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Regulation of Embryonic Stem Cell Self-renewal by Phosphoinositide 3-Kinase-dependent Signaling*

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The maintenance of murine embryonic stem (ES) cell self-renewal is regulated by leukemia inhibitory factor (LIF)-dependent activation of signal transducer and activator of transcription 3 (STAT3) and LIF-independent mechanisms including Nanog, BMP2/4, and Wnt signaling. Here we demonstrate a previously undescribed role for phosphoinositide 3-kinases (PI3Ks) in regulation of murine ES cell self-renewal. Treatment with the reversible PI3K inhibitor, LY294002, or more specific inhibition of class I_A PI3K via regulated expression of dominant negative Δp85, led to a reduction in the ability of LIF to maintain self-renewal, with cells concomitantly adopting a differentiated morphology. Inhibition of PI3Ks reduced basal and LIF-stimulated phosphorylation of PKB/Akt, GSK3α/β, and S6 proteins. Importantly, LY294002 and Δp85 expression had no effect on LIF-induced phosphorylation of STAT3 at Tyr^{705}, but did augment LIF-induced phosphorylation of ERKs in both short and long term incubations. Subsequently, we demonstrate that inhibition of MAP-Erk kinases (MEKs) reverses the effects of PI3K inhibition on self-renewal in a time- and dose-dependent manner, suggesting that the elevated ERK activity observed upon PI3K inhibition contributes to the functional response we observe. Surprisingly, upon long term inhibition of PI3Ks we observed a reduction in phosphorylation of β-catenin, the target of GSK-3 action in the canonical Wnt pathway, although no consistent alterations in cytosolic levels of β-catenin were observed, indicating this pathway is not playing a major role downstream of PI3Ks. Our studies support a role for PI3Ks in regulation of self-renewal and increase our understanding of the molecular signaling components involved in regulation of stem cell fate.

Murine embryonic stem cells, derived from the inner cell mass of preimplantation embryos (1, 2), are pluripotent, retaining the ability to differentiate into cells of all three germ layers of the developing mouse embryo. The pluripotency of stem cells relies on their ability to self-renew, and the cytokine leukemia inhibitory factor (LIF)[1] has been shown to be important for maintaining the pluripotency of murine ES cells (3, 4). LIF signals through a heteromeric receptor, comprised of gp130 and the low affinity LIF receptor, to induce activation of STAT3, which has been shown by several groups to play an essential role in maintaining self-renewal (5–8). LIF also induces activation of a number of additional signaling proteins, including ERKs (9, 10), ribosomal S6 kinases (11), cyclic AMP response element-binding proteins (11), and Src family tyrosine kinases (12). The activation of ERKs appears to promote differentiation (9) and has led to the suggestion that the balance between LIF-induced STAT3 and ERK signals is important in determining the fate of a dividing undifferentiated ES cell (13). The Src family of tyrosine kinases appears to play a role in maintaining self-renewal (12).

Recently, a number of publications have identified additional pathways that also appear to contribute to the regulation of self-renewal of murine ES cells. Forced expression of homeodomain protein Nanog can maintain ES cells in an undifferentiated state in the absence of LIF (14, 15). The bone morphogenic proteins BMP2 and BMP4 also participate in maintenance of self-renewal by cooperating with LIF (16, 17). It appears that BMP-induced expression of Id genes, via Smad signaling, suppresses expression of genes, which normally induce neuronal differentiation (16). Alternatively, it has also been reported that BMP4-mediated inhibition of p38 MAPK activity may play a role in regulation of self-renewal (17).

Other recent reports have implicated glycogen synthase 3 (GSK-3) α/β and the Wnt signaling pathway in regulation of self-renewal of both murine and human ES cells (18, 19). This work stemmed from the development of novel GSK-3 inhibitors (18) although the selectivity of these novel inhibitors has not been comprehensively evaluated (18). Additionally, Wnt3a has been shown to play a role in maintaining self-renewal of hemopoietic stem cells (20).

PI3Ks are a family of lipid kinases, whose products, phosphoinositide 3,4-bisphosphate ($PI(3,4)P_2$) and phosphoinositide 3,4,5-trisphosphate ($PI(3,4,5)P_3$) act as intracellular second messengers (21, 22). Members of the three distinct classes of PI3Ks have been implicated in the regulation of an array of physiological processes, notably the control of proliferation, cell survival, cell migration, and trafficking (21, 22). Members of the class I_A family of PI3Ks, comprising a regulatory subunit (typically 85 or 55 kDa) and a 110 kDa catalytic subunit (21, 22) are known to be activated via gp130, the signaling component of the LIF receptor (23, 24). The role of phosphoinositide signaling in ES cells is currently limited to reports implicating PI3Ks in the control of ES cell proliferation (25–28). Here we

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[1] The abbreviations used are: LIF, leukemia inhibitory factor; BMP, bone morphogenic protein; ERK, extracellular signal-regulated kinase; ES, embryonic stem cell; GSK, glycogen synthase kinase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; STAT, signal transducer and activator of transcription; Tet, tetracycline; MAPK, mitogen-activated protein kinase.

report that PI3Ks are also involved in regulation of self-renewal of murine ES cells. Using both pharmacological and molecular tools we demonstrate that PI3K signaling is required for efficient self-renewal in the presence of LIF. Loss of self-renewal upon inhibition of PI3K signaling is associated with an increase in ERK phosphorylation, which appears to play a functional role in this response.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of Transfectants—E14tg2a (4), CCE (29, 30), and IOUD2 (31) murine ES cell lines were routinely cultured on tissue culture plates (Nunc) coated with 0.1% (v/v) porcine gelatin (Sigma) in knock-out Dulbecco's modified Eagle's medium (Invitrogen, Scotland) in the presence of 15% (v/v) knock-out serum replacement (Invitrogen), 0.1 mM mercaptoethanol, 1 mM sodium pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids, and 500–1000 units/ml murine LIF (Chemicon). Cells were trypsinized and replated or re-fed every second day. The Tet-off-regulated expression system was used to express a dominant negative p85 regulatory subunit of class I_A PI3Ks. E14tg2a expressing the Tet-sensitive transactivator, tTA (E14 tTA (32)), was a kind gift from Dr. O. Witte (UCLA). 1×10^7 E14 tTA cells were electroporated at 800 mV/3.0 microarad with 10 mg of linearized response plasmid encoding the dominant negative form of class I_A p85a regulatory subunit, termed Δp85. Δp85 lacks the p110 interaction site, so acting as a competitive inhibitor, and we have described its use previously (33). Selection in the presence of 1000 units/ml LIF, 500 ng/ml Tet, and 200 mg/ml G418 was initiated 48 h following transfection, and individual clones were picked following 6 days in selection media. For screening, 1×10^5 cells were plated into 60-mm plates in the presence or absence of 500 ng/ml Tet. Following 24 h, lysates were prepared and immunoblotted for expression of Δp85. Independent clones (termed E14Δp85) exhibiting very low to undetectable basal expression and good inducible expression of Δp85 upon Tet removal were selected for further analyses. For induction of expression of Δp85, E14Δp85 clones were washed three times with Hank's-buffered saline solution and then incubated in LIF-containing media in the absence of Tet, to induce expression of Δp85, or were maintained in the presence of 500 ng/ml Tet for differing lengths of time, dependent on the experiment.

Self-renewal Assays—ES cells were plated at 3×10^3 to 1×10^4 cells per gelatin-coated 60-mm tissue culture dish in Glasgow-modified Eagle's medium (Invitrogen) containing 10% (v/v) fetal bovine serum (ES-tested Hyclone, Perbio, Cheshire, UK), 0.1 mM mercaptoethanol, 1 mM sodium pyruvate, 2 mM glutamine, and 0.1 mM nonessential amino acids. Plates were cultured without LIF or with the addition of LIF (500–1000 units/ml depending on the experiment). E14tg2a, IOUD2, or CCE cells were additionally supplemented with 5 μM LY294002, 10 μM U0126, 50 μM PD98059, or Me_2SO alone. Clones expressing Δp85 were additionally plated in the presence or absence of 500 ng/ml Tet. To detect cells expressing alkaline phosphatase, a marker of pluripotency, after 3–6 days of culture, dishes for each treatment were washed, fixed, and then stained for 15 min with a solution containing 1 mg/ml Fast Red TR salt™ (Sigma) dissolved in 0.1 M Tris, pH 9.2, containing 200 mg/ml naphthol AS-MX phosphate (34). Alkaline phosphatase-positive colonies were counted in triplicate for each treatment.

Proliferation and Cell Viability Assays—XTT (sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-*bis*(4-methoxy-6-nitro) benzenesulfonic acid hydrate) bioreduction assays were used to assess growth of ES cells. 1×10^3 ES cells were plated in triplicate in 96-well plates, previously coated with 0.1% (w/v) gelatin in the presence or absence of different doses of LY294002 (Calbiochem) or Me_2SO alone in media containing 1000 units/ml murine LIF. 25 μl of a solution containing 1 mg/ml XTT and 25 μM phenazine methosulfate was added per well for the final 4 h of the 48-h incubation. The soluble formazan product was measured at 450 nm. For cell growth curve analyses, cells were plated at 1×10^4 cells per 60-mm dish and counted in duplicate at defined times. Viability was determined using trypan blue exclusion assays. Duplicate plates were set up per treatment, and duplicate counts from each plate performed.

Short Term Stimulations with LIF and Cell Lysates—ES cells and transfectants were washed three times in Hank's-buffered saline solution and incubated for 4 h in media lacking serum replacement and LIF. When required, 10 μM LY294002 or Me_2SO alone were added for the last 30 min of this preincubation period. Cells were stimulated for the times indicated in minutes with 1×10^4 units/ml LIF prior to preparation of cell lysates. Dishes were placed on ice and washed three times with ice-cold phosphate-buffered saline prior to lysis directly in the dish with solubilization buffer, as described previously (35). Insoluble material was removed by centrifugation for 3 min at full speed in a microcentrifuge at 4 °C. Protein concentrations of clarified supernatants were determined using the Bio-Rad protein assay kit according to the manufacturers' instructions.

Immunoblotting, Immunoprecipitation, and Antibodies—20 μg of each cell lysate were fractionated by SDS-PAGE and immunoblotted onto nitrocellulose (35). For immunoprecipitation, 500 μg of protein were used per cell extract sample and anti-p85 precipitates prepared as described previously (36). Primary antibodies were used at the following dilutions for immunoblotting: 0.1 μg/ml mouse monoclonal antibody recognizing phosphotyrosine 4G10 (Upstate Biotechnology, 05–321); 1:1000 for rabbit polyclonal antibodies recognizing dual phosphorylation of ERK1 and 2 at Thr^{202}/Tyr^{204} (anti-pERK, Cell Signaling Technology 9101), phosphotyrosine 705 of STAT3 (anti-pSTAT3, CST 9131), phosphoserine 473 of PKB (anti-pPKB, CST 9271), phosphoserines 21 or 9 of GSK-3α/β (anti-pGSK3α/β, CST 9331), anti-phospho(Ser/Thr)PKB substrate (anti-pPKBSub, CST 9611, which was used for detection of phosphorylated S6 protein), phosphoserine 33/37 phosphothreonine 41 β-catenin (anti-p β-catenin, CST 9561), anti-pan β-catenin (CST 9562), anti-pan PKB (CST 9272); 1:2000 anti-total ERK (panERK, Santa Cruz Biotechnology, sc-93), anti-STAT3 (panSTAT3, sc-482), anti-STAT5 (sc-835), anti-SHP-1 (sc-287), anti-SHP-2 (sc 293), anti-Oct4 (sc-9081), and anti-p85 (Upstate Biotechnology, 06–195). Goat anti-rabbit or goat anti-mouse secondary antibodies, conjugated to horseradish peroxidase (Dako), were used at 1:10,000 dilution, and blots were developed using ECL (Amersham Biosciences). Blots were stripped and reprobed as described previously (37).

RESULTS

LIF Induces Activation of the PI3K Signaling Pathway—A fundamental property of all stem cells is their ability to undergo self-renewal. This process, defined as proliferation accompanied by the suppression of differentiation, is essential for maintenance of pluripotency (38). A thorough understanding of the signals regulating stem cell fate is critical if the therapeutic potential of embryonic and adult stem cells is to be fulfilled in the future, and progress is being made toward this goal (5–9,12,14–17,19). To expand our knowledge and understanding of the signaling pathways regulating self-renewal of stem cells we have investigated whether PI3K-dependent signaling, recently implicated in the control of ES cell proliferation (25–28), is also involved in control of murine ES cell self-renewal. Although previous reports have demonstrated that gp130 receptors couple to the PI3K pathway (10, 23), and that PI3K pathways are active in ES cells (25, 26), the ability of LIF to directly regulate this pathway in ES cells has not been reported.

We investigated LIF-induced activation of PI3K-dependent signals by assessing phosphorylation of serine 473 on PKB (also known as Akt), serine 21, or serine 9 of GSK3 α and β, and phosphorylation of the S6 protein. As shown in Fig. 1, *A* and *B*, LIF treatment transiently increased phosphorylation of PKB/Akt, GSK-3, and S6 protein. Interestingly, we did not detect changes in phosphorylation of threonine 308 of PKB/Akt upon LIF stimulation, the reasons for which are not clear (data not shown). The dependence of these effects on PI3Ks were examined by pretreating ES cells with the broad specificity PI3K inhibitor, LY294002, an extremely useful and widely used tool to investigate the involvement of PI3Ks in functional responses, because it reversibly inhibits all three classes of PI3Ks (39). As shown in Fig. 1*A*, LY294002 significantly reduces LIF-induced phosphorylation of PKB, GSK-3, and S6 protein.

The class I_A PI3K subfamily are most commonly activated by cytokines signaling via gp130 (23, 24), and to examine their role we expressed a Myc-tagged dominant negative version of the p85α regulatory subunit of class I_A PI3Ks in ES cells using Tet-off-regulated expression. This mutant, termed Δp85, which we have used successfully in the past (33, 40), lacks the p110 binding site (41) and acts as a competitive inhibitor for all class I_A catalytic subunits (p110 α, β, and δ). A number of independent E14tTA ES cell transfectants that expressed Δp85 in the absence of Tet were isolated, characterized, and referred to as E14Δp85. Expression of Δp85 reduced phosphorylation of

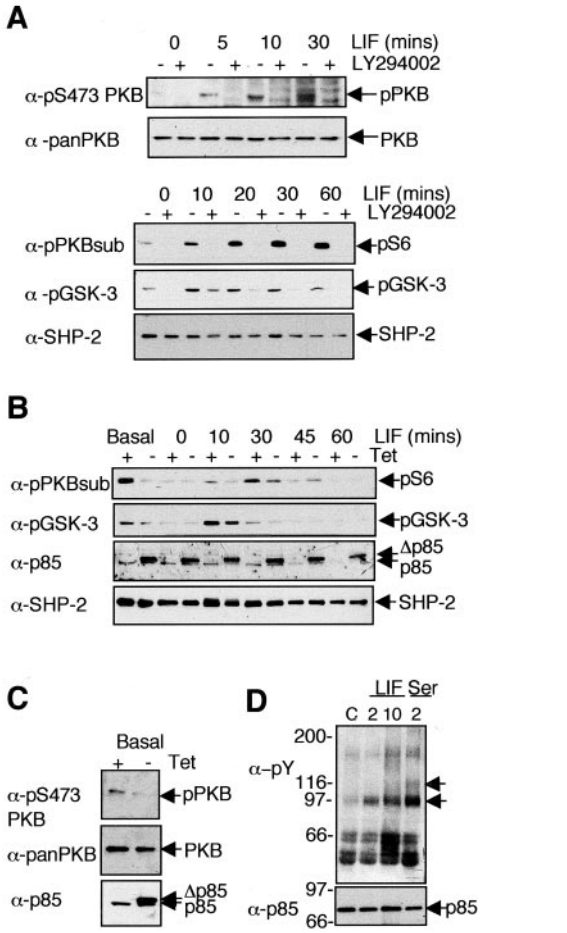

FIG. 1. **LIF-induced activation of PI3K-dependent signaling in undifferentiated ES cells.** *A*, E14tg2a ES cells were starved of LIF and serum for 3.5 h and preincubated for 30 min with 10 μM LY294002 or Me_2SO alone prior to stimulation with 1×10^4 units/ml LIF for the times indicated. *B*, E14Δp85 ES cells were induced to express Δp85 by the removal of tetracycline (*−Tet*) for 24 h. As a control, cells were maintained in the presence of Tet (*+ Tet*). After 24 h, cells were starved of LIF and serum for 4 h prior to stimulation with 1×10^4 units/ml LIF for the times indicated. *Basal* refers to cells induced to express Δp85 for 24 h and used directly to prepare cell lysates, *i.e.* with no further stimulation. *C*, E14Δp85 ES cells were induced to express Δp85 by the removal of tetracycline (*−Tet*) for 24 h prior to preparation of cell lysates. *A–C*, 20 μg of each protein sample were separated through 7.5% or 10% acrylamide gels by SDS-PAGE, and immunoblotting was carried out with the antibodies indicated (see "Experimental Procedures"). In *A* and *B* duplicate blots were prepared and probed separately with either anti-phospho-PKB substrate antibody or anti-phospho-GSK-3 antibody. The same blots were stripped and reprobed with SHP-2 or p85 antibodies to assess loading. A loading control for one set of samples is shown in each case. *D*, E14tg2a ES cells were starved of LIF and serum for 4 h prior to stimulation with 1×10^4 units/ml LIF for 2 or 10 min or with 20%(v/v) serum (*Ser*) for 2 min. Precipitates were prepared with an anti-p85 antibody, and after washing separated on a 7.5% acrylamide gel. Immunoblotting was carried out first with the anti-phosphotyrosine monoclonal antibody, 4G10 (*α-pY*). The same immunoblot was stripped and reprobed with the anti-p85 antibody. The two *arrows* on the *right hand side* of the panel indicate the position of the phosphotyrosine-containing proteins co-immunoprecipitating with p85 following treatment with LIF or serum. Molecular mass standards are indicated (in kDa) on the *left side* of the panel.

Ser^{473} of PKB/Akt, $Ser^{21/9}$ of GSK-3α/β, and S6 proteins upon LIF stimulation and in cells maintained in normal culture conditions prior to lysis here termed *Basal* levels (*−Tet samples*, Fig. 1, *B* and *C*), although not to such an extent at LY294002. As an additional measure of the coupling of LIF signaling to the PI3K pathway, we performed immunoprecipitations with antibodies recognizing the p85 adaptor subunit of class I_A family members. As shown in Fig. 1*D*, treatment of E14tg2a ES cells with LIF led to increased co-precipitation of endogenous p85 with a phosphotyrosyl-containing protein of ~90–95 kDa, indicating engagement of the class I_A PI3K pathway upon LIF stimulation. These results, combined with the ability of LIF to induce PI3K-dependent phosphorylation of known downstream effectors, are consistent with LIF activating the PI3K signal transduction cascade in undifferentiated ES cells.

Inhibition of PI3K-dependent Signaling Reduces Murine ES Cell Self-renewal—Given that LIF activates PI3K-dependent signals, we investigated whether the PI3K pathway plays a role in regulating self-renewal. Self-renewal was quantified using clonal assays and staining for the presence of alkaline phosphatase using Fast Red™, which stains only undifferentiated cells. Culturing E14tg2a ES cells in the presence of LIF maintained undifferentiated colonies at between 80 and 90% (Fig. 2*A*, *panel i*). Addition of 5 μM LY294002, a dose close to the reported IC_{50} value for inhibition of class I_A PI3Ks (39) but five times lower than the dose used in previous studies on PI3K action in ES cells (25), significantly reduced the proportion of undifferentiated colonies observed in the presence of LIF (Fig. 2*A*, *panel i*). This effect was partially reversible because washout of LY294002 after 24 or 48 h led to a partial recovery in self-renewal (see Fig. 2*A*, *panel ii*). However, 24–48 h of incubation with LY294002 was sufficient to result in commitment of a proportion of the cells to differentiation. Similar results were observed using IOUD2 ES cells, which are derived from E14tg2a ES cells (31) and with a second independent ES cell line, CCE (data not shown). In the absence of LIF, ES cells spontaneously differentiated, and little effect of LY294002 treatment was noted. In LIF alone, largely spherical, alkaline phosphatase-positive (red) colonies predominated. However, with the addition of LY294002 the colonies formed were more irregular and flattened appearance and with many fewer red-stained cells, highly suggestive of a more differentiated phenotype (Fig. 2*A*, *panel iii*). With increased culture times, the morphology of cells in the presence of LIF plus LY294002 began to resemble cells from which LIF had been withdrawn (data not shown).

Using the E14Δp85 cell clones, we examined the effect expressing Δp85 on self-renewal. Induction of Δp85 expression by removal of Tet, shown in Fig. 2*B*, *panel ii*, also significantly reduced the ability of LIF to maintain self-renewal in three independent clones tested, see Fig. 2*B*, *panel i* and induced a similar change in morphology as LY294002 treatment, as shown in Fig 2*B*, *panel iii*. The presence of Tet had no significant effect on self-renewal of parental E14tTA cells (data not shown). These results are consistent with PI3Ks, including the class I_A subfamily, playing a role in regulation of self-renewal of murine ES cells.

Inhibition of PI3K Signaling Leads to Only Modest Effects on ES Cell Survival and Proliferation—The change in cell morphology that occurs following inhibition of PI3Ks is highly suggestive of cells undergoing differentiation. However, while undertaking this study, it was reported that PI3Ks play a role in controlling proliferation of ES cells (25–27), with small alterations in apoptosis also noted (25, 26). In our system, viability in the presence of LIF after 4 days of incubation was, on average 90.5% (± 1.5%) compared with 84.5% (± 0.5%) in the presence of LIF plus 5 μM LY294002. In 2-day XTT-based proliferation assays (Fig. 3*A*) it was only at doses of 10 μM and above that LY294002 partially inhibited proliferation of E14tg2a ES cells, consistent with the report where 25 μM LY294002 was used (25). When we examined growth rates of adherent cells in the presence and absence of LY294002 over longer periods of time, LY294002 at 5 μM (the same dose we

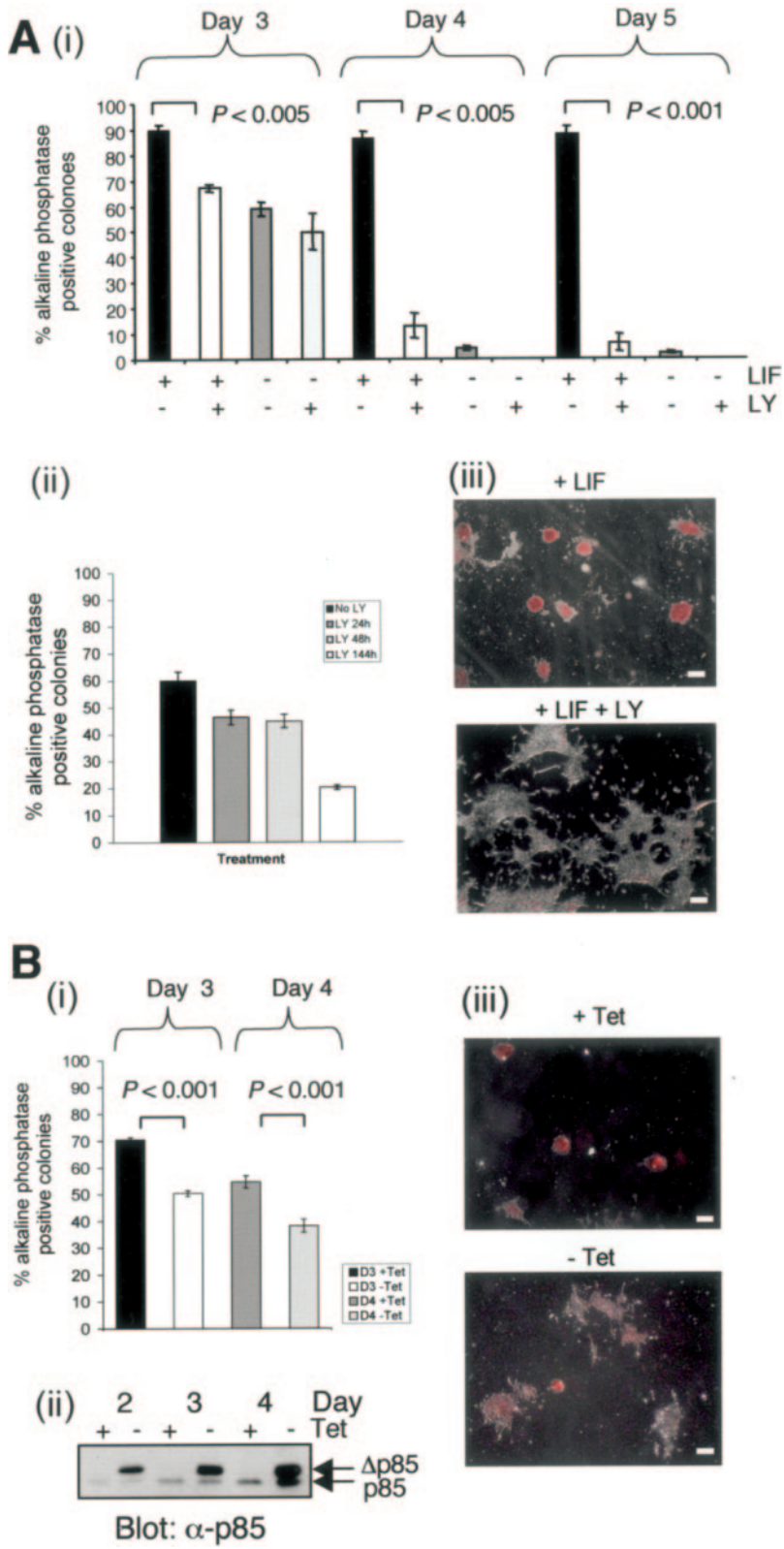

FIG. 2. **Inhibition of PI3Ks reduces the ability of LIF to promote self-renewal.** *A*, *panels i* and *ii*, E14tg2a ES cells were cultured in the presence or absence of 1000 units/ml LIF in the presence of 5 μM LY294002 or vehicle alone (Me_2SO, −) for the times indicated. In *panel ii*, LY294002 was washed out after 24 or 48 h or maintained for the entire culture period (144 h). Samples were fixed and stained after 6 days in this analysis. Triplicate counts were performed, and the percentage of alkaline phosphatase (Fast Red™)-positive colonies were determined for each treatment. In *panel i*, a representative experiment is shown, and S.D. are included. Paired Student's *t* tests were performed, and the significance values indicated by *P*. In *panel ii*, the combined data of three independent experiments, with S.E., are shown. *Panel iii*, photographs of representative colonies were taken on day 4 of the treatments shown. The *white bar* represents 250 μm. *B*, E14Δp85 transfectants were incubated in the presence of 500 ng/ml Tet (+), or without Tet (−), to induce expression of Δp85 in the presence of 500 units/ml LIF. *Panel i*, 3 and 4 days after plating, colonies were stained with alkaline phosphatase, and triplicate counts performed. The data represent the combined results, with S.E. from at least four independent experiments using three independent Δp85-expressing clones. *Panel ii*, immunoblot shows the levels of p85 and Δp85 expressed by E14Δp85 clone 4. Δp85 incorporates a double Myc epitope tag, hence its reduced migration through the gel. *Panel iii*, photographs of representative colonies were taken on day 4 of the treatments shown. The *white bar* represents 250 μm.

used for self-renewal assays) led to a small reduction in total cell numbers (Fig. 3*B*) although rates of proliferation appeared similar. Thus, our results are consistent with PI3K-dependent signaling being required for efficient self-renewal of murine ES cells, with only small effects on proliferation and apoptosis.

STAT3 Is Not a Target of PI3K Action—Our next aim was to define the mechanisms involved in regulation of ES cell self-renewal by PI3Ks. The Oct-4 and STAT3 transcription factors are known to be involved in maintenance of pluripotency (5–8,42). Therefore, we first investigated whether the protein levels of these factors were altered significantly in E14tg2a cells treated with LY294002, compared with cells cultured in LIF alone (Fig. 4*A*). The levels of Oct-4 and STAT3 were not altered upon inclusion of LY294002. However, levels of STAT5 were elevated in LY294002-treated cells. STAT5 has previously been reported to be an early marker of ES cell differentiation (43)

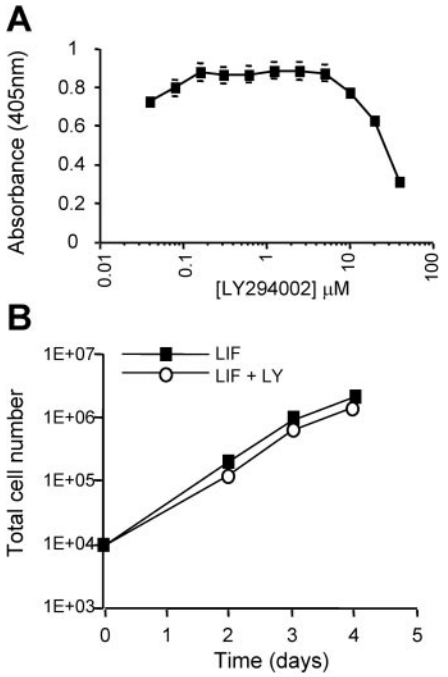

FIG. 3. **Inhibition of PI3Ks by LY294002 has only limited effects on proliferation.** *A*, E14tg2a parental ES cells were plated in triplicate in gelatin-coated wells of a 96-well plate in media containing 1000 units/ml LIF. Different doses of LY294002 were added, or cells were incubated with Me_2SO alone. Plates were incubated at 37 °C for 48 h before being developed using XTT. Mean and S.D. are shown. *B*, E14tg2a cells were plated at 1×10^4 cells per 60-mm dish and incubated with 1000 units/ml LIF, with or without addition of 5 μM LY294002. At the times indicated, adherent cells were harvested and duplicate cell counts performed, the average of which is shown.

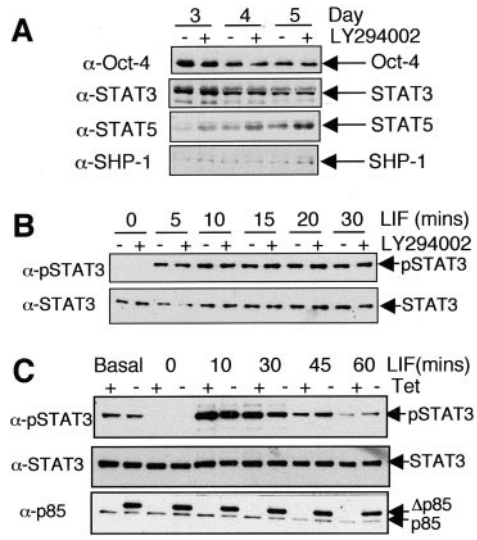

FIG. 4. **LY294002 pretreatment does not affect LIF-induced activation of STAT3.** *A*, E14tg2a ES cells were cultured in the presence of 1000 units/ml LIF in the presence of 5 μM LY294002 or vehicle alone (Me_2SO, −) for 3–5 days. *B*, E14tg2a ES cells were starved of LIF and serum for 3.5 h and preincubated for 30 min with 10 μM LY294002 or Me_2SO alone prior to stimulation with 1×10^4 units/ml LIF for the times indicated. *C*, E14Δp85 ES cells were induced to express Δp85 by the removal of tetracycline (*−Tet*) for 24 h. As a control, cells were maintained in the presence of Tet (*+Tet*). After 24 h, one sample for each condition was lysed directly (*Basal*), and the remaining samples were starved of LIF and serum for 4 h prior to stimulation with LIF as described in *B*. 20 μg of each protein sample were separated through 7.5 or 10% acrylamide gels by SDS-PAGE. *A*, immunoblotting was carried out with the antibodies indicated. The anti-SHP-1 immunoblot shows equivalence of loading. *B* and *C*, phosphorylation of STAT3 on tyrosine 705 was detected in the *upper panel* (α-*pSTAT3*). The blot was stripped and reprobed with an antibody to detect total levels of STAT3 (α-*STAT3*). In *C* a duplicate immunoblot was probed with anti-p85 antibodies to illustrate levels of Δp85 expression achieved.

and this, coupled with the alteration in cell morphology is consistent with the ES cells differentiating upon inhibition of PI3Ks.

Previously, it has been proposed that the balance between LIF-induced activation of STAT3 and ERKs may determine the efficiency of self-renewal and hence stem cell fate (9, 13). Neither pretreatment with LY294002 (Fig. 4*B*) nor expression of Δp85 (Fig. 4*C*) affected LIF-induced phosphorylation of STAT3 at tyrosine 705, the site important for STAT3 activation.

Inhibition of PI3K-dependent Signaling Increases Activation of Erk MAP Kinases—We also examined the effect of PI3K inhibition on phosphorylation of the MAP kinases ERK 1 and ERK2 in response to LIF stimulation. Pretreatment with LY294002 enhanced the ability of LIF to induce phosphorylation of ERK1 and 2 in short term stimulation assays (Fig. 5*A*). Expression of Δp85 also led to an increase in the level of ERK phosphorylation observed in cells under basal conditions, *i.e.* in the absence of acute treatment with LIF, as shown in Fig. 5*B*.

The increase in LIF-induced activation of ERK1 and 2 in the presence of LY294002 prompted us to investigate whether this alteration contributes functionally to the effects on self-renewal we observe. Self-renewal assays were performed in the presence of LIF with addition of either LY294002 alone (5 μM), the MEK inhibitor U0126 alone (10 μM), or both inhibitors together. As shown in Fig. 6*A* incubation with U0126 together with LY294002 led to an increase in the percentage of alkaline phosphatase-positive colonies compared with the effects of LY294002 alone. The effect was dependent on the dose of U0126, at 1 μM little effect was observed whereas 4 μM resulted in a partial recovery at day 4 (data not shown). Similar results were observed in the CCE ES cell line (data not shown). In such longer term cultures of both E14tg2a and CCE, the presence of U0126 reduced the increase in ERK phosphorylation caused by

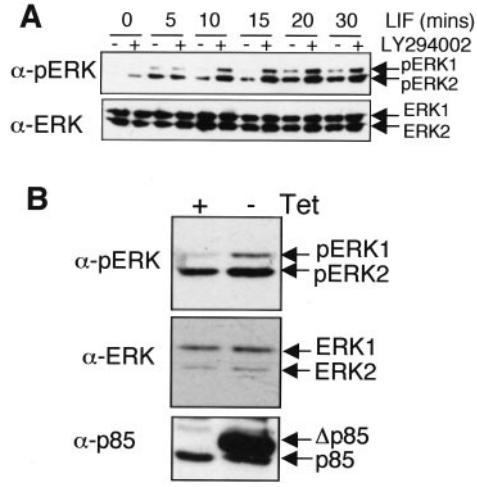

FIG. 5. **Inhibition of PI3Ks enhances LIF-induced activation of Erk MAPKs.** *A*, E14tg2a were treated as described in the legend to Fig. 4*B*. *B*, E14Δp85 ES cells were cultured in the presence (+) or absence (−) of Tet for 24 h prior to preparation of cell extracts. 20 μg of each protein sample were separated through 10% acrylamide gels by SDS-PAGE. *A* and *B*, dual phosphorylation of the ERK activation motif was detected (α-*pERK*). The same immunoblot was stripped and reprobed to detect total ERK protein (α-*ERK*). In the case of *B*, this blot was stripped again and reprobed with anti-p85 antibodies to illustrate the levels of Δp85 expression achieved.

incubation with LY294002, whereas the levels of STAT3 phosphorylation were unaffected (see Fig. 6*B*). We also examined the effects of MEK inhibitors, following expression of Δp85. The two unrelated MEK inhibitors, PD98059 and U0126 both led to

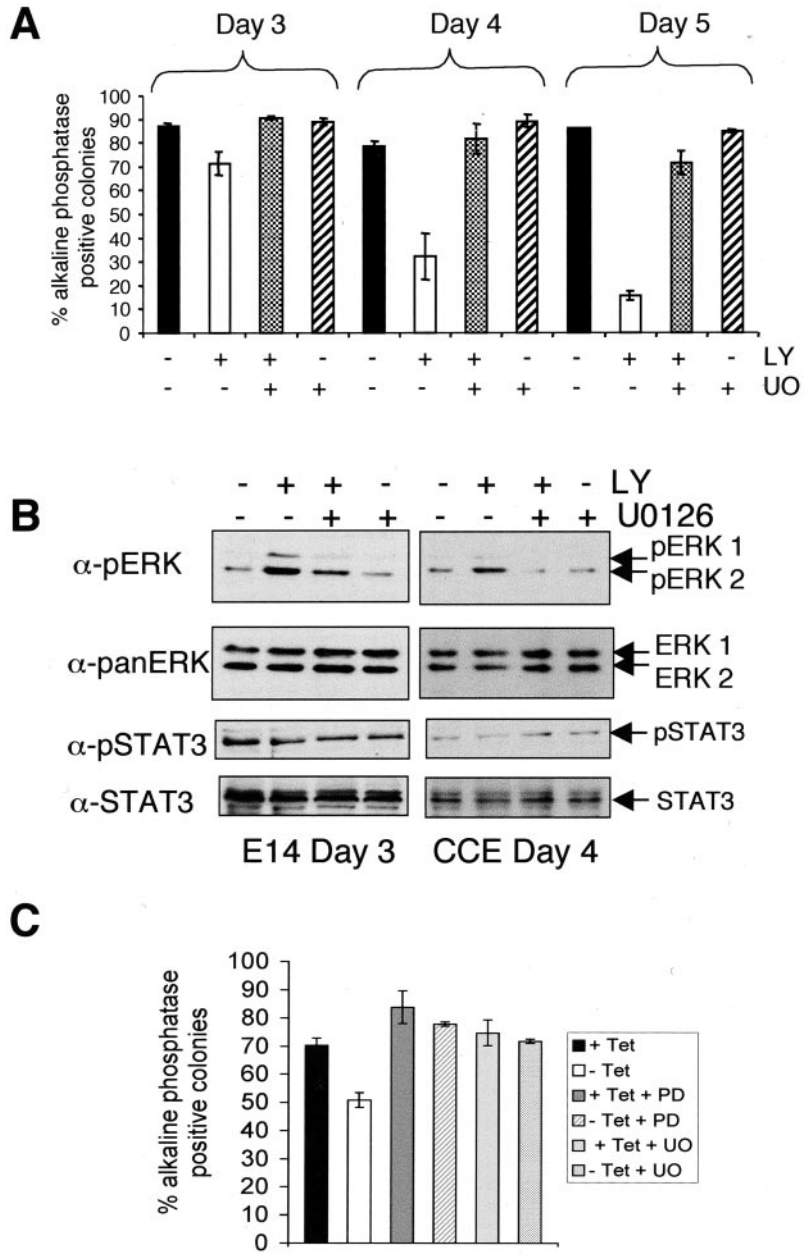

FIG. 6. **Inhibition of MEKs reverses the effect of inhibition of PI3Ks on ES cell self-renewal.** *A* and *B*, E14tg2a cells were plated in the presence of 1000 units/ml LIF in the presence or absence of 5 μM LY294002 (*LY*), 10 μM U0126 (*U0*), or both inhibitors together. Cultures were incubated for 3–5 days. *A*, cultures were stained using Fast Red™ to detect alkaline phosphatase-positive colonies. Cultures were counted in triplicate, and the percentage of alkaline phosphatase-positive colonies determined. S.D. are shown in each case. A representative experiment is shown. *B*, cell lysates were prepared from either E14tg2a or CCE ES cell cultures at the times shown, and 20 μg separated per sample through 10% acrylamide gels by SDS-PAGE. *Upper panel*, dual phosphorylation of the ERK activation motif was detected (*α-pERK*). The same immunoblots were sequentially stripped and reprobed to detect total ERK protein (*pan-ERK*), phospho-STAT3 (*α-pSTAT3*) and total STAT3 (*α-STAT3*), as indicated. *C*, E14Δp85 ES cells were incubated in the presence of 500 units/ml LIF and in the presence or absence of Tet to induce expression of Δp85. Cells were additionally plated in the presence or absence of 50 μM PD98059 (*PD*) or 10 μM U0126 (*U0*). Cultures were incubated for 4 days and stained using Fast Red™ to detect alkaline phosphatase-positive colonies. Cultures were counted in triplicate, and the percentage of alkaline phosphatase-positive colonies determined. The data represent the combined results, with S.E., from two independent clones.

a recovery in self-renewal in cultures expressing Δp85, as shown in Fig. 6*C*. Thus, regulation of ERK activity by PI3K-dependent mechanisms at least partly contributes to determination of stem cell self-renewal.

Effects of PI3K Inhibition on β-Catenin—Sato *et al.* (19) recently reported that inhibition of GSK-3β enhances self-renewal of murine and human ES cells, in part caused by maintenance of β-catenin levels (19). Activation of PI3Ks leads to PKB/Akt-dependent phosphorylation of GSK-3α/β at serines 21 and 9, which inhibits GSK-3 activity. We predicted that if β-catenin-dependent events contribute to PI3K-dependent maintenance of self-renewal, then inhibition of PI3Ks should result in an increase in β-catenin phosphorylation at GSK-3 target sites coupled with a decrease in β-catenin levels, as a result of enhanced β-catenin degradation. Our results, shown in Fig. 7A, demonstrate that acute treatment with LIF did not alter β-catenin phosphorylation at GSK-3β-dependent sites or levels of β-catenin and that treatment with LY294002 or expression of Δp85 also had little influence. In long term cultures, see Fig. 7*B*, Δp85 expression had little effect on β-catenin phosphorylation or levels. However, we unexpectedly observed a reduction in phosphorylation of β-catenin at Ser^{33}, Ser^{37}, and Thr^{41} in cells treated with LY294002, although the overall levels of β-catenin were not dramatically altered. LY294002 was effective over this time course, indicated by the consistent reduction in phosphorylation of S6 protein phosphorylation (Fig. 7*B*, *left hand side*, *lower panels*).

DISCUSSION

In this study we have characterized a role for PI3Ks in the regulation of self-renewal of murine ES cells. We demonstrate that LIF induces the PI3K-dependent phosphorylation of PKB/Akt, GSK-3α/β, and S6 proteins. Uniquely, we show that inhibition of PI3Ks with LY294002, for periods as short as 24 h, results in a decline in the ability of murine ES cells to self-renew. The importance of PI3Ks in this response was confirmed by the finding that expression of a dominant negative form of the class I adaptor subunit p85, which more specifically inhibits class I_A PI3Ks (33, 40, 41), also led to a reduction in self-renewal. Correspondingly, when PI3Ks were inhibited, ES

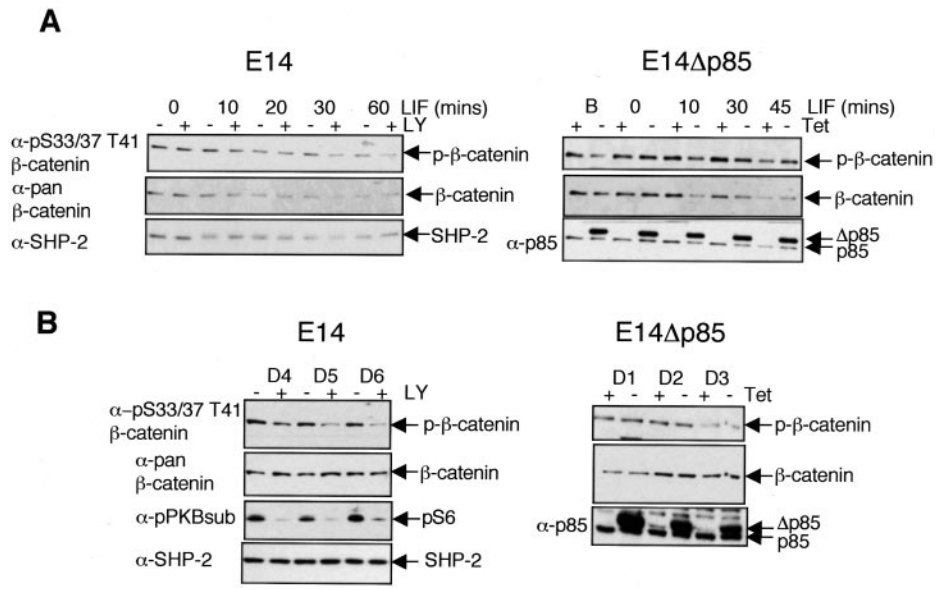

FIG. 7. **Inhibition of PI3Ks does not dramatically alter β-catenin phosphorylation or levels.** *A*, E14tg2a ES cells (*left hand panel*) and E14Δp85 ES cells (*right hand panel*) treated as described in the legend to Fig. 1 (*A* and *B*). *B*, cell lysates were prepared from E14tg2a (*left hand panel*) incubated in the absence or presence of 5 μM LY294002 for 4–6 days or E14Δp85 ES cells (*right hand panel*) that had been induced to express Δp85 by the removal of tetracycline (−*Tet*) for the times shown or maintained in Tet as a control. 20 μg of each protein sample were separated through either 7.5% (for anti-β-catenin blots) or 10% (for anti-pPKBsub blotting) acrylamide gels by SDS-PAGE. Immunoblotting was carried out first with antibody specific for β-catenin phosphorylated at serines 33 and 37 and threonine 41. Blots were stripped and reprobed with anti-pan β-catenin antibody and then stripped again and probed with either SHP-2 or p85 antibodies to assess equivalence of loading and expression of Δp85. Immunoblotting of the same samples shown in *B* (*left hand panel*) with the pPKBsub antibody was used to the demonstrate the sustained inhibitory effect of LY294002 over the time course.

cells adopted a more differentiated phenotype, and STAT5 expression was elevated, consistent with commitment of the cells to differentiation. Inhibition of PI3Ks also led to enhanced LIF-induced activation of the ERK MAPKs, which appeared to play a functional role because inhibition of ERK activity reversed the effects of PI3K inhibition on self-renewal. Based on these findings we propose that PI3Ks are important for maintenance of efficient self-renewal of murine ES cells and when inhibited cells commit to a program of differentiation.

PI3Ks have been implicated in the regulation of a plethora of functional responses in a wide variety of cell systems including migration, survival, cell cycle progression, and proliferation (22). Previous studies examining the role of PI3K signaling in ES cells have implicated PI3Ks in regulation of proliferation (25, 26), possibly because PI3Ks operate downstream of ERas to promote growth (27). At low doses of LY294002, we observed only small effects on proliferation, although at doses of greater than 20 μM our results are consistent with those published (25). We also found that apoptosis only increased by ~6% upon inhibition of PI3Ks, similar to levels previously reported (25, 26). We do not believe that these small effects on viability and proliferation are sufficient to account for the decline in self-renewal we report. Whereas our findings may at first appear at odds with those of Jirmanova *et al.* (25), we believe that they are consistent. IOUD2 ES cells, which express the neomycin resistance gene under the regulation of the *Oct-4* promoter (44), were used in the earlier study, and only undifferentiated IOUD2 ES cells are resistant to G418. When examining the effects of LY294002 on ES cell proliferation, Jirmanova *et al.*, maintained their IOUD2 cells in G418, and under these conditions very little increase in cell number was observed. Whereas this is consistent with reduced proliferation, it is also consistent with the cells having differentiated, so becoming susceptible to G418. In our studies, LY294002 was effective in reducing self-renewal of IOUD2 ES cells leading us to suggest that the effects previously attributed to proliferation (25) are also consistent with PI3K signaling contributing to efficient self-renewal.

In characterizing the mechanism of action of PI3Ks in regulation of ES cell pluripotency we concentrated initially on STAT3 and ERKs. Our data demonstrate an enhancement of ERK 1 and 2 phosphorylation, consistent with their enhanced activity upon inhibition of PI3Ks. The fact that inhibition of MEK, an upstream regulator of the ERK cascade by two structurally unrelated MEK inhibitors, overcomes the effects of PI3K inhibition on self-renewal argues in favor of this effect being functionally important. Based on these results, we propose that upon PI3K inhibition, the balance between STAT-3 and ERK signals, believed to be important in determining cell fate (13), is altered in favor of the prodifferentiation effects of the ERK cascade. It is interesting that a recent report demonstrating a role for BMP4 in maintenance of self-renewal of murine ES cells implicated BMP4-induced inhibition of p38 and ERK MAPKs in the response (17). Others have reported elevated ERK activation in ES cells lacking the p85α regulatory subunit of class I_A PI3Ks compared with their wild-type counterparts in response to IGF-1 (26). ES cells lacking functional phosphoinositide-dependent kinase 1, a downstream effector of PI3Ks, also have elevated levels of basal ERK activity, which increases further after treatment with LY294002 (45). These two reports, along with our study, are consistent with PI3Ks playing a role in negative regulation of ERK activity in ES cells. This is distinct from findings in many terminally differentiated cells, where inhibition of PI3K activity results in reduced activation of the ERKs (40, 46). Importantly, in differentiated myotubes PKB/Akt negatively regulates the Raf-MEK-ERK pathway via actions on Raf (47). One intriguing possibility is that similar mechanisms of cross-talk may be active in murine ES cells and contribute to determination of cell fate.

While our data support a functional role for ERKs in the PI3K dependence of self-renewal, we cannot rule out the possibility that PI3K signaling also regulates additional pathways. BMP2/4 signaling via Smads, leading to increased expression of the Id transcriptional repressors, contributes to maintenance of self-

renewal by effectively blocking differentiation to neuronal lineages (16). Recent reports suggest that members of the TGF-β family, including BMP2, can activate PI3K-dependent signaling in certain cell systems (48–50). In addition, these reports suggest that PI3K-dependent pathways are involved in regulation of Smad protein transcriptional activity, either via modulation of phosphorylation or location (48–50). Therefore, it will be interesting to address whether PI3Ks regulate BMP2/4 activation of Smads in murine ES cells, which could also contribute to their role in regulating self-renewal.

Sato *et al.* (19) have reported that the GSK-3β inhibitor termed BIO maintains self-renewal of murine ES cells in the absence of LIF, implicating the canonical Wnt/β-catenin signaling in maintenance of self-renewal. If PI3Ks also acted via effects on this pathway then we would have expected to measure alterations in β-catenin phosphorylation and levels, but we did not. Therefore, our results suggest that PI3K-dependent regulation of self-renewal occurs independently of effects on β-catenin. However, it is possible that BIO mimics the effects of PI3K activation, by inhibiting GSK-3 activity, and contributes to self-renewal via pathways not involving Wnt/β-catenin. Additional experimentation is required to further define the mode of action of BIO.

Based on our studies we propose that PI3K-dependent signaling is required for efficient self-renewal of murine ES cells, and regulation of ERK activity is functionally important in this response. Further investigations should reveal how PI3K inhibition alters the genetic program of undifferentiated ES cells thereby leading to loss of self-renewal. It will also be interesting to evaluate if PI3K-dependent signaling is important for self-renewal of human ES cells.

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