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Phosphoinositide 3-kinase inhibitor LY294002 but not serum withdrawal suppresses proliferation of murine embryonic stem cells

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Abstract

Mouse embryonic stem (mES) cells have short duration of their cell cycle and are capable of proliferating in the absence of growth factors. To find out which signaling pathways contribute to the regulation of the mES cell cycle, we used pharmacological inhibitors of MAP and PI3 kinase cascades. The MAP kinase inhibitors as well as serum withdrawal did not affect mES cell cycle distribution, whereas the inhibitor of PI3K activity, LY294002, induced accumulation of cells in G_1 phase followed by apoptotic cell death. Serum withdrawal also causes apoptosis, but it does not change the content and activity of cell cycle regulators. In contrast, in mES cells treated with LY294002, the activities of Cdk2 and E2F were significantly decreased. Interestingly, LY294002 had a much stronger effect on cell cycle distribution in low serum conditions, implying that serum can promote $G_1 \rightarrow S$ transition of mES cells by a LY294002-resistant mechanism. Thus, proliferation of mES cells is maintained by at least two separate mechanisms: a LY294002-sensitive pathway, which is active even in the absence of serum, and LY294002-resistant, but serum-dependent, pathway.

Keywords: Mouse ES cells; Cell cycle; PI3 kinase; LY294002; Serum withdrawal

1. Introduction

Proliferation of mammalian cells *in vitro* is regulated by extracellular mitogenic signals provided by growth factors. Cells are sensitive to growth factors signals in the G_1 phase of the cell cycle. Growth factors activate cytoplasmic signaling cascades which promote release of E2F transcription factors from Rb/E2F complexes. The released E2F factors stimulate transcription of S phase-specific genes and genes involved in regulation of $G_1 \rightarrow S$ transition, including cyclins D, E and A and cyclin-dependent kinases 2 and 4 (Cdk2, 4) (Pardee et al., 2004).

It is known that two waves of growth factors-triggered signaling are needed for cells to overcome G_1. The first is mediated mainly by the ERK pathway, and the second is mediated by PI3K. On this basis, G_1 is subdivided into two periods: G_1 early (G_{1E}) and G_1 late (G_{1L}). ERK pathway stimulates cyclin D mRNA expression, whereas the PI3K pathway stabilizes the cyclin D protein and eliminates $p27^{kip}$ inhibitor of cyclin/cdk complexes. Accumulated cyclin D forms complexes with either Cdk4 or Cdk6. The cyclin D/Cdk4,6 complexes phosphorylate Rb, releasing a small amount of E2F, which in turn drives the formation of cyclin E/Cdk2 complexes. The cyclin E/Cdk2 complexes further phosphorylate Rb releasing additional E2F transcription factors, initiating S phase entry (Jones and Kazlauskas, 2001). In the absence of growth factors cells exit cell cycle from the G_1 phase into the quiescent G_0 state (Pardee, 1989).

Murine embryonic stem (mES) cells are derived from the inner cell mass (ICM) of the blastocyst. ICM gives rise to the whole embryo body, implying that these cells can differentiate into any cell type. ICM cells demonstrate very rapid proliferation—between day 4.5 and day 7.0 they expand from 20–25 to over than 4000 cells, indicating a generation period of <10 h. This is a much shorter than time than for many other cell types. Being ICM derivates, mES cell lines retain their

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Nomenclature

Cdk	cyclin-dependent kinase
CK2	casein kinase 2
ERK	extracellular-regulated kinase
FCS	fetal calf serum
GSK3β	glycogen synthase kinase-3β
ICM	inner cell mass
IGF	insulin-like growth factor
MEK	mitogen activated protein kinase kinase 1
mES cells	mouse embryonic stem cells
mEC cells	mouse embryonic carcinoma cells
PI3K	phosphoinositide 3-kinase
PKB/Akt	protein kinase B/Akt
TBB	4,5,6,7-tetrabromobenzotriazole

main characteristics of ICM: when maintained *in vitro* in undifferentiated state they are pluripotent and proliferate rapidly.

Proliferation of mES and mEC cells reveals rather unusual features as compared with other cell types; they are incapable of undergoing cell cycle arrest, they have very short G_1 phase of the cell cycle, and up to 70% of cells are concurrently in S phase (Savatier et al., 1994; Malashicheva et al., 2000, 2002). The ability of ES cells to proliferate is driven by permanently hyperphosphorylated Rb and active E2F, which are accompanied by high levels of Cdk2 activity (Savatier et al., 1996; Stead et al., 2002). Both mouse and primate ES cells continue to proliferate in the absence of growth factors in culture medium (Schratt et al., 2001; Fluckiger et al., 2006); they seem to lack for growth factor-dependent periods of the cell cycle because of the shortened G_1 phase.

Probably mitogenic signals from growth factors in ES cells are substituted by constantly hyperphosphorylated Rb and free E2F. Which cytoplasmic cascades contributes to activation of these cell cycle regulators is not yet clear. The top priority candidates for such intrinsic cell cycle triggers are the ERK and PI3K pathways, since they conduct signals from growth factors to the cell cycle machinery in G_1 phase of somatic cells. The PI3K-dependent pathway is the more probable candidate, because it is known that undifferentiated ES cells express an unique isoform of Ras protein—Eras—which is constantly active and stimulates preferably PI3 Kinase. Cells lacking Eras demonstrate reduced growth rate (Takahashi et al., 2003). We have suggested that PI3K-dependent signaling is an intrinsic stimulator of $G_1 \rightarrow S$ transition in mES cells. In this work, we have applied the pharmacological PI3 kinase inhibitor, LY294002, to study the effects of PI3K-dependent pathways on the $G_1 \rightarrow S$ transition of mES cells.

2. Materials and methods

2.1. Cell culture and differentiation

ES cells were cultured on tissue culture dishes (Corning) coated with 0.2% porcine gelatin (Sigma) in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum (PAA), 0.1 mM 2-mercaptoethanol, and 50 units/ml of murine LIF (Sigma). Cells were trypsinized and split or re-fed every second day. IOUD2 and E14.1, the feeder-independent mES cell lines, were used. NIH 3T3 fibroblasts were cultivated similarly but without LIF and gelatin coating. Differentiation of mES cells in monolayer was induced by 24 h of LIF withdrawal followed by retinoic acid (1 μM) treatment. After 2 days of RA treatment medium was replaced with both RA and LIF-free medium and cells were cultivated 1 further day. LY294002, PD98059, SB203580, SP600125, BIO ((2′Z,3′E)-6-Bromoindirubin-3′-oxime) and roscovitine were purchased from Calbiochem.

2.2. Transfection

To establish a cell line expressing the luciferase reporter gene under the E2F promoter, IOUD2 cells were cotransfected with 3 μg of the reporter plasmid and 0.3 μg of pBabe-puro plasmid using 9 μl Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). Resistant clones were selected with 1 μg/ml of puromycin for 1 week. In obtained clones luciferase activity was measured with Luciferase Assay System (Promega).

2.3. FACS analysis of cell cycle distribution

For analysis of DNA content, cells were harvested, washed with PBS and incubated at room temperature during 30 min in buffer containing 0.1% BSA, 0.01% of saponin in PBS. The cells were washed twice with PBS and incubated with RNase A (100 μg/ml) and propidium iodide (40 μg/ml) for 15 min at 37 °C. Probes were analyzed by flow cytometry on a Becton Dickinson FACscan. Data acquisition was performed with the CellQuest software, and cell cycle phase distribution analysis was performed with MODFIT software.

2.4. Immunoblotting

For immunoblotting, cell lysates were obtained in RIPA buffer containing PBS solution, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors and phosphatase inhibitors. Proteins were separated in 10% or 12.5% polyacrylamide gels and blotted on Immobilon-P membranes (Millipore). The following antibodies were used: non-phosphorylated ERK (sc-94), phosphorylated ERK (sc-7383), cyclin D1 (sc-717), cyclin A (sc-751), cyclin E (sc-481), Cdk4 (sc-260), p27 (sc-528) from SantaCruz Biotechnology Inc., non-phosphorylated PKB (#9272), phosphorylated (Ser473) PKB Antibody (#9271), cleaved caspase 3 (#9661), Cdk2 (#2546) from Cell Signaling Technology. Antibody to GAPDH was from HyTest Ltd. Goat anti-rabbit and rabbit anti-mouse were used as second antibodies. Proteins on membranes were detected by means of ECL.

2.5. In vitro kinase assay

For immunoprecipitation, cells were lysed in buffer, containing 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0,5% NP-40, 1% Triton X-100, protease and phosphatase inhibitors. Immunoprecipitation was conducted during 3 h or overnight incubation at 4 °C (500 μg of cell extract/μg of antibody), the complexes were collected on Protein A-Sepharose beads (Sigma). After washing the bound kinases, the activity was determined in a kinase buffer in the presence of 20 mM $MgCl_2$, 25 μM ATP, 1,5 μCi ^{32}P-α- ATP (Amersham) and 1.5 μg histone H1 as a substrate during 15–20 min at 30 °C. After incubation, Laemmli sample buffer was added and samples were boiled for 2 min. Probes were resolved in 10% SDS–PAAG with following drying. Dry gels were exposed to X-ray sensitive film.

2.6. RT–PCR

Total cellular RNA was isolated with TRIzol®. RT was conducted with 2 μg RNA and 1 μg random hexaprimers. PCR was performed in the presence of 50 ng primers to different mouse cDNAs: *p27*—5′-acttggagaa gcactgcc-3′/5′-ttcctcatccctggacac-3′; *cyE*—5′-accctccaaagttgcaccag-3′/5′-ccatctcccggataaccatg-3′; *cyA2*—5′-gcatgagggcgatccttgtg-3′/5′-acagtttgcaggctgcaggtg-3′;