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

Received for publication, November 27, 2006 · Published, JBC Papers in Press, January 4, 2007, DOI 10.1074/jbc.M610906200

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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) 2 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 morphogenic 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).

* This work was supported by European Community FP6 integrated project FunGenES Grant LSHG-CT 2003-503494 (to M. J. W. and P. S.), by the Wellcome Trust and Biotechnology and Biological Sciences Research Council (BBSRC) (to M. J. W.), by the INSERM AVENIR program (to P. S.), and by the Biotechnology and Biological Sciences Research Council (BBSRC) (to M. J. Welham) and the Engineering and Physical Sciences Research Council (EPSRC) fellowship (to A. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2, Table S1, and supplemental data.

2 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 I, 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/H9004 p85 (35) (see supplemental data, Note 1) and ligated into AscI cut pINSHygro (34) to generate pUHD10-3neo-H9004 p85 (35) (see supplemental data, Note 1). The region containing the tTA-interaction site, and we have previously described its use as a coaction system incorporating chromatin insulator sequences (34) whose products PI(3,4)P₂ and PI(3,4,5)P₃ act as intracellular

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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 (E14 tTA) (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 E14Δp85) 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

3 P.-Y. Bourillot and V. Savatier, unpublished information.
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), phosphoserine 235/236 (pS 6, CST 2211), phospho-S33/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) 

*α*-Nanog

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*α*-pS6

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(ii) 

*α*-Nanog

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*α*-p-β-catenin

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*α*-β-catenin

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B

(i) 

Normalized Ratio

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Normalized Ratio

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C

(i) 

% alkaline phosphatase positive colonies

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(ii) 

% alkaline phosphatase positive colonies

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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 IC_{50} 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 IA PI3Ks are involved in regulation of Nanog expression, we expressed a dominant negative p85 cDNA insert to transiently express mutated forms of GSK-3β, acts as a dominant negative glutamic acid residue (R96E), acts as a dominant negative inhibitor of mTORC1 (43), indicating that the observed decrease in Nanog expression led to inactivation of GSK3β, 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 blots 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 day3 + LIF 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 using 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 (LY) and TD114-2 (TD, 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, (i)) 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 lefty1 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-ERT2) that is only active in the presence of 4OHT and derived ES cell transfectants expressing Nanog-ERT2. Levels of Nanog-ERT2 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-ERT2 (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-ERT2. We next examined whether activation of Nanog-ERT2 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-ERT2 prevents 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-ERT2 in the presence of both LIF alone or LIF plus PI3K inhibitor. In the case of silver, Nanog-ERT2 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 IA PI3Ks in this process. We further show that regulation of GSK-3 activity downstream of PI3Ks plays a critical role in regulating Nanog expression.
PI3Ks Regulate Nanog Expression

In addition, our data showing that activation of a Nanog-ERT2 ES cell transfectant upon inhibition of PI3Ks, an effect reversed upon induction of Nanog-ERT2 activity, indicating that Nanog may normally repress its own 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 \(\beta\)-catenin transcription factor partner, were up-regulated upon inhibition of PI3Ks, an effect reversed upon induction of Nanog-ERT2 activity, indicating that Nanog may normally repress expression of these genes. Indeed, in both cases RNA levels were reduced simply upon induction of Nanog-ERT2 activity. During the preparation of this manuscript, Loh 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 \textit{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 \(\beta\)-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{lefty2} expression by PI3Ks, because \textit{Lefty2} antagonizes Nodal signaling (52) involved in tissue patterning (53). Throughout our studies we noted that expression. In addition, our data showing that activation of a Nanog-ERT2 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-ERT2 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.
PI3Ks Regulate Nanog Expression

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 signaling is 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 activity also plays an important role. The GSK-3 inhibitor BIO has previously been used as a surrogate activator of Wnt signaling, suggesting that PI3K- and GSK-3α/β activity play opposing roles in controlling 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.

Acknowledgements—We thank A. Konstantinos and F. Stewart (Dresden, Germany) for pNShyg vector and T. Dale (Cardiff, Wales) for the GSK-3β mutants.

REFERENCES