

## Analysis of the Cell Cycle in Mouse Embryonic Stem Cells

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### 1. Introduction

The molecular mechanisms underlying self-renewal of pluripotent embryonic stem (ES) cells is still poorly understood. Deciphering these mechanisms is of prime importance for at least two reasons: (1) ES cells derive from, and are closely related to, the pluripotent stem cells of the blastocyst, the founder cells of the whole embryo proper. Hence, they constitute a unique model for studying embryonic development at the time of implantation, when embryos are inaccessible to experimental manipulation; and (2) Isolating and manipulating ES cells in species of economic or therapeutic interests is more difficult than in the mouse. It is likely that better defining their growth requirements will lead to major improvements in their culture conditions.

During the past 6 yr, intrinsic features of mouse ES cells regarding the regulation of their growth cycle have been pinpointed. These features may serve not only to understand how the cell cycle machinery of ES cells works, but also to better characterize ES cells isolated from embryos of other species. Hence, a striking feature of mouse ES cells is their unusual cell cycle distribution. The three phases of the cell cycle, G1, S, and G2/M, represent 15, 75, and 10%, respectively, of the total cell cycle, with a G1 phase of approx 1 h. Hence, ES cells reenter the S-phase very shortly after exit from mitosis (1,2). These preliminary observations have paved the way to the analysis of cell cycle control in ES cells, focusing on the regulation of G1  $\rightarrow$  S transition.

#### 1.1. Retinoblastoma Pathway

The proliferation of mammalian cells is controlled largely during the G1 phase of their growth cycles. The decision to initiate a new round of DNA synthesis is largely dependant upon the phosphorylation and functional inactivation of the retinoblastoma (RB) protein. This phosphorylation is driven by components of the cell cycle apparatus, specifically cyclins and cyclin-dependent kinases (CDKs). Of prime importance are complexes of D-type cyclins (cyclin D1, D2, and D3) and CDK4 or CDK6 (3). Moreover, the cellular machinery that is organized to collect extracellular signals and transduce them via tyrosine kinase receptors and the SOS-RAS-MEKK-MAPK pathway seems to be dedicated largely to driving RB phosphorylation (4). This control

circuitry appears to be operative in virtually all cell types. In contrast, the control of the ES cell mitotic cycle is likely to be markedly different. First, ES cells seem to lack the CDK4-associated kinase activity that characterizes all RB-dependent cells. They express very low levels of D-type cyclins, as a result of the very poor activity of the respective promoters. This is somewhat surprising as hypophosphorylated RB remains undetectable during the M→G1→S transition, indicating that RB is rapidly rephosphorylated in G1 (2). Secondly, ES cells appear to be resistant to the growth inhibitory effect of the cyclin D:CDK4-specific inhibitor p16<sup>ink4a</sup>, further suggesting that RB phosphorylation may not rely on proper CDK4-associated kinase activity in ES cells. Not surprisingly, induction of differentiation restores the expression of all three D-type cyclins, strong CDK4-associated kinase activity, and the sensitivity to the growth-inhibitory activity of p16<sup>ink4a</sup> (1,2, and unpublished results), suggesting that differentiating ES cells resume a normal cell cycle control.

Another important aspect of G1 control lies in the regulation of cyclin D1 expression by the Ras→MAPK pathway. Phosphorylated ERKs activate cyclin D1 expression through fos and ets transcription factors (5). In ES cells, inhibition of ERK phosphorylation by wortmannin (an inhibitor of Ras activation) or by PD98059 (an inhibitor of MEK) neither inhibits background expression of cyclin D1 nor induces growth retardation. Induction of differentiation up-regulates the steady-state level of cyclin D1, whose expression then becomes sensitive to the inhibitors of the Ras→MEK→ERK cascade (Jirmanova et al., unpublished results). Hence, cyclin D1 expression seems not to be regulated by Ras in ES cells. This regulation is likely to be restored upon differentiation.

Recently, it has been shown that Rb-E2F forms a transcriptional repression complex by recruiting histone deacetylase and SWI/SNF subunits (6). These large complexes are capable of blocking the transcription of cell cycle genes and remodeling chromatin (7). However, it is unclear if these large complexes have a specific role in chromatin organization of ES cells. Thus far, our preliminary immunoprecipitation experiments suggest that HDAC1 binds to the low amount of RB in ES cells. This could be a key aspect in the ES renewing cell cycle that should be investigated.

## 1.2. p53 Pathway

In somatic cells, cell cycle checkpoints limit DNA damage by preventing DNA replication under conditions that may produce chromosomal aberrations. The tumor suppressor p53 is involved in such control as part of a signal transduction pathway that converts signals emanating from DNA damage, ribonucleotide depletion, and other stresses into responses ranging from cell cycle arrest to apoptosis (8). Stress-induced stabilization of nuclear p53 results in the transactivation of downstream target genes encoding, for example, the cyclin-dependent kinase inhibitor p21<sup>cip1/waf1/sdi1</sup> or Mdm2. p21<sup>cip1/waf1/sdi1</sup> inhibits RB phosphorylation, thereby preventing transition from G1 to S (8). ES cells do not undergo cell cycle arrest in response to DNA damage (caused by  $\gamma$ -radiations, UV light, genotoxic agents) or nucleotide depletion (9,10). ES cells express abundant quantities of p53, but the p53-mediated response is inactive because of cytoplasmic sequestration of p53. Moreover, enforced expression of nuclear p53 still fails to induce cell cycle arrest, suggesting that, in addition to its cytoplasmic sequestration, p53 cannot activate the downstream targets required for growth arrest

(9). One of these targets is p21<sup>cip1/waf1/sdi1</sup>. ES cells do not express p21<sup>cip1/waf1/sdi1</sup> (2), suggesting that the p21<sup>cip1/waf1/sdi1</sup> promoter is not responsive to p53 in ES cells. Therefore, it appears that ES cells have a very effective mechanism for rendering them refractory to p53-mediated growth arrest. Induction of differentiation restores the p53-mediated cell cycle arrest response (9).

Taken together, these results suggest fundamental differences in the regulation of cell proliferation in ES cells as compared to somatic cells. Firstly, they suggest that the complex apparatus that operates in most cells with extracellular mitogens, transducing signals through the SOS-RAS-RAF-MEKK-MAPK pathway and that ultimately leads to pRB phosphorylation is not engaged in ES cells. Induction of differentiation would reactivate this mechanism. Secondly, ES cells do not seem to have a p53-mediated checkpoint control. This control would also become operative when differentiation occurs.

In the second part of this chapter, we describe experimental procedures used to synchronize ES cells and to analyze their cell cycle distribution. These procedures have been developed to characterize the growth cycle of mouse ES cells.

## 2. Materials

1. Feeder-independent ES cell line: CGR8 (11).
2. Complete medium: Glasgow's Modified Eagle's Medium (GMEM) (BioMedia, cat. no. GMEMSPE2052) supplemented with 10% fetal calf serum (FCS) (PAA, cat. no. A15-043), 2 mM L-glutamine (BioMedia, cat. no. GLUN2002012), 1% nonessential amino acid solution (BioMedia, cat. no. AANE0002012), 1 mM sodium pyruvate (BioMedia, cat. no. PYRU0002012), 0.1 mM 2-mercaptoethanol (Sigma, cat. no. M7522), 100 U/mL penicillin, 100 mg/mL streptomycin, and 1000 U/mL human leukemia inhibitory factor (LIF). For LIF preparation and testing (*see ref. 12*).
3. 0.25% (w/v) Trypsin in Phosphate-Buffered Saline (PBS).
4. 20 ng/mL Demecolcine (Sigma, cat. no. D6165).
5. 0.1% and 0.2% Gelatin (Sigma, cat. no. G9391) dissolved in H<sub>2</sub>O.
6. 5 mM BrdU (Sigma, cat. no. B9285) (100× stock solution).
7. 1 mg/mL RNase dissolved in PBS + 0.13 mM EGTA.
8. PBT: PBS + 0.5% Bovine Serum Albumin (BSA) (Sigma, cat. no. A2153) + 0.5% Tween-20 (Sigma, cat. no. P7949).
9. Anti-BrdU (Becton Dickinson, cat. no. 347583).
10. 100 µg/mL Propidium iodide (Sigma, cat. no. P4170) (100X stock solution).
11. Sterile flasks and Petri dishes: sterile 5- and 10-mL pipets.
12. FACScan (fluorescence-activated cell sorter) (Becton-Dickinson), equipped with a 15-mW 488-nm air-cooled argon-ion laser. Filters used: 530 nm fluorescein isothiocyanate (FITC), 585 nm (propidium iodide). Data acquisition and analysis are performed using CellQuest (Becton-Dickinson) software.

## 3. Methods

### 3.1. Synchronization of ES Cells by Mitotic Shake-Off

This protocol is intended to generate large numbers of synchronized ES cells exiting from mitosis, entering into G<sub>1</sub>, and then into S phase, synchronously.

1. At d 1, trypsinize ES cells and seed at a density of 20–30 million cells in 25 mL complete medium in T160 flasks coated with 0.2% gelatin (gelatin is added to flasks at least 2 h

before seeding the cells. Gelatin is thoroughly removed by aspiration just before seeding the cells). Incubate at 37°C in 7.5% CO<sub>2</sub> (*see Note 1*).

2. At d 2, add 50 mL complete medium (removing the exhausted medium is not necessary) and incubate overnight.
3. At d 3, ES cells should form a confluent layer. Check that each flask is confluent. Discard those in which empty spaces are visible, as isolated clumps of cells are likely to detach from the flasks during the shake-off procedure. Then:
  - a. Remove the loosely attached cells by preshaking the flasks 5 times by hitting the flasks against the palm of the hand.
  - b. Quickly aspirate the medium and replace it with 25 mL complete medium containing 20 ng/mL demecolcine (*see Note 2*). Incubate for 3–4 h at 37°C in 7.5% CO<sub>2</sub>.
  - c. Shake the flasks 5 times by hitting them against the palm of the hand. Collect the medium in 50-mL disposal plastic tubes. From this step on, sterile manipulation is not required.
  - d. Spin mitotic cells at 500g for 5 min. Aspirate the medium. Invert the tubes on absorbing paper for 5 min.
  - e. Gently resuspend each pellet with 1 mL of prewarmed demecolcine-free medium using a P1000 Gilson pipet. Do not pipet the cells up and down more than required to get a single-cell suspension. Fill the tubes with complete medium.
  - f. Spin at 500g for 5 min. Discard the medium. Invert the tubes onto absorbing paper to dry.
  - g. Gently resuspend each pellet with 1 mL of prewarmed medium and pool into a single tube. Count the cells. This procedure yields approx  $2 \times 10^6$  mitotic cells/T160 flask (i.e., approx 1% of the total number of cells).
  - h. Prepare a cell suspension containing approx  $10^6$  mitotic cells/mL. Seed 6-cm dishes (coated with 0.1% gelatin as described in **step 1**) with 5 mL cell suspension. Incubate at 37°C in 7.5% CO<sub>2</sub>.
  - i. Collect the cells at various time points and analyze them for cell cycle distribution as described in **Subheading 3.2**. Since mitotic cells usually take 4–5 h to attach to the dish, do not aspirate the medium. Any supplements should be added dropwise using 10X stock solutions (*see Note 3*).

### 3.2. Analysis of DNA Content in Synchronized ES Cells

As mitotic ES cells usually take 4–5 h to attach strongly to the Petri dish, the following protocol must be used to prepare a single-cell suspension suitable for flow cytometry:

1. Collect the cells by pipetting up and down approx 10 times with a P1000 Gilson to dissociate the loosely adherent cells. Trypsinization is not required (*see Note 4*). Transfer the suspension (>1 million cells) into a conical 15-mL tube.
2. Spin for 5 min at 500g. Discard the medium and wash in PBS. Repeat once.
3. After the last spin, resuspend cells in 100 µL of PBS. Pipet up and down with a P200 Gilson until clumps are no longer visible. Dropwise, add 1 mL of 70% ethanol at –20°C (1 drop/s to avoid formation of clumps of cells). Store the fixed cells at 4°C.
4. To analyze the DNA content, add 10 mL PBS directly to cells in ethanol. Incubate for 5 min at room temperature to allow cells to rehydrate.
5. Spin for 5 min at 500g. Resuspend the pellet in 100 µL of PBS. Add 10 mL PBS. Incubate for 5 min at room temperature.
6. Resuspend the pellet in 100 µL of 1 mg/mL RNase. Incubate for 20 min at room temperature. Store at 4°C if required (<24 h).

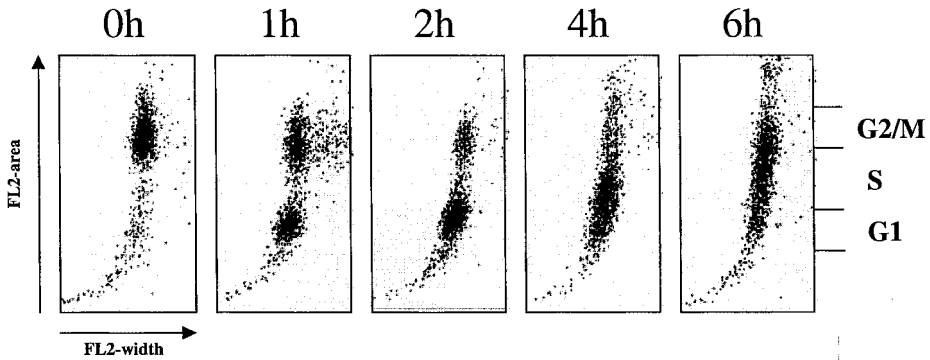


Fig. 1. Analysis of DNA content of ES cells synchronized by mitotic shake-off, determined according to the protocol described in **Subheading 3.2**. FL2-width indicates the size of the cells. FL2-area quantitates fluorescence associated to propidium iodide. 0h, 1h, 2h, 4h, and 6h indicate the time after release from the mitotic block.

7. Add propidium iodide to a final concentration of 1  $\mu\text{g}/\text{mL}$ . Incubate for 5 min at room temperature.
8. Analyze fluorescence using conventional setups. The result of a representative synchronization experiment is given in **Fig. 1**.

### 3.3. Analysis of Cell Cycle Distribution in Exponentially Growing ES Cells

1. Refeed exponentially growing ES cells with complete medium. Incubate for 1 h at 37°C.
2. Add BrdU at a final concentration of 50  $\mu\text{M}$  and incubate for 30 min.
3. Trypsinize the cells and take 5 million cells for analysis of BrdU incorporation.
4. Spin for 5 min at 500g. Discard the medium and wash in PBS. Repeat once.
5. After the last spin, resuspend the pellet of cells in 100  $\mu\text{L}$  of PBS. Dropwise, add 1 mL of 70% ethanol at  $-20^\circ\text{C}$  (1 drop/s to avoid formation of clumps of cells). Store the fixed cells at 4°C for up to several weeks.
6. Add 10 mL PBS to ethanol-fixed cells. Incubate for 5 min at room temperature to allow cells to rehydrate.
7. Spin for 5 min at 500g. Resuspend the pellet in 200  $\mu\text{L}$  of 2 N HCl and incubate for 20 min at room temperature.
8. Wash 3–4 times in 10 mL PBT.
9. Resuspend the pellet in 100  $\mu\text{L}$  FITC-conjugated antibody raised to BrdU (Becton Dickinson), diluted 1:10 in PBT, and incubate for 30 min at room temperature.
10. Wash 3–4 times in 10 mL PBT.
11. Resuspend the pellet in 100  $\mu\text{L}$  of 1 mg/mL RNase. Incubate 20 min at room temperature. Store at 4°C if required (<24 h).
12. Add propidium iodide to a final concentration of 1 mg/mL. Incubate for 5 min.
13. Analyze fluorescence associated to FITC and to propidium iodide using conventional setups. The result of a representative experiment is given in **Fig. 2**.

## 4. Notes

1. Check carefully that T-flasks are horizontal in the incubator, as uniformity is essential for recovery of pure populations of mitotic cells.

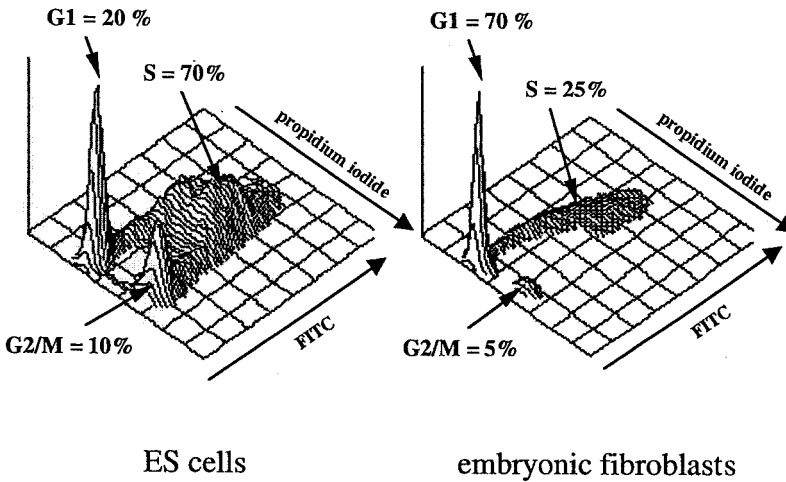


Fig. 2. Cell cycle distribution of ES cells and mouse embryonic fibroblasts, determined according to the protocol described in **Subheading 3.3**.

2. Do not leave the cells free of medium for more than 1 min, as this will lead to clumps of cells detaching during the shake-off procedure.
3. Following this protocol, one can obtain a population of ES cells containing >95% mitotic cells. Ninety percent of those cells will resume cell cycle progression within 1 h following incubation in demecolcine-free medium. ES cells will start entering the S-phase within 2 h, and the vast majority of them will be replicating their DNA at 4 h post-release from the mitotic block. Note that increasing the incubation time with demecolcine will result in a larger proportion of mitotic cells not being released from the mitotic block.
4. Collecting post-mitotic cells by pipetting up and down may lead to cell damage. Pipetting must be done very gently to avoid this, but for a long enough time to obtain a single-cell suspension suitable for cell cycle analysis. In any case, cell debris will be discarded during FACS analysis.

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### References

1. Savatier, P., Huang, S., Szekely, L., Wiman, K. G., and Samarut, J. (1994) Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. *Oncogene* **9**, 809–818.
2. Savatier, P., Lapillonne, H., van Grunsven, L. A., Rudkin, B. B., and Samarut, J. (1996) Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene* **12**, 309–322.
3. Weinberg, R. A. W. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**, 323–330.
4. Bartek, J., Bartkova, J., and Lukas, J. (1997) The retinoblastoma protein pathway in cell cycle control and cancer. *Exp. Cell Res.* **237**, 1–6.

5. Kerkhoff, E. and Rapp, U. R. (1998) Cell cycle targets of Ras/Raf signalling. *Oncogene* **17**, 1457–1462.
6. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X., et al. (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101**, 79–89.
7. Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98**, 859–869.
8. Hansen, R. and Oren, M. (1997) p53: from inductive signal to cellular effect. *Curr. Opin. Genet. Dev.* **7**, 46–51.
9. Aladjem, M. I., Spike, B. T., Rodewald, L. W., Hope, T. J., Klemm, M., Jaenisch, R., and Wahl, G. M. (1998) ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. *Curr. Biol.* **29**, 145–155.
10. Prost, S., Bellamy, C. O., Clarke, A. R., Wyllie, A. H., and Harrison, D. J. (1998). p53-independent DNA repair and cell cycle arrest in embryonic stem cells. *FEBS Lett.* **425**, 499–504.
11. Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., et al. (1998) Formation of pluripotent stem cells in the mammalian embryo depend on the POU transcription factor Oct4. *Cell* **95**, 379–391.
12. Smith, A. G. (1991) Culture and differentiation of embryonic stem cells. *J. Tiss. Cult. Methods* **13**, 89–94.