Published in final edited form as:

Exp Eye Res. 2006 July ; 83(1): 61–68. doi:10.1016/j.exer.2005.11.007.

Cell sorting but not serum starvation is effective for SV40 human corneal epithelial cell cycle synchronization

Sara J. Liliensiek^a, Kathleen Schell^b, Elise Howard^b, Paul Nealey^c, and Christopher J. Murphy^{a,*}

^a Department of Surgical Sciences, School of Veterinary Medicine, 2015 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

^b Comprehensive Cancer Center Flow Cytometry Facility, Clinical Science Center, University of Wisconsin, Madison, WI 53792, USA

^c Department of Chemical and Biological Engineering, School of Engineering, University of Wisconsin, Madison, WI 53706, USA

Abstract

SV40 human corneal epithelial cell (HCEC) populations are readily used as a substitute for primary corneal epithelial cells that are difficult to maintain in vitro. To initiate cell-cycle experiments with the SV40-HCEC cells, two separate methods of cell synchronization were compared including serum starvation and sterile cell sorting. We hypothesized that SV40 cells are synchronized at higher efficiencies into each cell cycle phase (G₁, S, G₂M) when cell sorting is performed when compared to alternative methods of synchronization. SV40 cells were synchronized by deprivation of serum over 96 h or labeled with Hoechst 33342 dye and sorted based on DNA content. Cells were synchronized using both methods and harvested at time points up to 72 h after release. To define more precisely the nature of sorted fractions, cells were pulsed with BrdU prior to sorting. SV40-HCEC cells exhibit a well-defined cell cycle profile. Serum deprivation up to 96 h was ineffective for cell synchronization of SV40-HCECs. In comparison, we achieved efficient synchronization of the SV40-HCECs with sterile cell sorting. SV40-HCEC cells gated into G₁, S and G₂M were synchronized up to 85% following the sort and maintained synchronization up to 24 h. Our findings indicate that serum starvation is not effective for synchronization of the SV40-HCEC cell line. We present a more effective approach, the use of cell sorting for cell synchronization of the SV40-HCEC cells.

Keywords

corneal epithelial cells; cell cycle; flow cytometry; synchronization

1. Introduction

The SV40 transformed human corneal epithelial cell (HCEC) line is commonly used as a replacement for primary corneal epithelial cells due to their ease of use and availability. These cells originally were immortalized via viral transduction of the SV40 T antigen into cultures of primary human corneal epithelial cells. Although immortalized, these cells maintain both morphological and biochemical characteristics of primary HCEC cells

* Corresponding author. Tel.: +1 608 263 3903; fax: +1 608 263 9713. sjlilien@wisc.edu (S.J. Liliensiek), kschell@wisc.edu (K. Schell), ehoward@wisc.edu (E. Howard), nealeyp@che.wisc.edu (P. Nealey), murphyc@svm.vetmed.wisc.edu (C.J. Murphy).

including expression of corneal specific keratins, the ability to differentiate, and the same population doubling time (Araki-Sasaki et al., 1995). Our aim was to utilize these cells in future experiments that explore the impact of cell cycle stage in response to biomechanical cues. In order to initiate our studies, we needed to develop the optimal procedure for synchronization of the SV40-HCEC cells.

The mammalian cell cycle has defined phases, including growth in G_1 , synthesis of DNA in S phase, and continued cell growth and mitosis in G_2 (Gray and Darzynkiewicz, 1987; Freeman, 2002). Due to the defined phases, synchronization can be achieved such that the complete cell population traverses the cell cycle at a similar rate (Cooper, 2003a,b). Although synchronization can be accomplished with some accuracy, cells do not cycle in discrete steps so it is not possible to synchronize cells to the exact same point within the cell cycle. Historically, the ability to synchronize a population of cells has allowed for studies involving pronuclei and the effects of proliferation (Tani et al., 2001), transplantation using synchronized donor and recipient cells (Galat et al., 1999; Niemann et al., 2000) and the development of new cancer treatments (Pawlik and Keyomarsi, 2004). Commonly used methods of cell synchronization include cytostatic and cytotoxic agents including treatment with aphidicolin, colcemid, or hydroxyurea (Grdina et al., 1984). Due to the potential disruption of cell function and cell-cycle associated proteins with addition of chemicals, these cytotoxic reagents were not utilized in our study (Gong et al., 1995).

Synchronization of cells with serum starvation is also commonly used and involves the deprivation of nutrients in cell culture for an extended period of time, often from 24–72 h (Gray and Darzynkiewicz, 1987). With deprivation of essential trophic factors, cells cannot continue through the G_1 phase of the cell cycle and cells are believed to enter into G_0 , a non-growth resting state. After enrichment of the population in G_1/G_0 is achieved, cells are released and continue through the cell cycle in synchrony (Cooper, 2003a,b). Serum starvation and synchronization yields around 70% of the cell population arrested in G_0/G_1 (Niemann et al., 2000; Tani et al., 2001). Although serum starvation can be effective, there are several drawbacks to the technique. Serum starvation can have many negative consequences including induction of DNA fragmentation (Niemann et al., 2000), decreased mitochondrial viability (Takeda et al., 2002), alterations or disruption of the cell cycle following serum starvation (Gray and Darzynkiewicz, 1987) and the induction of apoptosis or death (Magnani and Bettini, 2000; Oya et al., 2003; Tan et al., 2003). In addition, some transformed and primary cell lines, including primary rabbit corneal epithelial cells are resistant to the effects of serum starvation and will not synchronize with this method (Knehr et al., 1995; Cooper, 1998; Anderson and SundarRaj, 2001; Katska et al., 2002).

Due to these limitations, alternative approaches to synchronization have been developed. Recent advances in cell sorting technology now allow for sorting and enrichment of cells based on DNA content into G_1 , S, or G_2M (Herzenberg et al., 2002). The cell sorter allows for 1×10^7 cells to be sorted and analyzed per hour with high precision (Gray and Darzynkiewicz, 1987) and cell populations can be gated, which allows for specific regions and parameters to be looked at independently. In addition, a large population of cells can be identified and isolated at all phases of the cell cycle (G_1 , G_2M and S) compared to G_0/G_1 alone (Gray and Darzynkiewicz, 1987). Unlike other methods, the cell sorter can isolate all three phases simultaneously with only a single sample preparation. To our knowledge, the ability to sort corneal epithelial cells into each phase of the cell cycle simultaneously using flow cytometry and the characterization of the cells post-sort has not been previously reported. In summary, this methodology provides an alternative approach allowing for relatively quick synchronization of corneal epithelial cells into each cell cycle phase at the same time with identical treatment. We hypothesized that SV40-HCEC cells will be more effectively synchronized using flow cytometry versus serum starvation.

2. Materials and methods

2.1. Cell culture

SV40-HCEC cells were a generous gift from Dr. Kaoru Araki-Sasaki (Kumamoto University, Kumamoto, Japan (Araki-Sasaki et al., 1995). Cells were cultured in epithelial media containing 1:1 F12 to DMEM, 0.5% DMSO, 40 µg/ml gentamycin, and 10% fetal bovine serum (FBS). Cultures were maintained at 37 °C in 5% CO₂. SV40-HCEC cells were utilized between passages 15–30. For some sorting experiments cells were incubated with 20 µM BrdU for 45 min at 37 °C just prior to loading them with dye. For serum starvation experiments, SV40-HCECs were plated and cultured in SHEM with 0% (no serum) or 10% (normal serum) FBS. Cells were harvested by trypsinization after 24, 48, 72 and 96 h.

2.2. BrdU incorporation

Similar to tritiated thymidine ([³H]-TDR), bromo-deoxyuridine (BrdU), a thymidine analog, is incorporated into newly synthesized DNA. The extent of BrdU incorporated is proportional to the amount of newly synthesized DNA during the S phase of the cell cycle. BrdU is detected in ssDNA with the use of an anti-BrdU antibody tagged with a fluorochrome after the DNA has been partially denatured (Gratzner, 1982; Dolbeare et al., 1983; Darzynkiewicz et al., 2004). Simultaneous staining of total DNA content and labeling of incorporated BrdU will give a definitive measure of the cell cycle location. In these experiments cells were pulsed 30 min prior to sorting and then the purity of the sort was determined using two fluorochromes: one that identified the S phase (Anti-BrdU + anti-mouse FITC) and one that identified and quantitated the total DNA (propidium iodide). Because cells can have a DNA content consistent with G₁ or G₂M but are in S phase, this procedure more accurately assessed the purity of the sort. This technique is well described in flow cytometry and sorting (Melamed et al., 1979).

2.3. Cell sorting

For sterile cell sorting, 2×10^7 SV40-HCECs with or without pulsing with BrdU were resuspended at a density of 5×10^5 /ml in epithelial media + 10% FBS containing 20 µg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 30 min at 37 °C. After incubation, the cell preparation was centrifuged at $400 \times g$ for 5 min and a portion of the supernatant was removed to yield a final cell concentration of 1×10^7 /ml. Cells were then filtered through a 40 µm nylon mesh cell filter. To identify dead cells, propidium iodide (PI) was added at a concentration of 2 µg/ml from a 10 mg/ml solution just prior to placing the cells on the sorter, a triple laser FACSVantage SE equipped with the FACSDiVa digital electronics package. For these experiments the following laser excitation wavelengths and filter sets were used for light collection:

1. Propidium iodide: 100 mW of 488, water-cooled Innova 90 argon laser (Coherent, Santa Clara, CA); 630/30 bandpass filter.
2. Hoechst 33342: 100 mW of multi-line UV, Innova 302C krypton laser (Coherent); 450/50 bandpass filter.

Cells were sorted using a 100 µm ceramic nozzle tip with a pressure of 12 psi into 12 × 75 mm tubes containing 1 ml of epithelial media + 10% FBS. Fig. 1(A–C) shows the gating strategy used in sorting. First, non-viable cells were excluded based on exclusion of PI (A) and the absence of doublets based on pulse-width measurements (B). Next, cells were divided into different phases of the cell cycle based on forward scatter measurements and DNA content (C). In addition, as a control, a portion of each sample was sorted for cells that excluded PI only. Cells were centrifuged immediately following sorting and resuspended in epithelial media (FBS) and plated into 6-well plates.

2.4. Propidium iodide and anti-BrdU labeling

Cells were trypsinized, harvested and rinsed twice with 4 ml of 1× phosphate buffered saline (PBS) followed by a 5 min centrifugation at 1500 rpm. After the second rinse, cells were gently re-suspended in 100 µl of 1× PBS and placed on ice for 10 min. After incubation 900 µl of cold 100% ethanol was added slowly in a drop-wise fashion. Cells were stored at −20 °C. Ethanol-fixed cells were rinsed twice with 1× PBS followed by a 5 min centrifugation at 1500 rpm. To release the nuclei, cells were resuspended in 0.5 ml of 0.4% pepsin in 0.1 N HCl at room temperature for 30 min. After incubation, the nuclei were pelleted, re-suspended in 2 N HCl, and incubated for 30 min at 37 °C, shaking the tubes every 10 min. To inactivate the reaction, 0.1 M sodium tetraborate was added and cells were pelleted followed by two washes with PBS-TB (1× PBS + 0.5% Triton × (Sigma-Aldrich, St. Louis, MO) + 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO). Pellets were incubated with mouse anti-BrdU (Becton-Dickinson, San Jose, CA) overnight at 4 °C. Pellets were washed twice with PBS-TB followed by incubation with goat anti-Mouse FITC (Becton Dickinson, San Jose, CA) for 30 min at room temperature. Cells were washed and re-suspended in 50 µg/ml of propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO.), 10 mg/ml of RNase A (Qiagen, Valencia, CA) and incubated overnight at 4 °C. Samples were analyzed for BrdU labeling within 48 h of staining.

2.5. Flow cytometry and data analysis

Samples stained after serum starvation or sorting were fixed and stained as described above. A minimum of 10,000 events were collected for each time point with a benchtop (FACScan) cytometer using CellQuest software (BD Biosciences, San Jose, CA.). Doublets and aneuploid cells were excluded. The percentage of BrdU labeled cells was determined using FlowJo software (Treestar, Ashland, OR). In addition, using single parameter measurements of DNA content, the percentage of cells in each phase of the cell cycle was estimated and analyzed using ModFit software (Verity Software, Topsham, ME).

3. Results

3.1. Serum starvation and synchronization of SV40-HCEC cells

In order to determine if the SV40-HCEC cell line would be useful for our analysis of the cell cycle, we initially looked at the cell cycle profile of these cells in their normal media (Fig. 2, inset). Upon initial observation of the DNA histogram, it was apparent that the SV40-HCEC cells have a well-defined cell cycle profile. Quantitative analysis of the percentage of cells within each phase of the cell cycle show that 50.6% of cells are in G₁/G₀, 17.2% are in S phase and 32.1% in G₂M. We compared our profile to a pooled sample of primary human corneal epithelial cells as well as published reports and found that the profiles were similar (data not shown) (Burns et al., 1990; Nezu et al., 1992).

Serum starvation was utilized to determine if this method could synchronize the SV40-HCECs. Cells were plated into media containing no serum (0%) or complete serum (10%). Cells were harvested at 24, 48, 72, and 96 h time points and their cell cycle profile based on propidium iodide staining was analyzed using flow cytometry. Although we did note a decrease in proliferation (% S) in the serum-deprived cells based on DNA histograms, there was no marked shift of cell number into G₁/G₀ in the serum-deprived cells over 96 h (Fig. 2). Profiles in the serum-deprived SV40-HCECs were not qualitatively different from cells maintained in the normal amount of serum (10%).

When DNA content was quantified (Table 1) 24 h following serum starvation, 62.5% of cells were found in G₁, compared to 53.1% in G₁ with normal levels of serum. The percentage of cells in G₁ increased over time, with 58.7% in G₁ after 48 h, 62.7% after 72 h

and 69.2% after 92 h. After 4 days of complete serum deprivation, the percentage of cells synchronized in G₁ was only 10% higher than the number of cells in G₁ with normal serum levels. Our data demonstrate that serum starvation does not effectively synchronize SV40-HCECs.

3.2. SV40-HCEC synchronization with cell sorting

SV40-HCEC cell were harvested and synchronized utilizing sterile cell sorting. Our sorting strategy and separation of each cell cycle phase is described in Section 2 (refer back to Fig. 1). Because sorting by cell cycle is based on the DNA content of the cells (fluorescence of the DNA intercalating dye, Hoechst 33342) and because cells can be in S phase without increasing the amount of DNA, it was necessary to measure S phase to verify the purity of the sorted fractions accurately. In some sorting experiments, bromo-deoxyuridine (BrdU) was added 30 min prior to dye labeling. Table 2 demonstrates the efficiency of the sort immediately post-sorting. Cells were removed from the cytometer, fixed, stained, and analyzed for DNA content (ModFit) or % incorporation of BrdU (DNA content vs. BrdU incorporation). It is apparent that even though cells were sorted based on DNA content, accurate calculation of the sorted fraction required examination of the incorporation of BrdU. By analyzing DNA content alone 97.4% of the cells sorted as G₁ appeared to be in G₁, but by examining the incorporation of BrdU, 14.2% of the cells were in S phase. Examination of cells sorted into S and G₂M showed similar results. Calculations based on DNA content alone showed that 89.8% of the cells sorted for DNA content were in S phase but 78.9% was, in fact, actively synthesizing DNA. For cells sorted based on DNA content considered G₂M, 31.8% were actually in late S phase even though calculations based on DNA content revealed 92.3% had a G₂M amount of DNA.

The SV40-HCEC cells have a doubling time of 24 h (Araki-Sasaki et al., 1995). In order to follow the progression of these sorted populations in culture, we analyzed cell cycle profiles of SV40-HCEC cells (non-BrdU labeled) at 12 and 24 h following the sterile sort (Fig. 3). After 12 h, cells in each phase appeared to be synchronized. Cells sorted with G₁ DNA content have cycled to S phase (72.8%, Table 2), those sorted for S phase (based on amount of DNA) moved into G₂ (59.3%) and G₁ (29.8%), and the majority of cells with G₂M content had cycled to G₁ (63.2%). Twenty-four hours post-sorting, the SV40-HCECs continue to maintain some synchronization and have completed their cell cycle. Cells originally in G₁ were now back to G₁ (30%) and S phase (58.3%). SV40-HCEC in S phase had returned to S phase (82.3%). Cells originally in G₂M moved into S phase (64.7%) and G₂M (23.6%). Time points were also taken up to 72 h post-sort. However, DNA profiles indicated a loss of synchrony by 48 h (data not shown). Our data suggest that the SV40-HCEC cells can maintain cell synchronization up to 24 h following sorting based on DNA content.

The viability of the synchronized SV40-HCEC cells at 24 and 48 h post-sort was also analyzed (Table 3). At 24 h, cells synchronized into each phase maintained a range of 70.8–78.9% viability, with the lowest viability observed in cells synchronized into S phase. By 48 h, the range of viability has increased. Cells synchronized into S phase and the control sorted population (All) had the lowest viability of 49% and 67.5%. Our data suggests that cells in S phase may be in a fragile state and more sensitive to the sort. The control SV40-HCEC cells in G₁ and G₂M remained high with 73.9% and 80.4%, respectively. In conclusion, we have presented a more effective alternative approach to synchronization of the SV40-HCEC cells.

4. Discussion

Due to the complexity of the cell cycle, it is difficult to achieve high levels of synchronization. Acceptable levels of synchronization have been defined as 75% or above

with no known method yielding 100% synchrony (Krek and DeCaprio, 1995). For synchronization to be effective it must be reversible, non-toxic and large numbers of synchronized cells must be obtained (Keyomarsi et al., 1991). Inhibitors and serum starvation may be effective in achieving high levels of synchronized cells, however the blockage and subsequent release of cells alters their physiological state. The resulting synchronized cells do not truly represent normal cell division of exponentially growing cells in culture due to the additional metabolic reactions and resynthesis of proteins (Pardee and Keyomarsi, 1992; Cooper, 2003a,b). We demonstrate an effective non-toxic method to isolate and identify SV40-HCEC cells within each phase of the cell cycle with minimal perturbation of normal cell division. This methodology has clear advantages over other alternative strategies in that each phase of the cell cycle can be synchronized at the same time, without detrimental effects on the cell itself.

There are an increasing number of reports detailing the negative impact of serum starvation on cells. In some cell-types, by the time the cells are synchronized by serum deprivation, apoptosis is induced resulting in cell death (Kulkarni and McCulloch, 1994; Jayadev et al., 1995). For example, serum-deprived goat skin fibroblasts exhibited a three-fold increase in apoptosis after 48 h and a 10-fold increase after 5 days. During the course of our experiments, we did not observe increased cell death, although a thorough characterization of apoptosis was not performed on cells that were serum starved.

We observed that the serum-deprived SV40-HCEC cells appeared to have decreased proliferation compared to cells grown in their normal serum containing media (data not shown). However, our data suggest that SV40-HCECs had a normal cell cycle profile based on DNA content. We observed an increase (10%) of cells within G₁/G₀ indicating that a population of cells responded to the lack of serum and could not continue through the cell cycle. Another possibility to explain our results is that the deprivation of serum caused a “cell cycle freeze”. Reports from cell cycle experiments with B16F10 melanoma cells suggest that growth arrested cultures still maintain asynchronous growth even when their proliferation has been decreased (Rodriguez-Ayerbe and Smith-Zubiaga, 2000). Similar results were observed in primary rabbit corneal cells and human keratinocytes where up to 25% of cells remain in S phase 48 h after deprivation of serum (van Ruissen et al., 1994; Anderson and SundarRaj, 2001). Clearly, our results indicate that serum starvation is ineffective for synchronization of the SV40-HCECs.

We found that BrdU inhibited cells within the G₂M phase of the cell cycle conflicting our cell synchronization results (data not shown). As a result, we utilized SV40-HCEC cells that had been labeled with BrdU or left unlabeled. The inhibition of cell cycle in cells that have been BrdU labeled has been reported (Poot et al., 1988). Amniotic fluid fibroblast-like cells were delayed in G₂ after the addition of BrdU. BrdU may enhance formation of DNA strand breaks or increase sensitivity to free radicals causing the delay of cells in G₂ (Lett et al., 1964; Balin et al., 1978). Cells were sorted without BrdU and results were obtained based on Hoechst fluorescence and DNA content. The ModFit software that we utilized for analysis also has some disadvantages in that the results (percentages) are estimates of populations within each cell cycle phase. However, experiments were repeated multiple times with similar results supporting the accuracy of this method for determining percent cell cycle phase.

Our cell sorting results indicate that we can achieve up to 85% enrichment of cells within each cell cycle phase for up to 24 h with relatively high viability and no perturbation of the normal cell cycle. In summary, the above data and discussion show that cell synchronization of SV40-HCECs via cell sorting techniques was much more efficient and effective than

serum starvation. Future studies in our laboratory will incorporate this alternative approach into our studies that require synchronization of the cell cycle.

Acknowledgments

Special thanks to Joan Batchelder and Joel Puchalski for their assistance with flow cytometry and cell cycle analysis. This work was funded by NIH RO1 NEI12253-7 and NSF DMR-9632527 (IRG3) and NIH core grant CA14520.

References

- Anderson SC, SundarRaj N. Regulation of a Rho-associated kinase expression during the corneal epithelial cell cycle. *Invest Ophthalmol Vis Sci*. 2001; 42(5):933–940. [PubMed: 11274069]
- Araki-Sasaki K, Ohashi Y, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995; 36:614–621. [PubMed: 7534282]
- Balin AK, Goodman DBP, et al. Oxygen sensitive stages of the cell cycle of human diploid cells. *J Cell Biol*. 1978; 44:390–400. [PubMed: 690172]
- Burns ER, Roberson MC, et al. Flow cytometric DNA analysis of corneal epithelium. *Am J Anat*. 1990; 187:254–260. [PubMed: 2321558]
- Cooper S. Mammalian cells are not synchronized in G1-phase by starvation or inhibition: considerations of the fundamental concept of G1-phase synchronization. *Cell Prolif*. 1998; 31:9–16. [PubMed: 9666815]
- Cooper S. Reappraisal of serum starvation, the restriction point, G0, and G1 phase arrest points. *FASEB J*. 2003a; 17:333–340. [PubMed: 12631573]
- Cooper S. Rethinking synchronization of mammalian cells for cell cycle analysis. *Cell Mol Life Sci*. 2003b; 6:1099–1106.
- Darzynkiewicz Z, Crissman H, et al. Cytometry of the cell cycle: cycling through history. *Cytometry A*. 2004; 58(1):21–32. [PubMed: 14994216]
- Dolbeare F, Gratzner H, et al. Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc Natl Acad Sci USA*. 1983; 80(18):5573–5577. [PubMed: 6577444]
- Freeman, S. *Biological Science*. Prentice Hall; Upper Saddle River, NJ: 2002.
- Galat VV, Lagutina IS, et al. Influence of serum starvation of rabbit fetal fibroblasts on nuclear reprogramming efficiency. *Ontogenez*. 1999; 30:311–416.
- Gong J, Traganos F, et al. Growth imbalance and altered expression of cyclins B1, A, E, and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. *Cell Growth Differ*. 1995; 6:1485–1493. [PubMed: 8562487]
- Gratzner HG. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science*. 1982; 218(4571):474–475. [PubMed: 7123245]
- Gray, JW.; Darzynkiewicz, Z. *Techniques in Cell Cycle Analysis*. Humana Press; Clifton, NJ: 1987.
- Grdina DJ, Meistrich ML, et al. Cell synchrony techniques. I. A comparison of methods. *Cell Tissue Kinet*. 1984; 17(3):223–236. [PubMed: 6538812]
- Herzenberg LA, Parks D, et al. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem*. 2002; 48:1819–1827. [PubMed: 12324512]
- Jayadev S, Liu B, et al. Role for ceramide in cell cycle arrest. *J Biol Chem*. 1995; 270:2047–2052. [PubMed: 7836432]
- Katska L, Bochnenek M, et al. Flow cytometric cell analysis of somatic cells primary cultures established for bovine cloning. *Theriogenology*. 2002; 58:1733–1744. [PubMed: 12472143]
- Keyomarsi K, Sandoval L, et al. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. *Cancer Res*. 1991; 51:3602–3609. [PubMed: 1711413]
- Knehr M, Poppe M, et al. A critical appraisal of synchronization methods applied to achieve maximal enrichment of HeLa cells in specific cell cycle phases. *Exp Cell Res*. 1995; 217:546–553. [PubMed: 7698256]
- Krek W, DeCaprio JA. Cell synchronization. *Methods Enzymol*. 1995; 254:114–124. [PubMed: 8531680]

- Kulkarni GV, McCulloch AG. Serum deprivation induces apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. *J Cell Sci.* 1994; 107:1169–1179. [PubMed: 7929626]
- Lett JT, Parkins G, et al. Mechanisms of sensitization to X-rays of mammalian cells by 5-bromodeoxyuridine. *Nature.* 1964; 203:593–596. [PubMed: 14250964]
- Magnani E, Bettini E. Resazurin detection of energy metabolism changes in serum-starved PC12 cells and of neuroprotective agent effect. *Brain Res Brain Res Protoc.* 2000; 5(3):266–272. [PubMed: 10906492]
- Melamed, MR.; Lindmo, T., et al., editors. *Flow Cytometry and Sorting.* Wiley Liss; New York: 1979.
- Nezu E, Ohashi Y, et al. Flow cytometry cell cycle analysis for in vivo corneal epithelium. *Nippon Ganka Gakkai Zasshi.* 1992; 96:283–287. [PubMed: 1580209]
- Niemann H, Kues WA, et al. Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. *Biol Reprod.* 2000; 62:412–419. [PubMed: 10642581]
- Oya N, Zoelzer F, et al. Effects of serum starvation on radiosensitivity, proliferation and apoptosis in four human tumor cell lines with different p53 status. *Strahlenther Onkol.* 2003; 179:99–106. [PubMed: 12590320]
- Pardee AB, Keyomarsi K. Modification of cell proliferation with inhibitors. *Curr Opin Cell Biol.* 1992; 4(2):186–191. [PubMed: 1376129]
- Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys.* 2004; 59:928–942. [PubMed: 15234026]
- Poot M, Schindler D, et al. Bromodeoxyuridine amplifies the inhibitory effect of oxygen on cell proliferation. *Cytometry.* 1988; 9:332–338. [PubMed: 2456899]
- Rodriguez-Ayerbe C, Smith-Zubiaga I. Effect of serum withdrawal on the proliferation of B16F10 melanoma cells. *Cell Biol Int.* 2000; 24:279–283. [PubMed: 10805961]
- Takeda K, Akagi S, et al. Mitochondrial activity in response to serum starvation in bovine (*Bos taurus*) cell culture. *Cloning Stem Cells.* 2002; 4:223–229. [PubMed: 12398803]
- Tan JH, Zhao CB, et al. Studies of the cell cycle of in vitro cultured skin fibroblasts in goats: work in progress. *Theriogenology.* 2003; 59:1277–1289. [PubMed: 12527075]
- Tani T, Kato Y, et al. Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biol Reprod.* 2001; 64:324–330. [PubMed: 11133690]
- van Ruissen F, van Erp PE, et al. Cell kinetic characterization of growth arrest in cultured human keratinocytes. *J Cell Sci.* 1994; 107:2219–2228. [PubMed: 7983181]

Fig. 1.

Sorting strategy for synchronization of SV40-HCEC cells into G₁/G₀, S, and G₂M. Dot plots were generated from propidium iodide labeled cells against Hoechst fluorescence. Single cells were gated out as indicated in (A). The gated single population was plotted with forward scatter versus Hoechst fluorescence (B). Low levels of fluorescence were gated out at G₁/G₀, moderate levels as S phase, and high levels were gated as the G₂M population (C).

Fig. 2.

Serum starvation of SV40-HCEC cells. DNA histograms are generated by plotting Hoechst fluorescence against cells number. Cells were harvested at 24, 48, 72, and 96 h and were either deprived of serum, or cultured in media with 10% serum. The inset histogram represents the distribution of cycling cells and the various phases (G_1/G_0 , S, G_2M) as determined by flow cytometry. Single cells were gated and fluorescence against cell number was plotted.

Fig. 3.

SV40-HCEC maintain synchronization up to 24 h following the sterile sort. DNA histograms were generated to follow the cell cycle profile of cells originally synchronized into G₁/G₀, S, and G₂M at 12 (A) and 24 h (B) after sorting. The 12 h panels indicate that the cells are cycling through in synchrony. By 24 h, cells have completed one cell cycle and still maintain synchronization.

Table 1
Serum starvation does not synchronize SV40-HCEC cells

Serum starvation		G ₁	S	G ₂ M
24 h	0%	62.5	29.5	8.0
	10%	53.1	38.9	2.2
48 h	0%	58.7	33.3	3.0
	10%	56.5	35.5	1.0
72 h	0%	62.7	29.3	8.2
	10%	62.3	29.7	9.2
96 h	0%	69.2	22.8	3.3
	10%	59.4	32.6	11.1

Cells were harvested at 24, 48, 72 and 92 h and were either deprived of serum, or cultured in media with 10% serum. DNA histograms were analyzed with ModFit software to estimate the percentage of cells within each phase including G₁/G₀, S, and G₂M. The table is representative of experiments repeated in triplicate.

Table 2

Sort and release of synchronized SV40-HCEC cells

Sort and release of synchronized cells		0 h			12 h			24 h		
		ModFit			BrdU					
G ₁	G ₁	97.4	84	3.5	38.2					
	S	1.4	14.2	72.8	58.3					
S	G ₂ M	1.3	0.5	23.7	3.6					
	G ₁	0.80	2.13	29.8	9.4					
G ₂ M	S	89.8	78.9	10.9	82.3					
	G ₂ M	9.4	11.5	59.3	8.3					
G ₂ M	G ₁	1.2	1.0	63.2	11.7					
	S	6.5	31.8	19.6	64.7					
	G ₂ M	92.3	64.2	17.0	23.6					

Cells, with or without pulsing with BrdU, were sorted into G₁/G₀, S and G₂M phases of the cell cycle based on DNA content. Immediately following sorting, cells were assessed for DNA content (ModFit calculations) as well as BrdU incorporation. Percentage of cells not pulsed with BrdU in each phase at 0 h and 12 and 24 h after sorting were calculated with ModFit software. The data are representative of experiments repeated in triplicate.

Table 3
Viability of synchronized SV40-HCEC cells post sorting

Viability of synchronized cells	24 h	48 h
G ₁	78.9	80.4
S	70.8	49
G ₂ M	78.3	73.9
All	74.8	67.5

Cells, with or without pulsing with BrdU, were sorted into G₁/G₀, S and G₂M phases of the cell cycle based on DNA content. Twenty-four and 48 h following sorting, cells were assessed for viability. The table is representative of data repeated in triplicate.