Regulation of Nanog Expression by Phosphoinositide 3-Kinase-dependent Signaling in Murine Embryonic Stem Cells

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Embryonic stem (ES) cell pluripotency is regulated by a combination of extrinsic and intrinsic factors. Previously, we have demonstrated that phosphoinositide 3-kinase (PI3K)-dependent signaling is required for efficient self-renewal of murine ES cells. In the study presented here, we have investigated the downstream molecular mechanisms that contribute to the ability of PI3Ks to regulate pluripotency. We show that inhibition of PI3K activity with either pharmacological or genetic tools results in decreased expression of RNA for the homeodomain transcription factor Nanog and decreased Nanog protein levels. Inhibition of glycogen synthase kinase 3 (GSK-3) activity by PI3Ks plays a key role in regulation of Nanog expression, because blockade of GSK-3 activity effectively reversed the effects of PI3K inhibition on Nanog RNA, and protein expression and self-renewal under these circumstances were restored. Furthermore, GSK-3 mutants mimicked the effects of PI3K or GSK-3 inhibition on Nanog expression. Importantly, expression of an inducible form of Nanog prevented the loss of self-renewal observed upon inhibition of PI3Ks, supporting a functional relationship between PI3Ks and Nanog expression. In addition, expression of a number of putative Nanog target genes was sensitive to PI3K inhibition. Thus, the new evidence provided in this study shows that PI3K-dependent regulation of ES cell self-renewal is mediated, at least in part, by the ability of PI3K signaling to maintain Nanog expression. Regulation of GSK-3 activity by PI3Ks appears to play a key role in this process.

Embryonic stem cell pluripotency underpins their potential utility as a source of differentiated progeny for use in regenerative medicine. Leukemia inhibitory factor (LIF) plays an important role in maintaining self-renewal of murine ES (mES) cells (1, 2) via activation of STAT3 (3–6) and induction of c-Myc (7). LIF also activates additional signals including the Ras/ERK kinase pathway (8, 9), ribosomal S6 kinases (10), phosphoinositide 3-kinases (11), and Src kinases (12). However, whereas LIF-induced STAT3 activation promotes self-renewal, LIF-induced ERK activation appears to promote differentiation (8), leading to the proposal that the balance between STAT3 and ERK signals contributes to the determination of mES cell fate (13).

Other extrinsic factors that also play a role in maintenance of mES cell self-renewal include bone morphogenetic protein 4 (BMP4), which acts in synergy with LIF to maintain self-renewal via Smad-mediated induction of Id transcriptional repressor expression (14). A further report suggests BMP4 inhibition of p38 mitogen-activated protein kinase (MAPK) may also contribute to maintenance of self-renewal (15). Wnt signaling has been implicated in regulation of pluripotency of both mES and human ES (hES) cells, work stemming largely from use of the GSK-3 inhibitor 5-bromoindirubin-3-oxime (BIO) (16, 17).

A number of intrinsic regulators in the form of transcription factors have been identified that play important roles in regulation of pluripotency, among them Oct4, Sox2, and Nanog (18). Interestingly, expression of the homeodomain protein Nanog alone can overcome the requirement of mES cells for LIF (19, 20) and delays differentiation induced by retinoic acid. Insights into the transcriptional networks regulated by these transcription factors have recently been reported for both human and murine ES cells (21, 22). Significant overlap in the target genes for Oct4, Sox2, and Nanog have been revealed in hES cells (21), whereas in mES cells Oct4 and Nanog bind the promoter regions of many of the same genes (22). Further evidence suggests that Oct4-Sox2 complexes, at least in part, are involved in regulation of Nanog expression (23), whereas p53 has been implicated in repression of Nanog expression (24).

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§ The abbreviations used are: LIF, leukemia inhibitory factor; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; ES, embryonic stem; mES, murine ES; hES, human ES; GSK, glycogen synthase kinase; PI3K, phosphoinositide 3-kinase; STAT, signal transducer and activator of transcription; Tet, tetracycline; 4OHT, 4-hydroxy-tamoxifen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BIO, 5-bromoindirubin-3-oxime; RT-PCR, reverse transcription PCR; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
PI3Ks Regulate Nanog Expression

Clearly, revealing the mechanisms that regulate Nanog expression will further enhance our understanding of the pluripotent state.

Phosphoinositide 3-kinases are a family of lipid kinases whose products PI(3,4)P₂ and PI(3,4,5)P₃ act as intracellular second messengers (25, 26). PI3K-mediated signaling has been implicated in an array of physiological processes, notably proliferation, cell survival, cell migration, and trafficking (25, 26). As observed in many somatic cells, PI3Ks have been reported to control proliferation of mES cells (27–30), whereas we (11) and others (31, 32) have reported that PI3K-mediated signaling is important for maintenance of mES cell pluripotency and very recently that of hES cells (33). Here we have examined the mechanisms regulated by PI3Ks that contribute to maintenance of mES cell self-renewal. Using a combination of genetic and biochemical approaches, we have identified Nanog as a critical target whose expression is dependent, at least in part, on PI3K-mediated signals.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—A modified form of the Tet-off expression system incorporating chromatin insulator sequences (34) was used for expression of a dominant negative form of the p85 regulatory subunit of class 1, PI3Ks (Δp85). Δp85 lacks the p110 interaction site, and we have previously described its use as a competitive inhibitor (11, 35). The region containing the tTA-responsive promoter and encoding Δp85 was amplified from pUHD10-3neo-Δp85 (35) (see supplemental data, Note 1) and ligated into AscI cut pINSHygro (34) to generate pINSHygroΔp85-off. Nanog-ER<sup>T2</sup> vector was generated by a two-step procedure. First, a 5’-EcoRV/Xhol-3’ PCR fragment containing the entire coding sequence of mouse Nanog (see supplemental data, Note 2) was amplified from Cgr8 RNA and cloned between the EcoRV and Xhol sites in pBSK-STAT3-ER<sup>T2</sup> (36) to generate pBSK-Nanog-ER<sup>T2</sup>. Second, a blunt BamHI fragment containing Nanog-ER<sup>T2</sup> from pBSK-Nanog-ER<sup>T2</sup> was subcloned into the blunt EcoRI site in the pPyCAGIZ bicistronic supertransfection vector (5) (which contains an Ori sequence allowing extrachromosomal replication upon transfection into cells expressing polyoma large T antigen) (37), generating pPyCAGIZ-Nanog-ER<sup>T2</sup>. Cell Culture and Generation of Transfectants—E14tg2a (2) murine ES cell lines were cultured as previously described (11). E14tg2a expressing the Tet-sensitive transactivator tTA (E14tTA) (38) were a kind gift from Dr. O. Witte (University of California, Los Angeles) and electroporated as previously described (11) with pINSHygroΔp85-off. Independent clones were selected in 1000 units/ml LIF, 500 ng/ml Tet, and 200 μg/ml hygromycin and screened for expression of Δp85 following 24 h of incubation plus or minus Tet. Independent clones (termed E14AΔp85) exhibiting very low to undetectable basal expression and good inducible expression of Δp85 (−Tet) were selected for further analyses. Δp85 expression was induced by washing cells three times with phosphate-buffered saline and incubating in LIF-containing medium in the absence of Tet. 500 ng/ml Tet was added back to control samples. To generate Nanog-ER<sup>T2</sup> cells, ES cells expressing the polyoma large T antigen (E14/T cells) (37) were electrophorated (200 V, 960 μA) with 20 μg of pPCAGIZ-Nanog-ER<sup>T2</sup>. Cells were plated at 5 × 10<sup>5</sup> cells/10-cm dish and cultured in the presence of LIF (1000 units/ml) plus zeocin (1 μg/ml) for 8 days and resistant colonies pooled and expanded. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) at a final ratio of 1:500 with 1.6 μg of plasmid/well of ES cells in a 6-well tray. pcDNA3.1 was used as a control alongside pcDNA3.1 encoding either GSK-3β S9A or R96E mutants (kindly provided by T. Dale, Cardiff University, Wales). Transfection efficiency was monitored using a green fluorescent protein expression plasmid and flow cytometry and routinely found to be 35%. 16 h after transfection medium was replaced and cells incubated for a total period of 72 h.

Self-renewal Assays—To determine the ability of ES cells to retain an undifferentiated phenotype, self-renewal assays were performed as previously described (11). In principle, this assay is based on alkaline phosphatase expression, which is high in undifferentiated ES cells and lost upon differentiation. Where required, following a 4–6 h period of cell adherence, cultures were supplemented with 5 μM LY294002, 2 μM BIOC, 10 μM U0126, or Me₂SO alone (all Calbiochem). The additional GSK-3 inhibitor, termed here TD114-2 (10,11,13,14,16,17,19,20,22,23-decachydro-9,4:24,29-dimetheno-1H-dibenzo[n,t] pyrrolo[3,4-q] [1, 4, 7, 10, 13, 22]-tetraoxadiazacyclotetracosen-31,33(32H) dione) caclidion brneret was synthesized following the procedure of Kuo et al. (39) (with minor modifications) and was spectroscopically identical to compound 12 in their series. (For structures of BIO and TD114-2 see supplemental data, Note 3). Alkaline phosphatase-positive colonies, indicative of undifferentiated, self-renewing ES cell colonies and unstained, differentiated colonies, were counted in triplicate for each treatment following 3–5 days in culture.

Preparation of RNA and Quantitative PCR—RNA was extracted using RNasy kits (Qiagen) with on-column DNase digestion or TRIzol (Invitrogen) according to the manufacturer’s recommendations. Quantitative RT-PCR was carried out as follows. 1 μg of RNA was incubated at 65 °C for 5 min in the presence of 250 ng of oligo(dT)<sub>18</sub> RNasin Plus RNase inhibitor (Promega), and 200 μM dNTPs and then placed on ice for 1 min. Reverse transcription was carried out with Omniscipect (Qiagen) in the presence of 200 μM dNTPs at 40 °C for 1 h. This cDNA was then used as the template for quantitative PCR using LightCycler FastStart DNA Master SYBR Green 1 (Roche Applied Science) according to the manufacturer’s instructions. The gene-specific primers used in this study are available (supplemental data, supplemental Table S1). Reactions were carried out in a total volume of 20 μl, comprising 0.4 μM of each primer, 3.5 mM MgCl₂, 2 μl of SYBR Green master mix, and 2 μl of diluted cDNA. Each template was analyzed in duplicate within the same run. Amplification and on-line monitoring were performed using the LightCycler™ 1.5 system (Roche Applied Science). Following 40 amplification cycles, melt-curve analyses were performed to verify specific amplification. PCR efficiency of both the target and reference genes was calculated from the derived slopes of standard curves by LightCycler software (v4.0). These PCR efficiency values were used to calculate

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1 P.-Y. Bourillot and V. Savatier, unpublished information.
PI3Ks Regulate Nanog Expression

**FIGURE 1.** PI3K-dependent signaling regulates expression of Nanog. E14tg2a ES cells were cultured in the presence of LIF plus or minus 5 μM LY294002 or 20 nM rapamycin for the times indicated. A, quantitative RT-PCR was performed and Nanog RNA expression normalized relative to β-actin levels. Data are the average and S.E. of duplicate samples from each of three independent biological replicates (n = 6 in total; ***, p < 0.005). B and C, Nanog expression was analyzed by immunoblotting. 20 μg of protein/sample were immunoblotted using the antibodies indicated. All blots were stripped and reprobed with antibodies to GAPDH to assess loading. The values below the anti-Nanog blots represent the ratio of Nanog expression normalized to GAPDH expression. A value of 1 was given for the 2 h + LIF time point in panel B(i), the 48 h + LIF time point in panel B(ii), and the 24 h + LIF time point in panel C.

**FIGURE 2.** Class I PI3Ks are involved in regulation of Nanog expression. E14Δp85 transfectants were induced to express Δp85 by removal of Tet (−Tet) or maintained in 500 ng/ml Tet as a control (+Tet). RNA (A) or protein (B) were extracted at the times indicated. A, quantitative RT-PCR was performed and Nanog RNA expression normalized relative to β-actin levels. The data are the average and S.D. of quadruplicate samples. *, p < 0.05; ***, p < 0.005. B, 20 μg of protein/sample were immunoblotted with the antibodies indicated. The values below the anti-Nanog blot represent the ratio of Nanog expression normalized to GAPDH expression, where a value of 1 was given to 24 h + Tet, +LIF sample.

the relative quantification values for calibrator-normalized target gene expression by the LightCycler relative quantification software (v4.0). In all cases transcript levels were normalized to β-actin. Data were analyzed for statistical significance using two-tailed paired Student’s t-tests.

Preparation of Cell Lysates and Immunoblotting—Cells were placed on ice and washed three times with phosphate-buffered saline prior to lysis as described previously (40). 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 manufacturer’s instructions. 20 μg of each cell lysate was fractionated by SDS-PAGE and immunoblotted onto nitrocellulose (40). The following primary antibodies were used: 1:1000 for rabbit polyclonal antibodies recognizing phosphotyrosine 705 of STAT3 (anti-pSTAT3, CST 9131), phosphoserines 235/236 S6 (pS6, CST 2211), phospho533/S37/T41 β-catenin (anti-p-β-catenin, CST 9561), anti-β-catenin (CST 9562), anti-p85 (06–195; Upstate Biotechnology), anti-Nanog (ab21603; Abcam), and 1:4000 for goat polyclonal antibody recognizing GAPDH (sc-20357; Santa Cruz Biotechnology). Goat anti-rabbit or rabbit anti-goat secondary antibodies conjugated to horseradish peroxidase (Dako) were used at 1:20,000 dilution and blots developed using ECL (Amersham Biosciences). Blots were stripped and reprobed as described previously (41). Band intensities were determined using a GeneSnap instrument and levels of target protein (Nanog or phosphorylated β-catenin) normalized to levels of GAPDH or β-catenin as appropriate for each sample.

**RESULTS**

Phosphoinositide 3-Kinase-mediated Signaling Regulates Expression of Nanog—We have previously demonstrated a role for PI3K signaling in the efficient maintenance of self-renewal of mES cells (11) and were interested to investigate whether
PI3Ks Regulate Nanog Expression

A

(i) 

- Nanog
- pS6
- GAPDH

(ii) 

- Nanog
- pS6
- GAPDH

(iii) 

- p-β-catenin
- β-catenin

B

(i) 

- Normalized Ratio
- Day 3
- Day 4
- Day 5

(ii) 

- Normalized Ratio
- pcDNA3.1
- S9A
- R96E

C

(i) 

- % alkaline phosphatase positive colonies
- Day 3 PURE
- Day 3 ALL
- Day 4 PURE
- Day 4 ALL

(ii) 

- % alkaline phosphatase positive colonies
- LIF
- LIF + LY
- LIF + 2µM TD
- LIF + 5µM TD
- LIF + LY + 2µM TD
- LIF + LY + 5µM TD
PI3Ks Regulate Nanog Expression

PI3K signaling influenced expression of the intrinsic regulator of pluripotency Nanog. When assessed by quantitative RT-PCR, inhibition of PI3K-dependent signaling with the PI3K inhibitor LY294002 at a dose of 5 μM, close to its IC50 value (42, 43), led to a significant decrease in Nanog RNA levels within 48 h; see Fig. 1A. Nanog protein levels were similarly reduced (Fig. 1B, (i) and (ii)) within 8 h of PI3K inhibition. In contrast, and consistent with our previous findings (11), levels of Oct4 RNA and protein were not altered significantly (supplemental Fig. S1), indicating that the observed decrease in Nanog expression is not simply due to induction of differentiation upon treatment with LY294002. Assessment of S6 ribosomal protein phosphorylation at serines 235 and 236 (Fig. 1B), as an indicator of PI3K inhibition, demonstrated that 5 μM LY294002 effectively blocked S6 phosphorylation, whereas no effect on STAT3 Y705 phosphorylation was observed (supplemental Fig. S2). LY294002 has also been reported to inhibit mTORC1 (43), and whereas previous data have implicated mTORC1 in regulation of ES cell proliferation (44), it was important to examine whether direct inhibition of mTOR affected Nanog expression. ES cells were incubated with 5 μM LY294002 or 20 nM rapamycin and Nanog expression examined by immunoblotting (Fig. 1C). As demonstrated, rapamycin did not cause an alteration in Nanog expression after 24 h, although down-regulation of Nanog was observed after 48 h of rapamycin treatment. These results indicate that the effects of LY294002 on down-regulation of Nanog occur independently of mTORC1 inhibition during the initial phase of the response. The down-regulation of Nanog observed upon longer treatment with rapamycin is consistent with the view that multiple mechanisms are involved in regulation of Nanog expression.

To investigate whether class Iα PI3Ks are involved in regulation of Nanog expression, we expressed a dominant negative form of the p85 regulatory subunit of class Iα PI3Ks (Δp85) (used previously in Refs. 11, 35, 45), which lacks the p110 interaction domain and reduces activation of all class Iα PI3Ks, using a modified Tet-off expression system. In this modified Tet-off system chromatin insulators flanked the Δp85 cDNA insert to reduce the potential for gene silencing. Quantitative RT-PCR analyses, shown in Fig. 2A, revealed that expression of Δp85 reduced levels of Nanog RNA, with similar results observed in three independent clones. Δp85 expression also reduced Nanog protein levels, shown in Fig. 2B, supporting a role for class Iα PI3Ks in regulation of Nanog expression.

Glycogen Synthase Kinase 3 Plays a Role Downstream of PI3Ks in Mediating Regulation of Nanog—PI3Ks regulate a number of physiological responses via a complex network of downstream effector molecules (46). We have shown previously that inhibition of PI3Ks in mES cells enhances activation of ERKs and this plays a functional role in the loss of pluripotency observed (11). Sato et al. (17) have reported that inhibition of GSK-3 activity by BIO enhances self-renewal of mES and hES cells. Of relevance here is the fact that PI3K signaling also leads to inactivation of GSK3α/β, via protein kinase B-mediated phosphorylation of Ser-21/9. Therefore, we investigated whether inhibition of either ERK or GSK-3 signaling could overcome the effects of PI3K inhibition and restore Nanog expression. ES cells were cultured with LIF in the presence of either LY294002 alone or together with the MEK inhibitor U0126 or with either of two structurally distinct GSK-3 inhibitors BIO (17) or TD114-2 (compound 12 in Ref. 39). Inhibition of MEK/ERK signaling was unable to reverse the decrease in Nanog expression observed when PI3Ks were inhibited; see Fig. 3A, (i). In contrast, inhibition of GSK-3 by either BIO or TD114-2 reversed the effects of LY294002 treatment, and Nanog expression remained at a level similar or higher than that seen in cells cultured in LIF alone. The effects of GSK-3 inhibition on Nanog expression were also maintained over a longer time course; see Fig. 3A, (ii). These treatments had little effect on STAT3 phosphorylation (not shown), whereas S6 phosphorylation was reduced in all samples treated with LY294002, indicating effective inhibition of PI3Ks (Fig. 3A). It appeared that treatment with BIO or TD114-2 alone elevated Nanog expression (Fig. 3A, (i), right panel, and (ii)), suggesting GSK-3 may be basally active in ES cells. Indeed, β-catenin was phosphorylated at GSK-3 sites Ser-33/Ser-37/Thr-41 in ES cells cultured in LIF, and phosphorylation at these sites was reduced in a dose-dependent manner upon treatment with BIO or TD114-2 (see Fig. 3A, (iii)). In addition, quantitative RT-PCR demonstrated that Nanog RNA levels increased upon GSK-3 inhibition with BIO compared with cells cultured in LIF alone (shown in Fig. 3B, (i)); furthermore, inhibition of GSK-3 reversed the decrease in Nanog RNA observed when PI3Ks were inhibited. To verify that this is a GSK-3–specific effect, we transiently expressed mutated forms of GSK-3β in ES cells. The GSK-3β S9A mutant lacks the PI3K-dependent inhibitory phosphorylation site at Ser-9 and is therefore constitutively active. A second mutant, in which arginine 96 is replaced with a glutamic acid residue (R96E), acts as a dominant negative mutant by preventing recognition of phosphorylated substrates (47), thereby mimicking the effects of GSK-3 inhibitors. The effect of these mutants on Nanog expression was examined by quantitative PCR. As shown in Fig. 3B, (ii), the S9A GSK-3β mutant reduced Nanog expression, whereas the R96E GSK-3β

FIGURE 3. GSK-3 plays a functional role in regulation of Nanog expression and self-renewal via PI3Ks. E14tg2a ES cells were incubated for the times shown with either LIF alone, LIF plus 5 μM LY294002, LIF plus 5 μM LY plus 10 μM U0126, LIF plus 5 μM LY plus 2 μM BIO, LIF plus BIO, LIF plus 2 μM TD114-2, or LIF plus LY plus TD114-2 as indicated. Incubation in panel A (i) was for 48 h, in (ii) for the times indicated, and in (iii) for 30 min. A, 20 μg of protein/sample were immunoblotted with the antibodies indicated. All blots were stripped and reprobed to assess loading. In panel A (i) and (ii) the values below the anti-Nanog blot represent the ratio of Nanog expression normalized to GAPDH expression, where a value of 1 was given to the +LIF alone samples in (i) and +LIF alone sample in (ii). In panel A (iii) levels of β-catenin phosphorylation were normalized to total β-catenin levels and a value of 1 given to samples not treated with inhibitor. B, quantitative RT-PCR was performed, and relative levels of Nanog RNA (normalized to β-actin) are shown. (i), the average and S.D. of quadruplicate samples are shown and are representative of three independent experiments. (ii), ES cells were transiently transfected with either control expression plasmid (pcDNA3.1) or versions of pcDNA3.1 encoding the S9A GSK-3β mutant or the R96E GSK-3β mutant. The average and S.E. of quadruplicate samples are shown from two independent experiments. C, self-renewal was measured by clonal assays and alkaline phosphatase staining. (i), the proportions of highly self-renewing, pure red colonies (PURE) and the total number of self-renewing colonies (ALL) are shown for days 3 and 4. The combined averages, from three independent experiments, with S.E. (error bars) are shown. (ii), the effects of combined treatment with LY294002 (L) and TD114-2 (T, 2 or 5 μM) on the total number of self-renewing colonies were quantitated following 5 days of incubation.*, p < 0.05; **, p < 0.01; ***, p < 0.005.
mutant led to an elevation in Nanog expression. These results confirm the involvement of GSK-3 in regulation of Nanog expression. When we examined the effects of GSK-3 inhibition on self-renewal, using clonal assays and alkaline phosphatase staining, incubation with BIO or TD114-2 completely reversed the effects of PI3K inhibition on self-renewal, shown in Fig. 3C, (i) and (ii). Particularly noticeable were the number of compact, highly self-renewing colonies (PURE colonies in Fig. 3C, (ii)) present in cultures incubated with BIO. Collectively these results suggest that PI3K-mediated inhibition of GSK-3 activity contributes to maintenance of self-renewal, and this correlates with effects on Nanog expression.

**PI3K-mediated Signaling Regulates Expression of Nanog Target Genes**—To gain further insight into the relationship between PI3K signaling and Nanog expression, we examined the effect of inhibition of PI3Ks on expression of putative Nanog target genes. We selected the mouse orthologs of a number of the genes described by Boyer et al. (21), the promoters of which have been shown to bind either Nanog alone or with Oct4 and Sox2, and determined whether their expression is PI3K-dependent. As shown in Fig. 4, wt1 and left1 RNA levels are both up-regulated upon inhibition of PI3K signaling. In contrast, the levels of silver, lefty2, fry (function unknown), and of the transcription factor rfx4 were significantly down-regulated upon PI3K inhibition, suggesting a functional relationship between PI3K signaling and Nanog expression.

**Functional Role of Nanog in PI3K-mediated Signaling in ES Cells**—To explore the functional relationship between PI3K-dependent signaling and Nanog, we examined whether enforced expression of Nanog was sufficient to overcome the loss of self-renewal observed upon inhibition of PI3K activity. We constructed a Nanog estrogen receptor fusion protein (Nanog-ER<sup>T2</sup>) that is only active in the presence of 4OHT and derived ES cell transfectants expressing Nanog-ER<sup>T2</sup>. Levels of Nanog-ER<sup>T2</sup> fusion protein relative to endogenous Nanog are shown in Fig. 5A. Self-renewal assays (shown in Fig. 5B) were performed with these cells in the presence of LIF, the presence or absence of 4OHT, and in the presence or absence of LY294002. Importantly, activation of Nanog-ER<sup>T2</sup> (with 4OHT) significantly suppressed the loss of self-renewal observed upon inhibition of PI3Ks. Furthermore, self-renewal in the presence of LIF alone was enhanced upon activation of Nanog-ER<sup>T2</sup>. We next examined whether activation of Nanog-ER<sup>T2</sup> could overcome the effect of PI3K inhibition on expression of selected putative Nanog target genes. Quantitative PCR analyses, shown in Fig. 5C, demonstrated that activation of Nanog-ER<sup>T2</sup> counters the increase in wt1 expression observed when PI3Ks are inhibited and in the presence of LIF alone led to a reduction in wt1 levels, consistent with this gene being repressed by Nanog. Similarly, expression of left1 was significantly repressed upon activation of Nanog-ER<sup>T2</sup> in the presence of both LIF alone or LIF plus PI3K inhibitor. In the case of silver, Nanog-ER<sup>T2</sup> activation resulted in increased expression compared with LIF alone and partially alleviated the effects of PI3K inhibition. These results provide further evidence to support a functional link between PI3K signaling and maintenance of Nanog expression.

**DISCUSSION**

The identification of Nanog as a key intrinsic factor regulating ES cell pluripotency (19, 20) has generated significant interest in defining how Nanog expression is regulated. In this study we present novel data that functionally link PI3K signaling to regulation of Nanog expression. We show that inhibition of PI3Ks by pharmacological or molecular tools decreases Nanog RNA and protein levels and implicates class I<sub>i</sub> PI3Ks in this process. We further show that regulation of GSK-3 activity downstream of PI3Ks plays a critical role in regulating Nanog.
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expression. In addition, our data showing that activation of a Nanog-ER\textsuperscript{T2} fusion protein prevents the loss of self-renewal observed upon inhibition of PI3Ks provide evidence of an important functional relationship between PI3K-dependent signaling and maintenance of Nanog expression. This is further corroborated by our demonstration that a number of putative Nanog target genes are regulated in a PI3K-dependent manner and that induction of Nanog-ER\textsuperscript{T2} overcomes the effects of PI3K inhibition on expression of a number of these genes. Thus, our study demonstrates a novel functional relationship between PI3K-mediated signaling and regulation of Nanog expression.

Interestingly, our data indicate that down-regulation of Nanog protein (detected as early as 8 h) precedes the decline in Nanog RNA (detected at 24–48 h). Treatment of mES cells cultured on three-dimensional nanofibrillar structures with an unrelated PI3K inhibitor, wortmannin, has recently been reported to also lead to a decline in Nanog expression within 24 h, consistent with our data (48). The fact that rapamycin treatment does not affect Nanog expression at 24 h, whereas LY294002 and wortmannin treatment does, argues that the effects of LY294002 treatment are not being mediated via potential effects on mTORC1. Additionally, previous data implicate mTORC1 in regulation of ES cell proliferation (44), whereas at the doses of LY294002 used in this study we do not observe any perturbation in ES cell growth (11). PI3K signaling is known to be involved in translational regulation (49); thus, it is possible that upon inhibition of PI3Ks, translation of Nanog mRNA decreases, leading to the decline in Nanog protein levels observed. The Nanog promoter contains upstream Nanog binding sites (22), raising the prospect that Nanog can, at least in part, regulate its own expression. Thus, the preceding decline in Nanog protein could in turn lead to decreased Nanog transcription. It will be interesting to examine the mechanism of this regulation in greater detail in future studies.

From the perspective of genes regulated by Nanog we found that expression of a number of putative Nanog target genes (21) was sensitive to PI3K inhibition. For example, \textit{wt1}, encoding the Wilm tumor suppressor gene, and \textit{lef1}, encoding a β-catenin transcription factor partner, were up-regulated upon inhibition of PI3K, an effect reversed upon induction of Nanog-ER\textsuperscript{T2} activity, indicating that Nanog may normally repress expression of these genes. Indeed, in both cases RNA levels were reduced simply upon induction of Nanog-ER\textsuperscript{T2} activity. During the preparation of this manuscript, Loh \textit{et al.} (22) reported the mapping of Nanog binding sites in mES cells. Mining their combined Nanog RNA interference/expression profiling data revealed that \textit{wt1} expression is up-regulated upon Nanog knock down, consistent with our findings. Although little is known about the role of \textit{wt1} in very early development, the fact that Wt1 isoforms can act as both transcriptional regulators and co-regulators as well as RNA processing factors raises the possibility of some intriguing actions in ES cells (50). The Lef/TCF transcription factors, in combination with β-catenin, are involved in a range of differentiation events during embryogenesis (51), so their repression by Nanog could help suppress differentiation. Among the genes whose expression was down-regulated upon inhibition of PI3K signaling was \textit{rfx4}, a Nanog target identified in both mES and hES cells (21, 22). Clearly, investigation of the role of \textit{rfx4} in ES cells will be of further interest, as will the regulation of \textit{lef2} expression by PI3Ks, because Lefty2 antagonizes Nodal signaling (52) involved in tissue patterning (53). Throughout our studies we noted that

![FIGURE 5. Regulation of Nanog expression plays a functional role downstream of PI3Ks. A, expression of Nanog-ER\textsuperscript{T2} and Nanog in Nanog-ER\textsuperscript{T2} ES cells. Cells were cultured for 5 days in the conditions indicated. Immunoblotting was performed with 20 μg of protein/sample with the antibodies indicated. Blots were stripped and reprobed with anti-GAPDH antibodies to assess loading. The values below the anti-Nanog blot represent the ratio of Nanog normalized to GAPDH expression. A value of 1 was given for the +LIF alone sample. B, Nanog-ER\textsuperscript{T2} ES cell transfectants were incubated in the presence of LIF, plus or minus 5 μM LY294002 (LY) or plus or minus 100 nM 4OHT. The number of alkaline phosphatase-positive colonies was determined after 5 days in culture. The average values, with S.E., from three independent experiments are shown. C, Nanog-ER\textsuperscript{T2} ES cell transfectants were cultured for the times indicated with LIF plus or minus 5 μM LY294002 (LY) or plus or minus 100 nM 4OHT. RNA was extracted and expression of putative Nanog target genes analyzed by quantitative RT-PCR. Target gene expression was normalized to β-actin levels, and relative expression levels, as normalized ratios, are presented. The average and S.E. of duplicate samples from at least two independent biological replicates are shown in each case (n = 4). * p < 0.05; ** p < 0.01; *** p < 0.005.](https://www.jbc.org/content/282/9/6271/F5)
the relative expression of Nanog RNA and protein increased with time in culture for cells plated at the same initial density in LIF (see Fig. 1, A and B(i) and Fig. 2, A and B). This increase could be due either to effects mediated via cell-cell contact or due to the production of autocrine factors by ES cells that enhance Nanog expression. Further investigations are required to distinguish between these possibilities.

Maintenance of murine ES cell self-renewal and proliferation are intimately linked, and PI3Ks have been implicated not only in maintenance of self-renewal of ES cells (11, 31) but also in regulation of mES cell proliferation (27, 29, 30, 44) and more recently the survival (54) and pluripotency of hES cells (33, 54). Our initial report on the role of PI3Ks in self-renewal implicated enhanced activation of ERKs in this response (11), consistent with the view that the balance between STAT3 and ERK signals was key to determining ES cell fate (13). Our present study demonstrates that PI3K-mediated regulation of GSK-3α/β activity also plays an important role. The GSK-3 inhibitor BIO has previously been used as a surrogate activator of Wnt signaling (17), implicating Wnt-dependent signals in maintenance of self-renewal. PI3Ks, via protein kinase B-mediated phosphorylation of serines 21/9 of GSK-3 α/β, also activate GSK-3, but we have previously shown that inhibition of PI3Ks does not increase β-catenin phosphorylation at GSK-3 sites (11), suggesting there is little cross-talk between these pathways. Therefore, we believe it is significant that the ability of ES cells to self-renew in the presence of PI3K inhibition is restored effectively by either of two distinct GSK-3 inhibitors. Intriguingly, inhibition of both GSK-3 and PI3Ks restored Nanog RNA and protein expression, whereas inhibition of both MEK and PI3Ks did not. The opposing effects on Nanog expression of the constitutively active GSK-3β S9A mutant and the GSK-3β R96E dominant negative mutant provide further evidence that GSK-3 plays a key role in controlling Nanog expression. This leads us to propose that the effects of GSK-3 inhibition on enhancement of self-renewal may not be entirely due to activation of Wnt signaling but that GSK-3 plays additional roles in ES cells.

The ability of PI3K-dependent signaling to contribute to regulation of self-renewal via distinct molecular mechanisms, including regulation of Nanog expression, suggests that PI3K-dependent signaling pathways play important roles in regulating the networks of intrinsic factors that contribute to determination of ES cell fate. In view of the increasing evidence for a role of PI3Ks in regulation of human ES cell fate (33, 54), it will be interesting to determine whether similar mechanisms of regulation are conserved in these cells.

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REFERENCES
PI3Ks Regulate Nanog Expression