitor; and 10 units Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Eggenstein, Germany). The RT reactions were carried out in a PTC-200 thermocyler (MJ Research, Watertown, MA) at 25°C for 10 min, followed by 42°C for 1 h, and heat denaturation at 94°C for 10 min.

Multiplex PCR was carried out with optimized *Plk-p*- and GAPDH-specific primer pairs (Table 1) within the linear range of amplification using standard amounts of reverse-transcribed cDNA as template. The PCR reaction volume was 20 μl, consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM of each *Plk-p* primer, and 1 unit of Taq DNA polymerase (Gibco BRL). The primers specific for GAPDH (1 μM each) were added after the fifth cycle of the program was completed. PCR was performed as follows: initial denaturation at 94°C for 1 min, followed by 25 cycles of 30 sec at 94°C, 30 sec at 60°C, and 60 sec at 72°C. The program was finished by a 5-min extension at 72°C. PCR fragments were separated by standard gel electrophoresis in 1.5% agarose gels in Tris-borate-ETDA buffer containing ethidium bromide and visualized

by UV transillumination. Quantification was performed with use of the Image Master 1D elite program (Amersham-Pharmacia, Piscataway, NJ). The identity of the PCR fragments was confirmed by sequencing. The RT-PCR experiments were performed in at least three replicates with identical results. As internal controls for the RT, samples without RNA or without reverse transcriptase were prepared in parallel; these yielded no amplification products (data not shown). As negative controls for the PCR, samples without reverse-transcribed cDNA or without Taq enzyme were used. As positive PCR controls, samples containing the cloned *Plk-p* construct were performed.

Measurement of Cell Proliferation by BrdU Incorporation

DNA synthesis was assayed by BrdU incorporation using an in situ detection kit (Boehringer Mannheim). In brief, BrdU was added to the culture medium to give a final concentration of 10 μM for a 20-h labeling period. For determination of BrdU incorporation, the cells were fixed in −20°C cold 70% ethanol/50 mM glycine buffer (pH 2.0) for 30 min, washed with PBS, and incubated with a monoclonal antibody against BrdU and subsequently with an alkaline phosphatase-conjugated secondary antibody according to the supplied protocol. Alkaline phosphatase (AP) staining was performed in Tris-HCl buffer (pH 9.5) containing 325 ng/ml nitro blue tetrazolium salt and 166 ng/ml 5-bromo-4-chloro-3-indolyl phosphate for 30 min. Unlabeled cells were visualized by Hoechst 33342 (1 μg/ml) staining. Cells were counted using a Leica DM IRB (Milton Keynes, Bucks, UK) microscope equipped with brightfield (for AP precipitate detection) and epifluorescence optics (BP 340–380, LP 425 for Hoechst 33342 detection). Omission of BrdU or either the first or the second antibody in control samples did not result in background AP staining of nuclei. From each sample, at least 500 nuclei were counted, and data represent mean values from three independent experiments.

Measurement of DNA Fragmentation by the TUNEL Assay

For in situ detection of DNA fragmentation, the culture samples were fixed in 4% paraformaldehyde for 30 min and then permeabilized in a solution consisting of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min. A cocktail of terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-conjugated nucleotides, supplied in a cell death detection kit (Boehringer Mannheim), was employed to label DNA breaks during a 30-min incubation period [24]. After washing in PBS, the samples were incubated with a secondary anti-FITC antibody conjugated to AP. AP staining was performed as described above. In brief, unlabeled cells were visualized by Hoechst 33342 (1 μg/ml) staining. Cells were counted using a Leica DM IRB microscope equipped with brightfield and epifluorescence optics. From each sample, at least 500 nuclei were counted, and data represent mean values from at least two independent experiments. Control reactions were performed by omitting terminal transferase enzyme (negative control) and by preincubation of samples with DNase I (positive control). Negative controls did not yield any background staining of nuclei, whereas DNase I pretreatment resulted in high labeling of all nuclei (data not shown).

Statistical Analysis

Data were analyzed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. Parametric analysis of the means between two or more populations were tested by an ANOVA followed by multiple pair-wise comparisons using a Tukey test. Differences of $P < 0.05$ were considered significant.

RESULTS

Viability of Porcine Fetal Fibroblasts in Serum-Deprived Medium

Porcine fetal fibroblasts cultured in medium supplemented with 10% FCS proliferated continuously and reached confluency within 3–4 days (Fig. 1A, C), whereas in serum-deprived cultures (0.2% FCS) the cell divisions were terminated and a considerable loss of cells was observed (Fig. 1B, D). After 6 days of serum deprivation, fewer than 40% of the initial cell population was present; identical results were obtained in medium containing 0.5% FCS (data not shown). Measurements of DNA fragmentation by the TUNEL assay revealed that serum-deprived cultures of porcine fibroblasts underwent extensive DNA fragmentation after 3 days of serum starvation (Fig 1F). Up to 20% of the serum-deprived cells showed DNA fragmentation compared to less than 1% of the cells grown in serum-supplemented medium (Fig. 1E).

Cell Proliferation of Porcine Fetal Fibroblasts after Serum Deprivation

To determine the minimum time of serum deprivation required to arrest porcine fetal fibroblasts in cell cycle, DNA synthesis was measured via BrdU incorporation in either standard medium or serum-deprived medium (0.2% FCS). The two treatment groups showed nearly identical percentages of > 90% of dividing cells at the start of the experiment (Fig. 2A–E). Serum reduction led to a rapid decrease in the number of dividing cells. As soon as after 24 h of serum deprivation, no more than approximately 22% of cells were labeled. This proportion further decreased to 2% of cells after 48 h of serum deprivation; after 72 h of serum starvation, fewer than 0.5% BrdU-positive cells were found. In contrast, in medium containing 10% FCS, more than 90% of cells proliferated for at least 48 h (Fig. 2E). After 72 h these cultures reached confluency and the percentage of BrdU-positive cells decreased to 55%. Thus, 48 h of serum deprivation was sufficient to inhibit DNA synthesis in porcine fetal fibroblasts.

Cell Cycle Synchronization of Porcine Fetal Fibroblasts by Serum Deprivation

To determine the effects of serum deprivation on the cell cycle, the cellular DNA content was measured by FACS analysis, and the relative percentages of cells in the G0/G1 (2C DNA content), S (between 2C and 4C), and G2/M (4C DNA content) stages were calculated. FACS measurements showed that the relative percentage of cells in G0/G1 increased within 72 h in serum-deprived cultures to 80.2 ± 3.6% (Table 2), compared to 73.3 ± 4.4% in control cultures grown in the presence of 10% FCS (Table 3) ($P < 0.05$).

The relative percentage of cells in S phase decreased to 5–8% under serum deprivation. However, a constant percentage (approximately 15% of the cells) showed the 4C DNA content characteristic of the G2/M phase. Stimulation of 48-h-starved cells by serum addition (10%) triggered cell cycle progression from G1 to S phase (Table 2). After 16-h incubation in medium containing 10% FCS, the relative

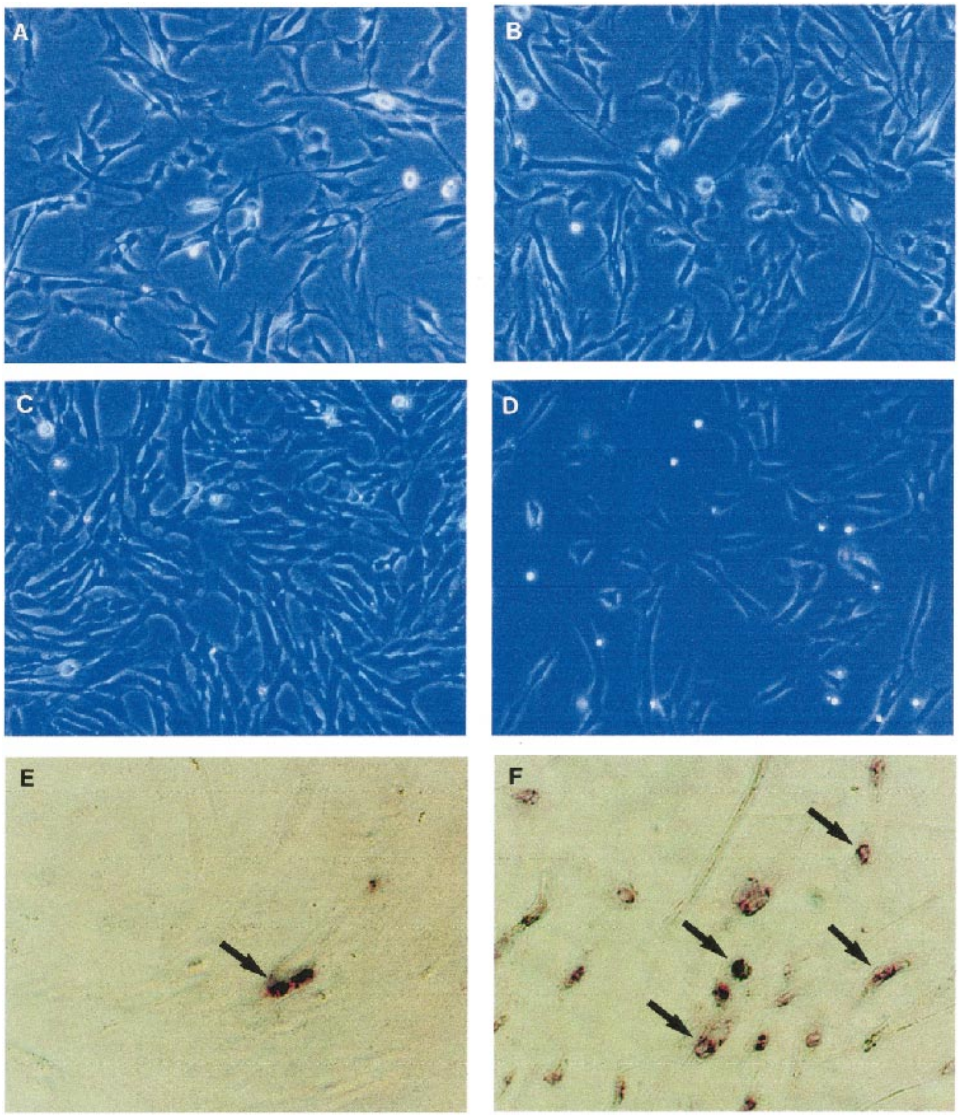

FIG. 1. Viability of porcine fetal fibroblasts under serum-deprived conditions. Cell morphology and cell density of porcine fetal fibroblasts cultured in medium containing either 10% FCS (**A,C**) or 0.2% FCS (**B,D**) at the start of the experiment (**A,B**) and after 72 h of culture (**C,D**). Note the low cell density of the serum-deprived culture (**D**). TUNEL assay of porcine fetal fibroblasts grown in medium containing 10% FCS (**E**) or 0.2% FCS (**F**) for 72 h. Arrows indicate some TUNEL-positive cells. ×200 (published at 85%).

percentage of cells in S phase increased from 4.6% to 40.0% ($P < 0.001$).

Cell Cycle Synchronization of Porcine Fetal Fibroblasts by Aphidicolin

For aphidicolin synchronization, porcine fetal fibroblasts were growth arrested by serum deprivation (0.2%) for 2 days and then returned to complete medium containing 6 μM aphidicolin for 14 h. Figure 1E shows that 72 h of serum deprivation induced considerable DNA fragmentation. As seen, serum deprivation for 48 h was sufficient to induce mitotic inhibition. Thus the synchronization schedule of 48-h serum deprivation followed by 14-h aphidicolin treatment was an attempt to obtain maximal presynchronization rates with minimum DNA fragmentation. FACS measurements revealed that > 80% of the cells could be maintained at the G1/S transition (Table 4), even in the presence of 10% FCS. Removal of the drug, followed by

TABLE 2. Percentages (± SD) of porcine fetal fibroblasts at G0/G1, S, and G2/M stages of cell cycle after culture in serum deprived (0.2% FCS) medium.

Cell cycle stage	24 h	48 h	72 h	Serum stimulated†
G0/G1	77.9 ± 4.8^{a*}	78.5 ± 4.4^{a*}	80.2 ± 3.6^{a*}	49.5 ± 6.5^{b}
S	8.1 ± 2.5^{a**}	5.1 ± 1.6^{ab**}	4.6 ± 0.8^{b**}	40.0 ± 5.7^{c}
G2/M	14.0 ± 6.6^{a}	16.4 ± 3.4^{a}	15.2 ± 1.5^{a}	10.8 ± 2.6^{a}

$^{a–c}$ Percentages with different superscripts within rows differ significantly ($P < 0.05$).
*, ** Percentages of the same cell cycle stage with symbols differ significantly ($P < 0.05$) between culture with 0.2% and 10% FCS.
† 48 h serum deprivation, followed by culture in medium containing 10% FCS for 16 h.

TABLE 3. Percentages (± SD) of porcine fetal fibroblasts at G0/G1, S, and G2/M stages of cell cycle after culture in complete medium (10% FCS).

Cell cycle stage	24 h	48 h	72 h
G0/G1	66.6 ± 4.0^{a*}	71.3 ± 3.4^{a}	73.3 ± 4.4^{a*}
S	15.2 ± 4.5^{a**}	12.9 ± 0.9^{a**}	9.3 ± 2.2^{b**}
G2/M	18.2 ± 2.9^{a}	15.8 ± 5.2^{a}	17.4 ± 6.8^{a}

^{a,b} Percentages with different superscripts within rows differ significantly ($P < 0.05$).
*, ** Percentages of the same cell cycle stage with symbols differ significantly ($P < 0.05$) between culture with 0.2% and 10% FCS.

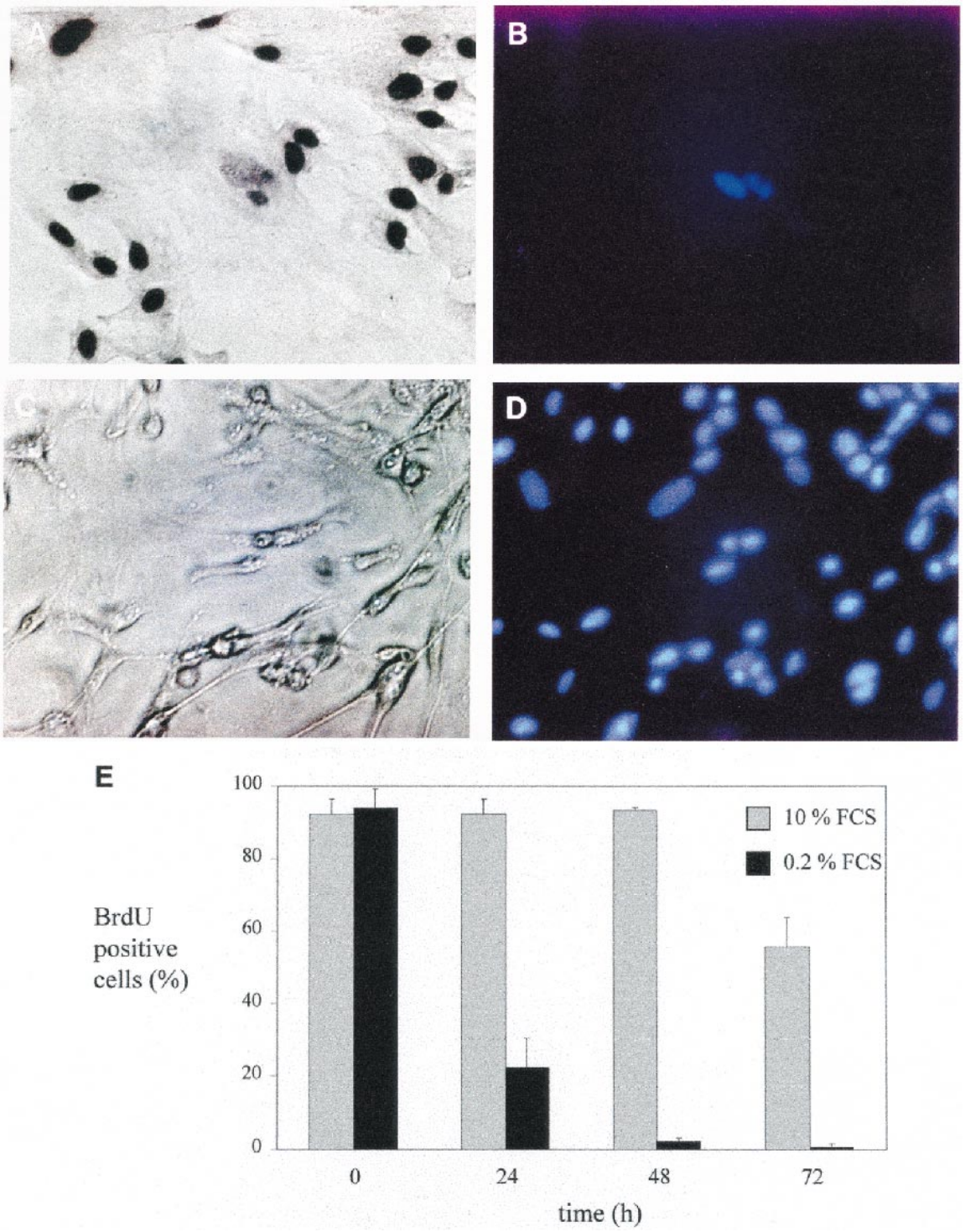

FIG. 2. Proliferation of porcine fetal fibroblasts. Mitotic activity of porcine fetal fibroblasts as assayed by BrdU incorporation. Cells were grown in medium containing 10% FCS (**A,B**) or in serum-deprived medium for 48 h (**C,D**). Incorporated BrdU was detected by AP staining (**A,C**), and nondividing cell nuclei were visualized with Hoechst 33342 (**B,D**). Note that nearly all serum-deprived cells were AP negative (no DNA synthesis). ×200. **E**) Time course of DNA synthesis in media containing 10% or 0.2% FCS.

culture in DMEM containing 10% FCS, triggered a rapid progression in the cell cycle (Table 4). Five hours after removal of aphidicolin, 38% of the cells had reached the G2/M phase (Table 4). Thus, aphidicolin blocked the cell cycle of porcine fetal fibroblasts effectively, and the effects of aphidicolin treatment were fully reversible.

Cell Cycle Synchronization by Butyrolactone I

To evaluate the effects of butyrolactone I on the cell cycle stage of porcine fetal fibroblasts, the cells were growth arrested by serum deprivation for 2 days and then cultured in medium containing 10% FCS and 118 μM bu-

TABLE 4. Percentages (± SD) of porcine fetal fibroblasts at G0/G1, S, and G2/M stages of cell cycle after treatment with aphidicolin (6 μM for 14 h) and after removal of the drug.

Cell cycle stage	0.2% FCS	Aphidicolin	Aphidicolin, followed by 10% FCS for 5 h	10% FCS for 19 h
G0/G1	78.5 ± 4.4[a]	81.9 ± 4.9[a]	38.4 ± 14.7[b]	31.1 ± 3.9[b]
S	5.1 ± 1.6[a]	3.9 ± 9.6[a]	23.8 ± 9.6[b]	46.9 ± 6.3[c]
G2/M	16.4 ± 3.4[a]	14.2 ± 3.8[a]	37.8 ± 7.6[b]	22.0 ± 2.8[c]

[a–c] Percentages with different superscripts within rows differ significantly ($P < 0.05$).

tyrolactone I for 5 h. This procedure led to accumulation of > 80% of cells at G1/S transition (Table 5). To synchronize fetal fibroblasts at the G2/M transition with butyrolactone I, the cells were growth arrested by serum deprivation and subsequently stimulated with serum for various time periods (Table 5) before butyrolactone I was added to the medium. With this schedule, 37% of the cells could be synchronized at the G2/M transition (Table 5). Removal of butryolactone I led to a progression in the cell cycle (Table 5). These data show that butyrolactone I was able to block cell cycle progression of porcine fetal fibroblasts at the G1/S transition (> 80%), and with lower success rates at the G2/M transition (37%), in a reversible manner.

Cell Cycle-Regulated Expression of Plk-p *in Porcine Fetal Fibroblasts*

To monitor the cell cycle synchronization at the molecular level, we determined the expression pattern of the strictly cell cycle-regulated *Plk* gene [16] via semiquantitative multiplex RT-PCR assay. The sequence of the porcine homologous gene, which we have designated *Plk-p* (unpublished results), was used to design specific primers. Semiquantitative multiplex RT-PCR, using the housekeeping GAPDH gene as an internal standard, showed that expression levels of porcine *Plk-p* were lowest at the presumptive G0 stage (Fig. 3). RT-PCR amplification from porcine fetal fibroblast samples, serum deprived for 48 or 72 h, resulted in faint bands that were quantified to 0.11 and 0.09 arbitrary units of the *Plk-p* mRNA levels in cycling cultures. In contrast to serum-deprived cultures, cultures arrested by aphidicolin or butyrolactone I at the G1/S transition yielded moderate *Plk-p* amplification products of 0.52 and 0.21 arbitrary units, respectively. Samples of cycling cultures in medium with 10% FCS produced moderate to high levels of *Plk-p* amplicons. The highest *Plk-p* mRNA levels were determined in cultures after release from the cell cycle block when cohorts of cells progressed through S and G2/M phases. The semiquantitative multiplex RT-PCR assay of *Plk-p* was found to be a reliable tool for monitoring the cell cycle stages of synchronized populations. Moreover, it allowed discrimination between G0 phase (low *Plk-p* mRNA levels) and G1/S transition (moderate *Plk-p* mRNA levels).

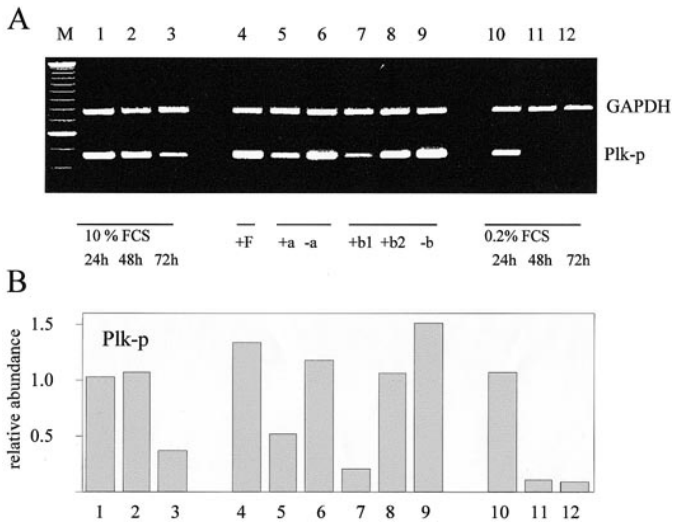

FIG. 3. Representative multiplex RT-PCR of *Plk-p* gene transcripts. **A**) Semiquantitative PCR of *Plk-p* using GAPDH as an internal standard. Labeling of lanes: M, 100-base pair (bp) DNA ladder with prominent 600-bp fragment; 1, 2, 3, cultures grown in DMEM with 10% FCS for 24, 48, and 72 h, respectively; 10, 11, 12, serum-deprived cultures for 24, 48, and 72 h, respectively; 4, serum-deprived culture after serum stimulation for 16 h (+F); 5, aphidicolin-arrested culture (+a); 6, aphidicolin-synchronized culture after removal of the drug for 5 h (−a); 7, butyrolactone I-arrested culture at G1/S (+b1); 8, butyrolactone I-arrested culture at G2/M (+b2); 9, butyrolactone I-synchronized culture after removal of the drug (−b). **B**) Relative abundance of Plk-p transcripts. Densitometric evaluation of gel image in **A.**

Reentry of Arrested Porcine Fetal Fibroblasts into the Cell Cycle

To follow the ability of synchronized porcine fetal fibroblasts to reenter into the cell cycle, the DNA synthesis was measured after release from the cell cycle block. Fetal fibroblasts synchronized either by serum deprivation or aphidicolin treatment were mitotically stimulated with 10% FCS in the presence of BrdU. The relative percentage of mitotic cells was determined at several time points after release from the cell cycle block by in situ detection of incorporated BrdU. Upon serum stimulation, starved fibroblasts and aphidicolin-synchronized fibroblasts showed distinct differences in their reentry rate into the cell cycle (Fig. 4). As expected, serum-deprived cultures reentered the S phase with a delay of 8–10 h in comparison to aphidicolin-synchronized cells. However, fewer cells from serum-deprived cultures showed DNA synthesis in comparison to those from aphidicolin-treated cultures. Maximally, 58% of serum-deprived cells showed BrdU incorporation as com-

TABLE 5. Percentages (± SD) of porcine fetal fibroblasts at G0/G1, S, and G2/M stages of cell cycle after different treatments with 118 μM butyrolactone I (B1; for 5 h).*

Cell cycle stage	0.2% FCS	10% FCS + B1	10% FCS for 8 h, then B1	10% FCS for 15 h, then B1	10% FCS for 20 h, then B1	10% FCS for 20 h	10% FCS for 15 h, then B1, then 10% FCS for 5 h
G0/G1	78.5 ± 4.4[a]	81.0 ± 5.8[a]	78.1 ± 7.1[a]	34.4 ± 6.4[b]	44.1 ± 8.7[b]	39.5 ± 4.3[b]	41.6 ± 2.2[b]
S	5.1 ± 1.6[ab]	3.0 ± 2.7[b]	12.4 ± 9.6[a]	46.1 ± 1.0[d]	19.6 ± 5.2[ac]	33.5 ± 8.2[c]	31.2 ± 9.7[c]
G2/M	16.4 ± 3.4[ab]	16.2 ± 3.4[ab]	9.6 ± 2.6[a]	19.6 ± 5.5[b]	37.0 ± 6.8[c]	27.0 ± 2.8[b]	27.3 ± 3.8[b]

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

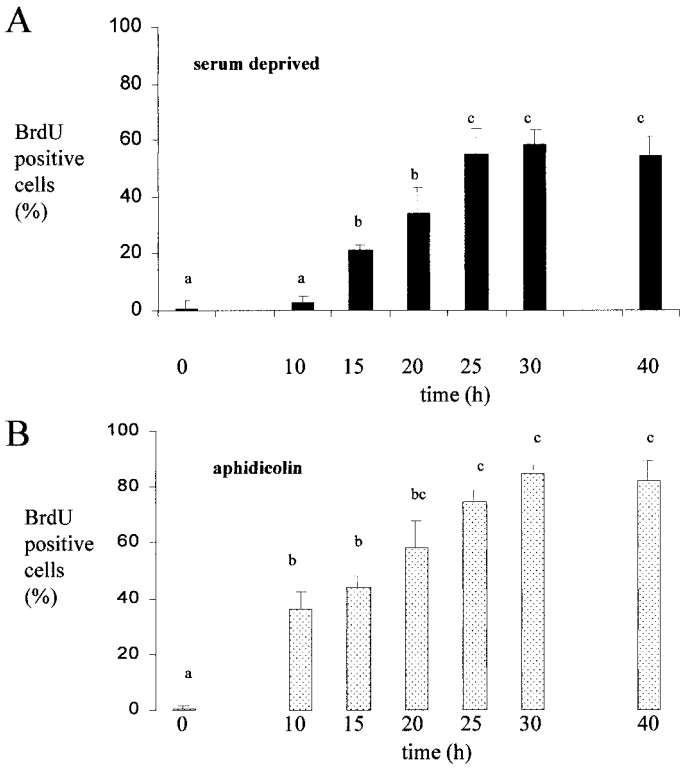

FIG. 4. Reentry of synchronized fibroblasts into the cell cycle. Percentages of DNA-synthesizing cells at different time points after release from cell cycle arrest as determined by BrdU incorporation. Cell cycle synchronization was achieved by (**A**) serum deprivation or (**B**) aphidicolin treatment (6 μM for 14 h) of presynchronized cells. Percentages with different superscripts differed significantly within the experiment ($P < 0.05$).

pared to > 80% of aphidicolin-treated cells. Thus a large portion of the starved cells seemed to be unable to leave G0 upon serum stimulation.

DISCUSSION

Our data indicate that porcine fetal fibroblasts can be successfully and reversibly arrested at G0 phase of the cell cycle by serum deprivation and at the G1/S transition by temporary aphidicolin or butyrolactone I treatment. Determination of the *Plk-p* expression pattern allows sensitive monitoring of the cell cycle status of porcine fibroblasts. BrdU incorporation assays confirmed that porcine fetal fibroblasts proliferate at high rates in serum-supplemented medium under our culture conditions. FACS measurements revealed that at least 66.6% of the cells have a 2C DNA content, showing that porcine fetal fibroblasts have an extended G1 phase.

This study demonstrated that serum deprivation has rapid and drastic effects on the cell cycle state of porcine fetal fibroblasts. The major effect of serum deprivation on the cell cycle is already achieved after 48 h, such that nearly 80% of cells are in G0/G1 stage. Recent flow cytometric analysis of porcine fetal fibroblasts showed similar synchronization efficiencies in G0/G1 stage after starvation for 5 and 10 days [25]. Here we demonstrate that extended serum deprivation periods of more than 48 h do not increase the proportion of cells at G0/G1 stage ($P < 0.05$), but exert deleterious effects on cell survival and lead to massive DNA fragmentation, a hallmark of apoptosis [26,27]. Whether the observed starvation-induced DNA fragmentation in fibroblasts could be attributed to apoptosis or necrosis requires further investigations; however, it has been shown that porcine granulosa cells undergo serum deprivation-induced apoptosis [28]. With respect to nuclear transfer, the high number of nuclei exhibiting DNA fragmentation after serum deprivation appears to be a critical factor. Several ovine and bovine cell types gave rise to offspring after nuclear transfer using donor nuclei subjected to serum deprivation for 4 to 10 days [1,6,29,30]. Growth factors, steroids, and other factors have been shown to be necessary for mitotic stimulation and survival of serum-starved fibroblasts [31–33], and retinol is thought to be a survival factor for fibroblasts after serum deprivation for more than 24 h [34]. Thus, among other factors, species-specific differences in metabolic pathways to synthesize retinol might contribute to the observed starvation-induced DNA fragmentation of porcine fibroblasts. Whether the high rate of late abortions and neonatal deaths of calves obtained from somatic nuclear transfer [30, 35, 36] might be related to culture conditions that induce DNA fragmentation of the donor nuclei requires a detailed analysis. Also it has been suggested that other factors such as micromanipulation, culture systems, and conditions after reconstruction affect the ontogenesis of cloned animals.

Recently, it has been shown that starvation for 48 h and subsequent serum stimulation of human primary fibroblasts lead to a well-orchestrated expression of at least 10 gene clusters with a total of 462 genes [11]. In particular, genes involved in wound healing were heavily up-regulated after serum stimulation. Thus, serum deprivation leads to a cell cycle arrest of fibroblasts and also initiates broad and complex alterations at the transcriptional level. In addition, our FACS data suggest that subpopulations of approximately 5% of porcine fetal fibroblasts were arrested at the S phase and that about 10–15% of the cells were blocked at G2/M phase by serum deprivation, indicating the necessity for improvements in cell cycle synchronization for nuclear transfer. Therefore, we have applied chemical cell cycle inhibitors to synchronize porcine fibroblasts at different points of the G1/S transition. Here we show for the first time that porcine fetal fibroblasts can be synchronized in an efficient manner by either aphidicolin or butyrolactone I. Both drugs lead to an efficient accumulation of > 80% of porcine primary fibroblasts at slightly different positions of the G1/S transition. Butyrolactone I arrests the cell cycle by inhibiting Cdks before phosphorylation of pRB takes place [14], whereas aphidicolin inhibits DNA polymerase activity in early S stage [13]. Additionally we show that the semiquantitative RT-PCR assay of the *Plk-p* mRNA can be used to monitor cell cycle synchronization and to discriminate between the G0 phase and the G1/S transition. However, cell cycle inhibition at the G2/M transition by butyrolactone I treatment arrests no more than 37% of the porcine fetal fibroblasts. One explanation seems to be a heterogeneity in the cell cycle progression of porcine fibroblast cells. In preliminary experiments, we found that significantly higher percentages of cells could be arrested in G2/M if a combination of aphidicolin and butyrolactone I was employed.

In summary, porcine fetal fibroblasts were effectively synchronized by serum deprivation, aphidicolin, or butyrolactone I at G0 and G1/S phases and extensively characterized for DNA content, DNA synthesis, *Plk-p* expression, and levels of DNA fragmentation. The synchronization protocols of primary cells need to be further optimized before they can be employed to study the significance of an in-

dividual cell cycle phase of somatic donor nuclei in the nuclear transfer technique. The application of specific and reversible cell inhibitors allows one to employ precisely tuned cell cycle stages and should lead to the identification of optimal cell culture conditions for donor cells in nuclear transfer.

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