2013

The Effects of Serum Starvation on Cell Cycle Synchronization

Negin Baghdadchi
California State University, San Bernardino

Follow this and additional works at: https://scholarworks.lib.csusb.edu/osr

Part of the Life Sciences Commons, Medicine and Health Sciences Commons, and the Physical Sciences and Mathematics Commons

The Effects of Serum Starvation on Cell Cycle Synchronization

The Effects of Serum Starvation on Cell Cycle Synchronization

Negin Baghdadchi

Abstract

Human pluripotent stem cells (hPSC) provide a valuable model for studying organogenesis and recapitulating human diseases. Notably, genetic modification techniques can be harnessed to realize potential of these cells. hPSC display the features of indefinite self-renewal and the potential to differentiate into three germ layers, which holds great promise for regenerative medicine and human disease research. Genetic manipulation continues to be a powerful to study the properties of hPSC. Despite current advances in optimization of transfection procedures, genetic manipulation of hPSC remains a capricious process. On the other hand, controlled differentiation also yielded a variable and unpredictable efficiency in different hPSC lines. Recent studies have shown that serum starvation-induced cell cycle synchronization significantly improved reprogramming efficiency in human fibroblast and increased transient gene delivery into mouse embryonic stem cells. Nevertheless, hPSC lines are routinely cultured in KnockOut Serum Replacement (KO-SR), a serum-free formulation that directly replaces serum in culture. In this regard, we hypothesized that reduction of KO-SR exerts a profound effect in transfection and differentiation efficiency of hPSC. Our procedure mirrors effect of serum starvation by synchronizing hPSC cultures in G1 phase. We showed that transient transfection efficiency could be increased by culturing hPSC in 5% KO-SR for 2 days. Furthermore, our procedure resulted in six-fold increment in the derivation of stably eGFP-expressing transfectants. Interestingly, KO-SR synchronized hPSC also differentiated in a more homogenous manner, as evidenced by a significantly stronger induction of lineage-specific progenitors and an absence of OCT4-expressing residual undifferentiated cells. These results demonstrate that cell cycle synchronization is a key milepost on the path to reducing heterogeneity of hPSC and should be useful in exploring the use of these cells in a predictable manner.

Author Interview

Which professors (if any) have helped you in your research?
Dr. Chee Gee Liew and Dr. Nicole Bournias.

What are your research interests?
My research interests are stem and cancer cell studies.

What are your plans after earning your degree? What is your ultimate career goal?
After earning my degree I plan on pursuing a PhD and become a researcher.

Keywords: *Human Pluripotent Stem Cells, Induced Pluripotent Stem Cells, Transfection, Differentiation, Serum Starvation.*

Introduction

Human embryonic stem (ES) cells are derived from inner cell mass of blastocyst and are capable of unlimited expansion and differentiation *in vitro* [10].The ability of these cells to differentiate and generate cell types corresponding to all three embryonic germ layers makes them as an important target in all different research arenas. Furthermore, these cells are capable of retaining a normal karyotype for prolonged periods in culture [7]. Ectopic expression of reprograming factors can result in the derivation of induced pluripotent stem cell (iPSCs) from somatic cells. The first establishment of murine iPSCs was reported by Takahashi and Yamanaka (2006) in 2006 [5]. This was done by transducing mouse embryonic fibroblast with retroviruses encoding cMyc, Oct3/4, Sox2 and klf4. Subsequently, the first hiPSC lines were derived in 2007 (Yu et al. 2007; Takahashi et al., 2007) [10, 12]. Similar to ES cells, iPSC lines can be maintained in their undifferentiated state and yet be differentiated into various cell types, providing a resource of considerable promise for the future of disease modeling and drug discovery. To date, however, reprograming of human adult cells is still challenging and inefficient. To realize the potential of hESCs, efficient methods are required to manipulate their genomes. Progress in certain conditions resulted in poor transfection and low single-cell cloning efficiencies. Although recent studies have suggested robust methods of transfection of hESCs, routine production of stable transfectants are still indefinable [9]. This applies for both transient assays as well as the generation of stable lines.

In order to increase the differentiation and nucleofection efficiency of the ESC, it is important to understand their cell cycle pattern. It is revealed that cell cycle is a key parameter driving epigenetic reprogramming to pluripotency [7]. Recent studies have shown that serum starvation and chemical inhibitors have caused cell cycle synchronization of the somatic cells. A summary of these studies is shown in figure 1 [5]. However, unlike the somatic cell cycle, ESCs have a very short G0 phase as the cells proliferate more rapidly throughout their life time. A comparison between the cell cycle of somatic cells and stem cells is shown in figure 2.
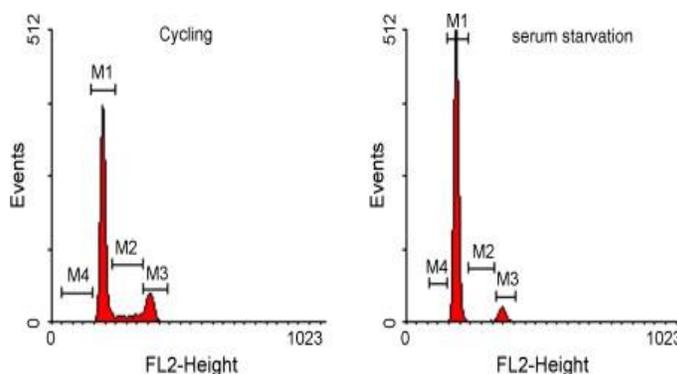

Figure 1: Representative Histograms of DNA

Figure 1. Representative histograms of DNA content using flow cytometry of canine dermal fibroblasts cultured under various conditions: cycling, serum starvation (24 h). M1: G0/G1 (2C DNA content), M2: S (between 2C and 4C), M3: G2/M (4C), and M4: apoptosis (2C) [5].
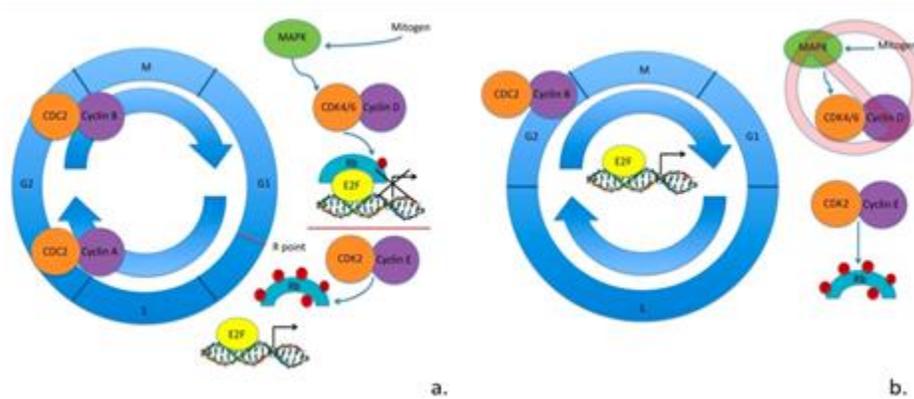
Figure 2: Cell Cycle in Somatic Cells vs. ESCs

Figure 2. Cell cycle in somatic cells vs. ESCs. (a) Cell cycle regulation in somatic cells: (b) Cell cycle regulation in ESCs as is currently understood [9].

The normal cell cycle distribution of the ESC can be demonstrated using flow cytometery analysis, shown in figure 3. As it is presented majority of the ES cells (50%) are in the S phase, leaving 30% for G1 and 19% for the G2 phase.
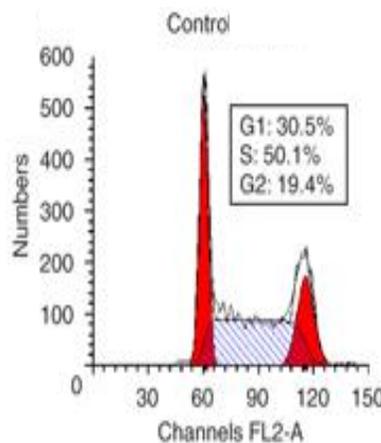

Figure 3: Flow Cytometry Analysis of Cell Cycle of hESCs (H9) [7]

It is well known that the cell cycle plays an essential role in the differentiation and nucleofection efficiency. The cell cycle stages of cultured cells can be synchronized by serum starvation, contact inhibition, and chemical treatments. Serum starvation is widely used for synchronizing donor cells by arresting them in the G0/G1 phase of the cell cycle, but it often reduces cell survival and increases the DNA fragmentation. In this study, using iPSCs and ESC, we have shown that starving the cells with 5% KOSR for the duration of two days can significantly increase the differentiation and nucleofection efficiency.

OSR Journal of Student | Inaugural Volume, Spring 2013

45

Methods and Materials

H9 human ESC and Riv9 human iPSC were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for hESC cultures and every 3-4 days during differentiation, depending on the differentiation protocol. For the study purpose, the ESC and iPSC were starved with different concentrations of Knock out Serum Replacement (KOSR); 20% (control), 5% and 0.5%. The starvation period was set into 1 day, 2 days and 3 days.

Immunocytochemistry analysis: Cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS and blocked with PBS with 0.1% Triton-X and 1% blocking serum for 30 min. Following blocking, cells were incubated with primary antibodies in blocking solution overnight: anti-BLIMP1 (1:100), anti-OCT 4 (Santa Cruz ; 1:50) and anti-FOXA2 (cell signaling technology 1:100). Cells were then washed in blocking solution and incubated with Alexa488 or Alexa 598 secondary antibodies (Molecular Probes; 1:500) for one hour at room temperature, followed by two washes with blocking solution and third wash with PBS. Cells were mounted with DAPI mounting reagent (Vectashield). Images were captured using Nikon Eclipse Ti microscope.

Differentiation of HESC and iPSCs: For the germ cell differentiation, cells were cultured in human embryonic stem cell (HES) media with bone morphogenetic protein-4 for 5 days.

Cell cycle analysis: Cells were harvested using 0.25% trypsin/EDTA and resuspended in DMEM at a concentration of $1X10^6$ cells per tube. After centrifugation at 700 X g for 10 min, the supernatant solution was removed and, while vortexing gently, cell were fixed by drop-wise addition of 0.8 ml cold methanol to the tube containing 0.2 ml of cell suspension. After fixation, fixed cells were again centrifuged and washed with cold PBS. Then, 0.25 mL of propidium iodide solution (PBS) containing 50 µg/ml propidium iodide and 0.1% Triton X-100 was added. After incubating in 37°C for 30 min, cells were ready for flow cytometry.

Nucleofection: Cells were pretreated with Rock inhibitor for one hour, after which single cells were obtained by trypsinization. To nucleofect the cells, the Green Fluorescent Protein (GFP) plasmid was added to the cells and using program B16 in Lonza nucleofector, the plasmid was transferred into the nucleus. The cells were let recovered in the incubator for 10 minutes and cultured in mTeSR media.

Flow cytometry: Cells were harvested using 0.25% trypsin/EDTA and resuspended in DMEM at a concentration of $1X10^6$ cells per tube and subjected for flow cytometry analysis on a Beckman Coulter Sc Quanta flow cytometer.

Results

We demonstrate that serum starvation in hPSC resulted in the cell cycle arrest in the G0 phase and hPSC that have been starved for 2 days with 5% KOSR yielded a significant increase in transfection and nucleofection efficiency.

Establishment and culture of H9 and Riv9 cells: H9 and Riv9 cells both cultured on Geltrex-coated plates with mTeSR medium supplemented with conditioned media (CM) in the ratio of 4:1. The CM was collected from the irradiated mouse embryonic fibroblast (MEF) cells and contained necessary growth factors for feeder-free hPSC growth. The cells were passaged upon reaching 70%-80% confluency, every 4-5 days. Each experiment has been repeated at least 3 times and consistent results were observed.

PI staining analysis: We examined the effect of serum starvation on cell cycle distribution of hPSC. As shown in figure 4, there is a clear shift of the cell cycle toward G1 phase in cells starved with 5% KOSR for 2 days compared to the control unstarved cells.
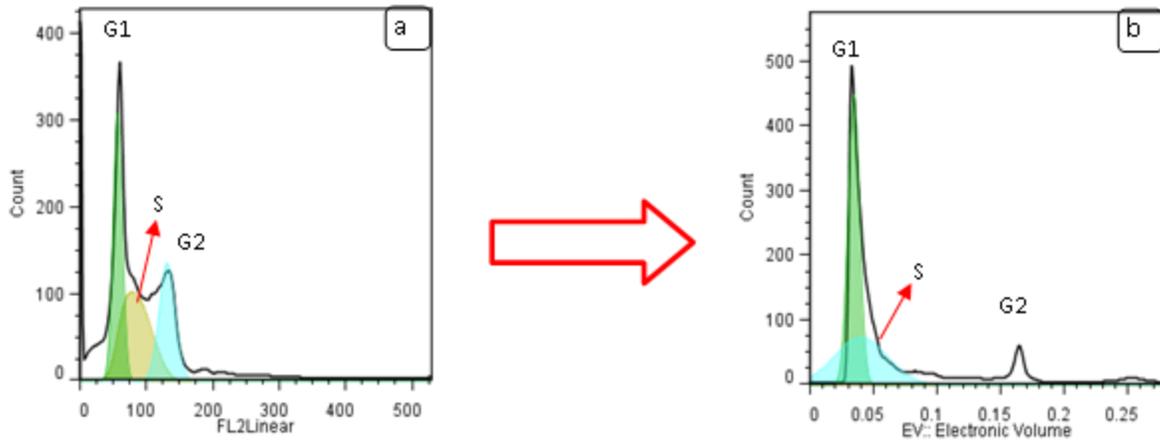
Figure 4: The PI staining results for Riv9

Figure 4, shows a significant increases in a G1 cell population was obtained following a 2-day starvation. *Nucleofection of the Stem Cells*: The H9 and Riv 9 cells were starved with 5% and 0.5% KOSR for the duration of 1, 2, and 3 days. The control cells were cultured with 20% KOSR. After nucleofection, the cells were cultured in mTeSR and the analyzed 24 hours following the nucleofection. Prior to flow cytometry analysis, microscopy images of the cells were taken (figure 5). All KOSR-starved cells yielded higher nucleofection efficiency compared to the non-starved control.
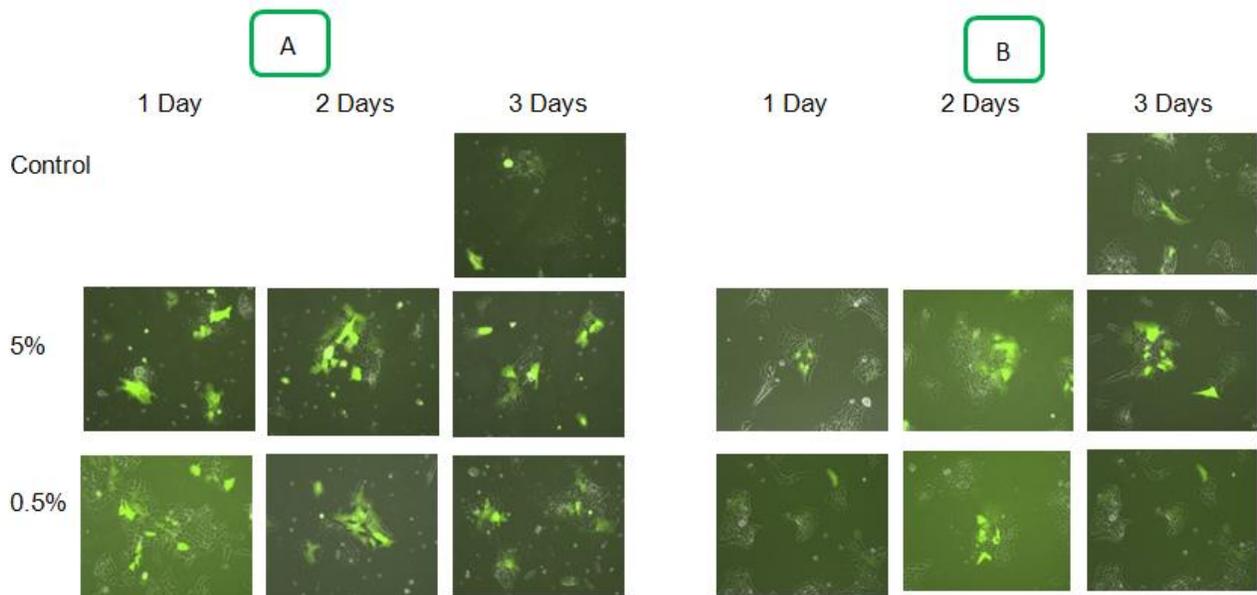

Figure 5: Transient Transfection Efficiency in hPSC

Figure 5, shows transient transfection efficiency in hPSC following 1, 2 and 3-day starvation with 5% and 0.5% KOSR is compared with control cells cultured in 20% KOSR. Nucleofection efficiency was higher in starved cells, A) GFP expression in Riv9 cells, B) GFP

OSR Journal of Student | Inaugural Volume, Spring 2013   47

expression in H9 cells. Flow cytometric analyses were then done on the nucleofected cells. In this regard the expression of GFP was measured and the data was analyzed using Flowjo software. Figure 6 shows the results of 6 treatments representing the highest expression of GFP. As it is seen, the red line is the negative control in which there was no nucleofection therefore there was no GFP expression. Figure 6a, displays the

Riv9 cells. In this group the blue line is representative of GFP expression in control cells, which were nucleofected without starvation. In same group, the dark green line represents the GFP expression in Riv9 cells that were nucleofected after 2 days being starved with 5% KOSR. As is seen, these cells show the highest expression of GFP in the group.
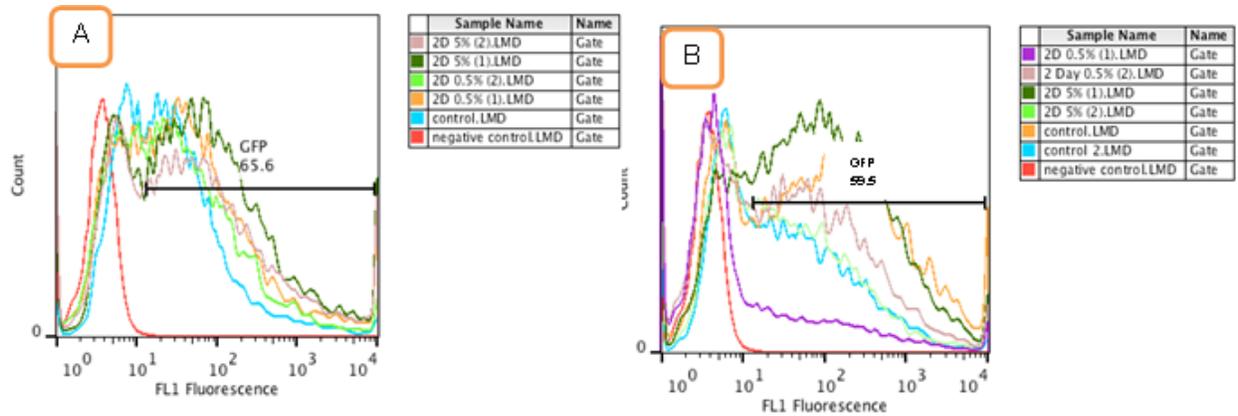

Figure 6: flow cytometric analyses of Riv9 and H9 cells

The Figure 6, shows the flow cytometric analyses of Riv9 and H9 cells. In this figure the highest 4 results are picked and compared to the positive and negative control. This means that all other groups had lower GFP expression compared to this group. a) shows the Riv9 cells and b) the H9 cells. 2D: 2 Days starvation, 5% and 0.5%: starvation with 5% and 0.5% KOSR respectively.

Figure 6b, shows the flow cytometric analysis on H9 cells. In this group, also, the blue line in representative of control cells that were nucleofected without being starved. It is clear that in H9 cells, the control population had lower nucleofection efficiency compared to other groups. However, there were variations in the results of the cells starved with 0.5% KOSR for 2 days. The average of GFP expression in H9 cells starved with 5% KOSR for 2 days was the highest among all other groups in H9 cells.

Differentiation efficiency of Riv9 and H9 cells: We next evaluated the effect of serum starvation on germ cell (GC) differentiation. hPSC were starved prior to treatment with BMP4. Experiments were done in triplicate. Differentiated cells were analyzed by immunocytochemistry analysis with anti-OCT and anti-BLIMP1 antibodies. OCT4 is a marker for undifferentiated hPSC and BLIMP1 is the GC marker. The number of cells expressing each marker was counted manually and the result is presented in figure 7. There was still a high percentage of cells expressing OCT4 in unstarved and 1-day starved cells, indicates the presence of residual undifferentiated stem cells. In addition, only a small percentage of cells expressed BLIMP1. Nonetheless, 2-day starvation resulted in a significant increase of BLIMP but a complete loss of OCT4 expression. Furthermore, serum starvation also resulted in a more consistent differentiation outcome, as indicated by the smaller error bars in 2- and 3-day starved cells.
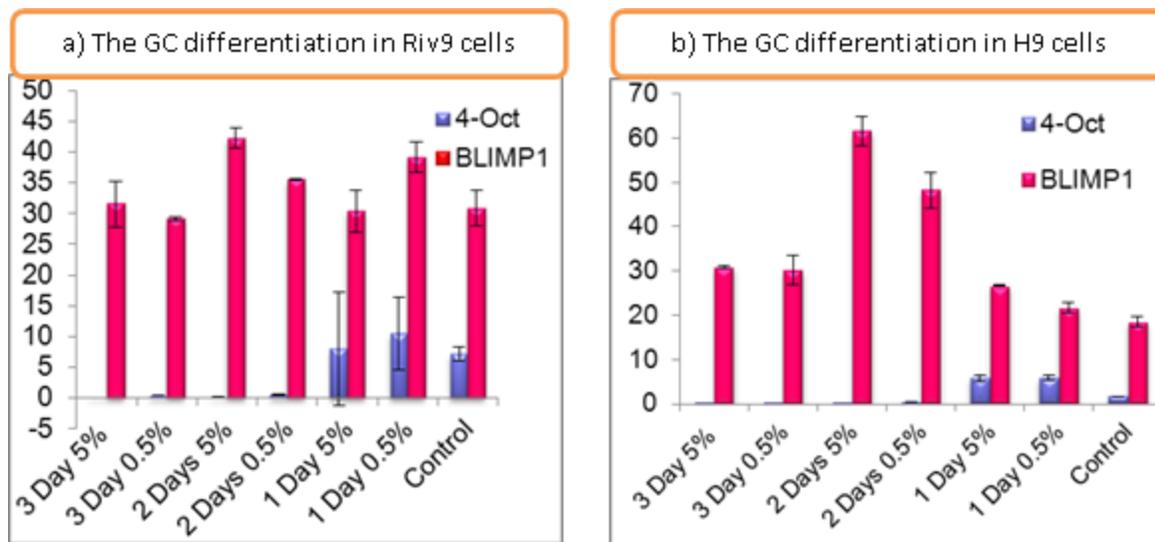
Figure 7: Triplicated Analyses

Figure 7, shows the expression of germ cell markers in Riv9 (a) and H9 cells (b) after being differentiated into these cells. The chart is representative of triplicated analyses.

Discussion

Here, in this study, we demonstrate that serum starvation synchronized hESC and iPSC in G0/G1 stage of the cell cycle. The synchronizing effect of serum starvation on the cell cycle was achieved by starving the cells for 2 days with 5% KOSR. Extended serum starvation periods (more than 48 hours) did not significantly increase the proportion of G0/G1 cells. The synchronized cells showed significantly higher nucleofection and differentiation efficiency compared to the non-starved cells.

References

Braam S. et al (2008), "Improved genetic manipulation of human embryonic stem cells", *Nature Methods*, Vol.5 No.5

Burrideg P. W. et al. (2007), "Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability", *Stem Cell*, 25,929-938

Chen M. et al. (2012), "Serum starvation induced cell cycle synchronization facilitates human somatic cells reprogramming", *PLoS ONE*, Vol7, Issue4.

Dick E. et al. (2011), "Two new protocols to enhance the production and isolation of human induced pluripotent stem cell lines", *Stem Cell Research",* Volume 6, Issue 2, March 2011, Pages 158–167

Khammanit R. et al (2008), "Effect of serum starvation and chemical inhibitors on cell cycle synchronization of canine dermal fibroblasts", *Theriogenology*, Volume 70, Issue 1, Pages 27–34

Liew C. G. et al (2007), "Transient and stable transgene expression in human embryonic stem cells", *Stem Cell*, 25, 1521-1528

Neganova I. et al. (2009), "Expression and functional analysis of G1 to S regulatory components reveals an important role for CDK2 in cell cycle regulation in human embryonic stem cellsAn important role for CDK2 in cell cycle regulation" Oncogene 28, 20-30

Ruiz S. et al. (2010), "High-efficient generation of induced pluripotent stem cells from human astrocytes", PLos ONE, Vol.5, Issue 12

Symonds C. E. (2009), "Aging of the inceptive cellular population: the relationship between stem cells and aging", *Aging,* Vol1, No.2

Takahashi K. et al. (2007), "Induction of pluripotent stem cells from adult human fibroblasts by defined factors"*, Cell,* 30;131(5):861-72

Thomson J. A. et al. (1998), "Embryonic Stem Cell Lines Derived from Human Blastocysts", *Science* 282, 1145

Yu J. et al. (2007), "Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells", *Science*, Vol. 318 no. 5858 pp. 1917-1920