

## ORIGINAL ARTICLE

## Activation of PDK-1 maintains mouse embryonic stem cell self-renewal in a PKB-dependent manner

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The phosphatidylinositol 3' kinase (PI3K) pathway is involved in many cellular processes including cell proliferation, survival and glucose transport, and is implicated in various disease states, such as cancer and diabetes. Although there have been numerous studies dissecting the role of PI3K signaling in different cell types and disease models, the mechanism by which PI3K signaling regulates embryonic stem (ES) cell fate remains unclear. It is believed that in addition to proliferation and tumorigenesis, PI3K activity may also be important for ES cell self-renewal. Paling *et al.* reported that the inhibition of PI3K led to a reduction in the ability of leukemia inhibitory factor to maintain self-renewal, causing cells to differentiate. Studies in our lab have revealed that ES cells completely lacking glycogen synthase kinase-3 (GSK-3) remain undifferentiated compared with wild-type ES cells. GSK-3 is negatively regulated by PI3K, suggesting that PI3K may have a vital role in maintaining pluripotency in ES cells through GSK-3. By using a modified Flp recombinase system, we expressed activated alleles of 3-phosphoinositide-dependent protein kinase-1 and protein kinase B to create stable, isogenic ES cell lines to further study the role of the PI3K signaling pathway in stem cell fate determination. *In vitro* characterization of the transgenic cell lines revealed a strong tendency toward the maintenance of pluripotency, and this phenotype was found to be independent of canonical Wnt signal transduction. In summary, PI3K signaling is sufficient to maintain the self-renewal and survival of stem cells. As this pathway is frequently mutationally activated in cancers, its effect on suppressing differentiation may contribute to its oncogenicity.

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## INTRODUCTION

The phosphatidylinositol 3-kinases (PI3Ks) are a family of conserved intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides, producing PtdIns(3)P, PtdIns(3,4)P<sub>2</sub> (PIP<sub>2</sub>) and PtdIns(3,4,5)P<sub>3</sub> (PIP<sub>3</sub>).<sup>1</sup> The various isoforms of PI3Ks are divided into three main classes. Class I PI3Ks are involved in many important physiological processes and are further subdivided into class IA: those with a p110 catalytic subunit ( $\alpha$ ,  $\beta$  or  $\delta$ ) and a p85 regulatory subunit ( $\alpha$ ,  $\beta$  or  $\gamma$ ) that are activated by growth factor receptor tyrosine kinases and class IB: those with a p110 $\gamma$  catalytic subunit and a p101 or p84 regulatory subunit that are activated by G-protein-coupled receptors.<sup>2</sup> Class II and III PI3Ks are further divided based on differences in substrate preference, mechanism of activation and structural homology.<sup>3</sup>

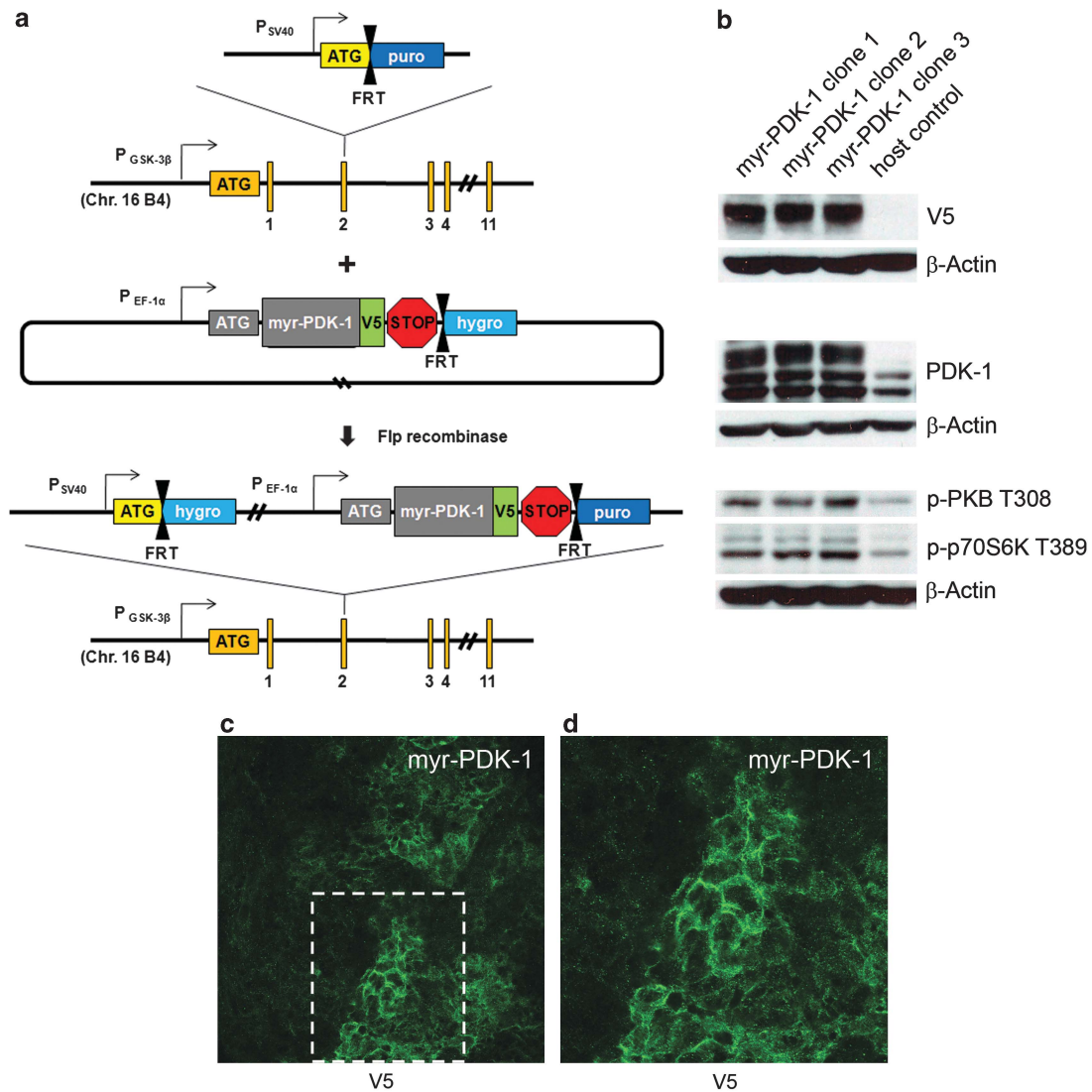
The PI3K signaling pathway is a complex pathway involved in the regulation of proliferation, cell growth, survival, glucose transport, vesicle trafficking, cell adhesion, cell motility and cytoskeletal organization. Insulin and other growth factors stimulate PI3K; the resulting lipid products act as second messengers and bind to N-terminal pleckstrin homology domains of proteins, such as 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase B (PKB), to allosterically modify their activity or translocate them to the plasma membrane where they can bind to receptors and activate signal transduction.<sup>4</sup> The PI3K pathway is one of the pathways most frequently activated in human cancers, and two point mutations of PI3K p110 $\alpha$  in particular, E545K and H1047R, lead to constitutive activation of the

pathway.<sup>5</sup> Previous studies have suggested that PI3K signaling may have a vital role in maintaining stem cell pluripotency and giving embryonic stem (ES) cells their tumor-like proliferative properties. A large-scale transcriptional study implicated components of the PI3K pathway in maintaining human ES cell viability and pluripotency.<sup>6</sup> Selective inhibition of class-IA PI3K catalytic isoforms increased differentiation and reduced proliferation.<sup>7</sup> Phosphatase and tensin homolog deleted on chromosome ten (PTEN) inhibits PI3K signaling and negatively regulates ES cell proliferation and teratoma formation.<sup>8</sup> PTEN-null ES cells have enhanced tumorigenicity and PI3K signaling with increased PKB activation and Cyclin D1 levels.<sup>9</sup> It has been suggested that the activation of PKB via myristoylation is sufficient to maintain the undifferentiated phenotype of ES cells without the addition of leukemia inhibitory factor (LIF).<sup>10</sup> Glycogen synthase kinase-3 (GSK-3) is negatively regulated by PI3K, and studies performed by Doble *et al.*<sup>11</sup> revealed that GSK-3 double knockout (DKO) mouse ES cells remained undifferentiated, further implicating the importance of the PI3K and Wnt pathways in regulating stem cell pluripotency.

Several studies have also employed the use of PI3K inhibitors. Paling *et al.*<sup>12</sup> reported that the inhibition of PI3K with the inhibitor LY294002 mitigated the ability of LIF to maintain mouse embryonic stem (mES) cell self-renewal and caused cells to differentiate. Transcriptome profiling revealed several markers of pluripotency to be downregulated, such as Nanog, Esrrb, Tbx-3 and Tcl-1.<sup>13</sup> Cell proliferation was also inhibited with the addition of LY294002, leading to the accumulation of mES cells in G1 phase

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**Figure 1.** Modified Flp-In system. (a) A targeting vector is introduced to knock out a single allele of GSK-3 $\beta$  and create a host cell line with a FRT-puro cassette. Co-transfection of Flp recombinase and a gene of interest previously cloned into a vector containing a V5 epitope and FRT-hygro cassette results in homologous recombination at the FRT sites. Positive colonies are resistant to hygromycin and sensitive to puromycin. (b) Western blot analysis of transgenic myr-PDK-1 cell lines. (c) Membrane localization of myr-PDK-1 by immunofluorescent staining of V5; confocal,  $\times 40$  magnification.

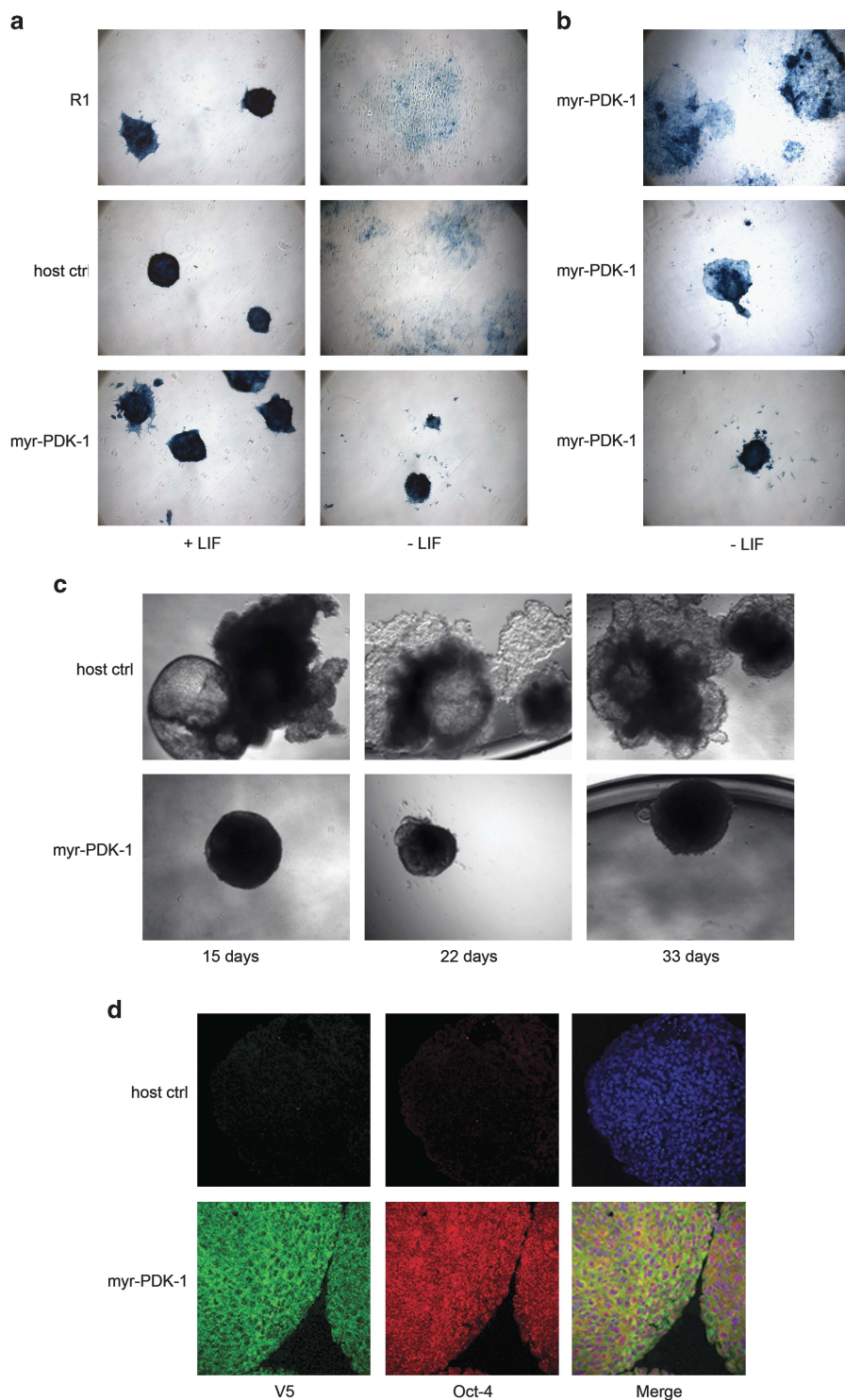
followed by apoptotic cell death.<sup>14</sup> Of note, LY294002 is a nonspecific PI3K inhibitor and also inhibits mammalian target of rapamycin (mTOR), which is crucial for ES cell proliferation.<sup>15</sup> Here, we analyzed the effects of expressing activated forms of components of the PI3K signaling pathway in ES cells on pluripotency. In order to further dissect the molecular mechanism of self-renewal under the control of PI3K signaling, we generated constitutively active isogenic mouse ES cell lines of PDK-1 and PKB, and characterized the pluripotency of the cell lines. In accordance with previous literature, activation of the PI3K signaling pathway appears to maintain stem cell self-renewal. We analyzed roles for PKB-mediated effects on Wnt signaling in mediating this pluripotent phenotype.

## RESULTS

**Role of PI3K pathway in maintenance of pluripotency of ES cells**  
To generate isogenic ES cell lines expressing activated alleles of genes of interest, we employed a modified Flp-In system (Figure 1a). The activated mutants of genes were inserted into

the GSK-3 $\beta$  locus that had been targeted by an FRT-puromycin cassette. In the generation of the host control cell line, almost 700 individual colonies were picked and screened following puromycin selection, and 4 positive colonies were identified. As expected, the host control ES cells (GSK-3 $\beta$ <sup>+/-</sup> ES cells) exhibited a decrease in GSK-3 $\beta$  protein expression compared with R1 ES cells (Supplementary Figure 1a). PDK-1 was made constitutively active by the addition of a Src myristoylation signal on the N-terminus, which localizes the protein to the cell membrane.<sup>16</sup> Three independent, activated PDK-1 ES cell lines were generated with a myc-his tag on the N-terminus and a V5 tag at the C-terminus resulting in the increased protein size in the transgenic cell lines (Figure 1b). All three transgenic cell lines showed increased phosphorylation of PKB on threonine 308 and p70S6K on threonine 389, demonstrating activation of the pathway (Figure 1b). In addition, proper localization of myr-PDK-1 at the cell membrane was visualized by confocal microscopy following immunofluorescent staining for the V5 epitope (Figure 1c).

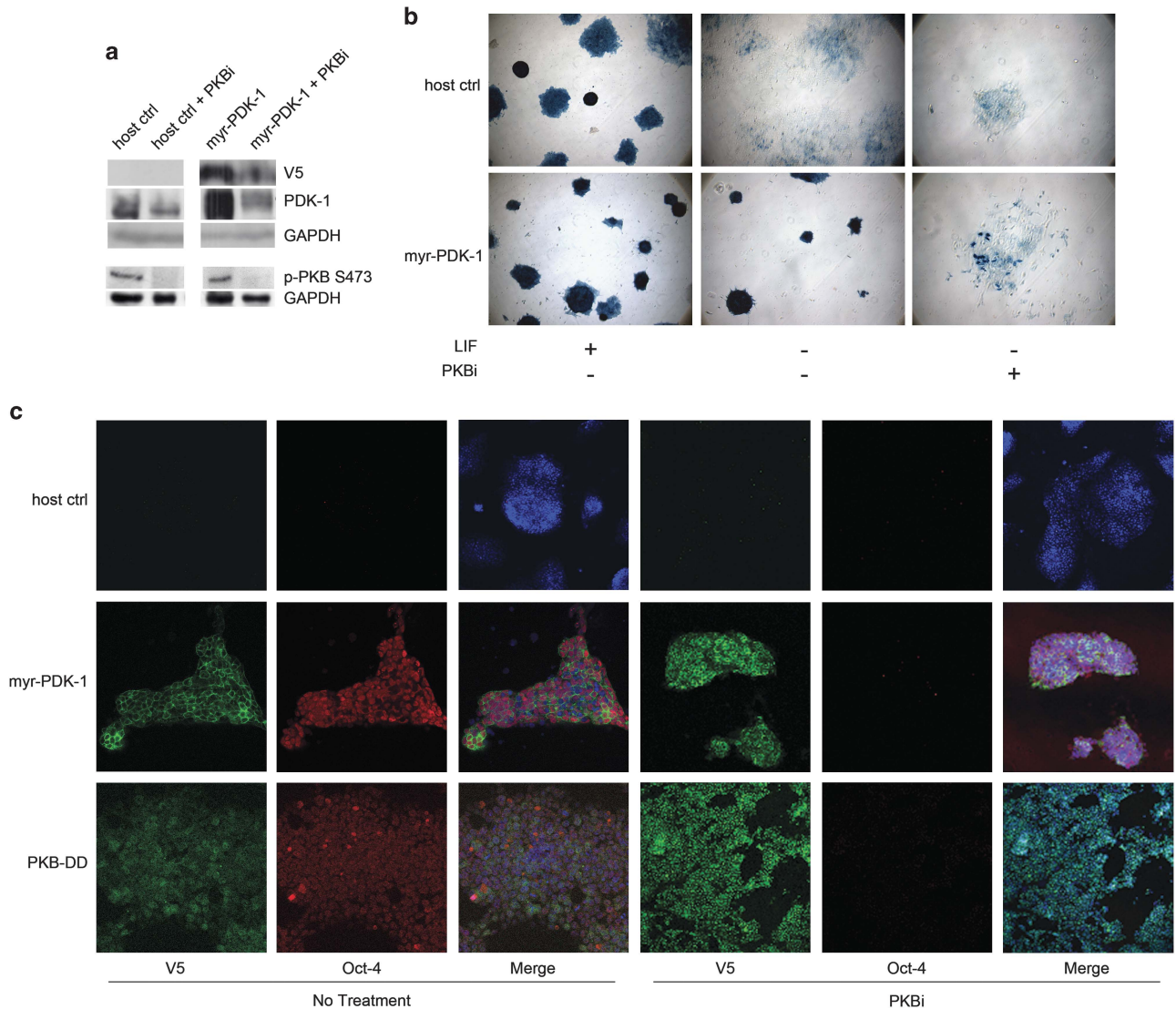
To assess the pluripotency of the myr-PDK-1 cells, we withdrew LIF from the media and observed the morphological changes of



**Figure 2.** Analysis of pluripotent markers on myr-PDK-1 cells. **(a, b)** Alkaline phosphatase activity. Cells were grown in monolayer and stained for alkaline phosphatase activity on day 5; phase contrast,  $\times 10$  magnification. **(c)** Gross morphology of embryoid bodies; phase contrast,  $\times 5$  magnification. **(d)** Oct-4 immunofluorescent expression of myr-PDK-1 transgenic embryoid bodies at day 10; confocal,  $\times 40$  magnification. Ctrl, control.

the cells grown in culture. Interestingly, the myr-PDK-1 cells tended to remain as distinct, round colonies in monolayer culture, whereas the host control ES cells, similar to R1 wild-type ES cells, showed evidence of morphological differentiation and spread throughout the dish (Figure 2a). The myr-PDK-1 colonies maintained positive staining for alkaline phosphatase (AP) activity even

in the absence of LIF for 5 days, a further indication that these cells remained pluripotent (Figure 2a). However, all cells comprising each colony did not always exhibit positive staining. For example, a number of colonies consisted entirely of AP-positive cells, whereas others consisted of both positive and negative cells (Figure 2b).



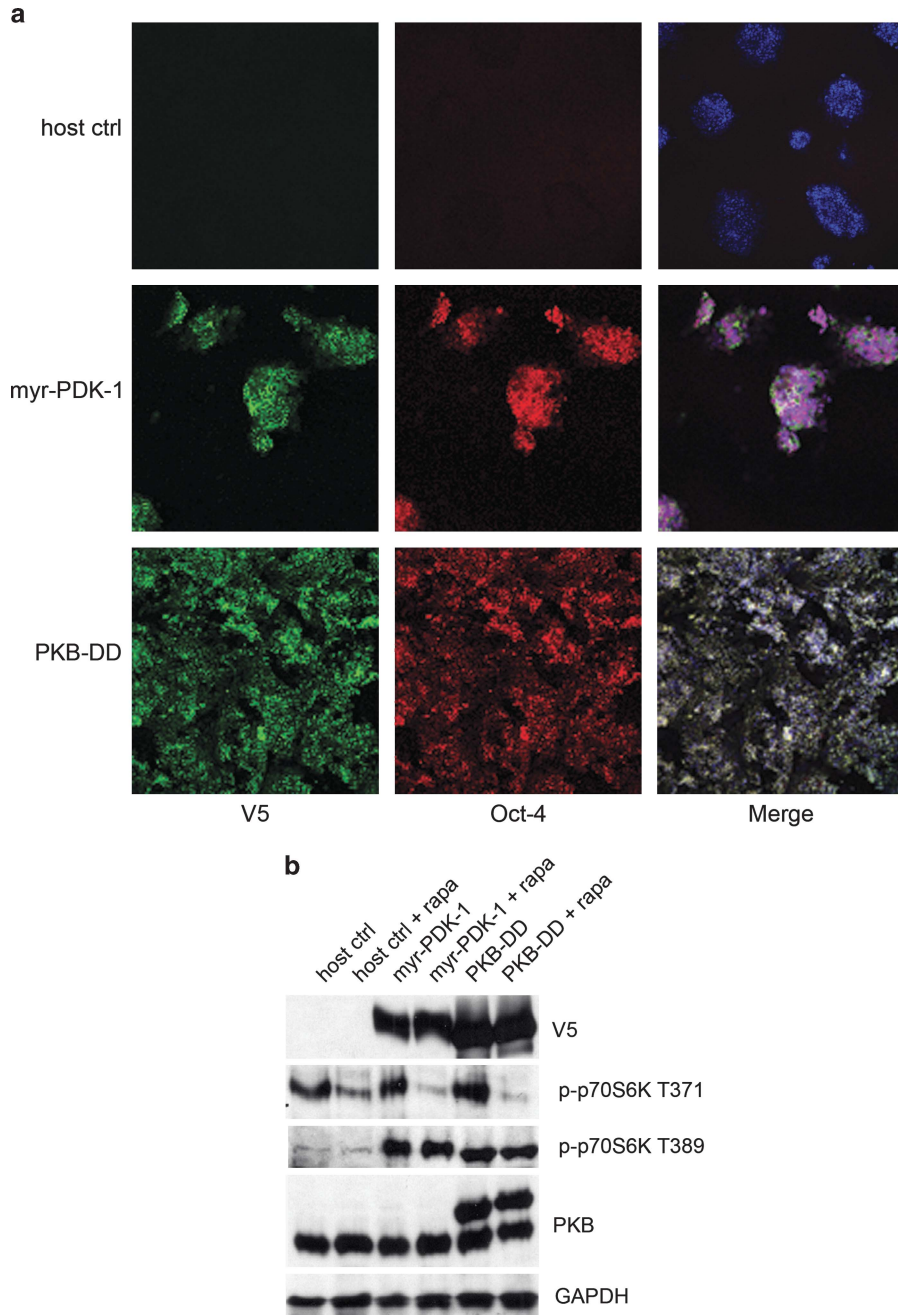
**Figure 3.** Treatment of myr-PDK-1 ES cells with PKB inhibitor. **(a)** Western blot analysis of whole-cell lysates after growing cells long term in inhibitor. **(b)** Cells grown in monolayer stained for alkaline phosphatase activity on day 5; phase contrast,  $\times 10$  magnification. **(c)** Immunofluorescent staining of cells grown in monolayer after 8 h treatment with PKBi; confocal,  $\times 40$  magnification. Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

We proceeded to induce differentiation of the cells by generating embryoid bodies using the hanging drop method. After 3 days as hanging drops, the embryoid bodies (EBs) were then each placed into the well of a 96-well tissue culture plate and maintained for weeks. After a few days in the plate, contractile beating was observed in both the myr-PDK-1 and control EBs. However, the myr-PDK-1 cells retained a tightly compact morphology even at 33 days, whereas the host control and R1 embryoid bodies grew in what appeared to be an uncontrolled manner and differentiated (Figure 2c). Immunofluorescent staining of the embryoid bodies for Oct-4, a marker of pluripotency, revealed that the V5-epitope-tagged myr-PDK-1 cells were positive for Oct-4 expression, whereas the control cells extinguished Oct-4 expression (Figure 2d).

Previously, Watanabe *et al.*<sup>10</sup> showed that myr-PKB expressing ES cells remain undifferentiated. We therefore generated transgenic ES cell lines expressing a constitutively activated PKB (PKB-DD) in which the two activatory phosphorylation sites are mutated to aspartic acid to partially mimic phosphorylation,<sup>17</sup> and detected a similar phenotype to the myr-PDK-1 cells in terms of

AP activity, overall EB morphology and immunofluorescent staining for Oct-4 (Supplementary Figure 2). These data are consistent with the notion that PDK-1 acts through PKB/Akt to promote maintenance of pluripotency.

To gain insight into the mechanism underlying the maintenance of de-differentiation of the myr-PDK-1 cell lines, we employed a downstream block and used a PKB inhibitor that specifically inhibits its activity by targeting the pleckstrin homology domain.<sup>18</sup> Cells grown for several weeks in the presence of the PKB inhibitor (long-term treatment) showed a marked decrease in PKB serine 473 phosphorylation and in PDK-1 expression (Figure 3a and Supplementary Figure 3). Interestingly, addition of the PKB inhibitor eliminated AP activity observed in untreated myr-PDK-1 cells (Figure 3b), and similarly ablated Oct-4 expression from the myr-PDK-1 and PKB-DD cells within 8 h of inhibitor treatment (Figure 3c), suggesting that the inhibition of PKB led to the loss of pluripotency. In initial experiments, long-term treatment with the PKB inhibitor appeared to significantly suppress the translation of transgenic and endogenous PDK-1, complicating the interpretation of the data. However, experiments

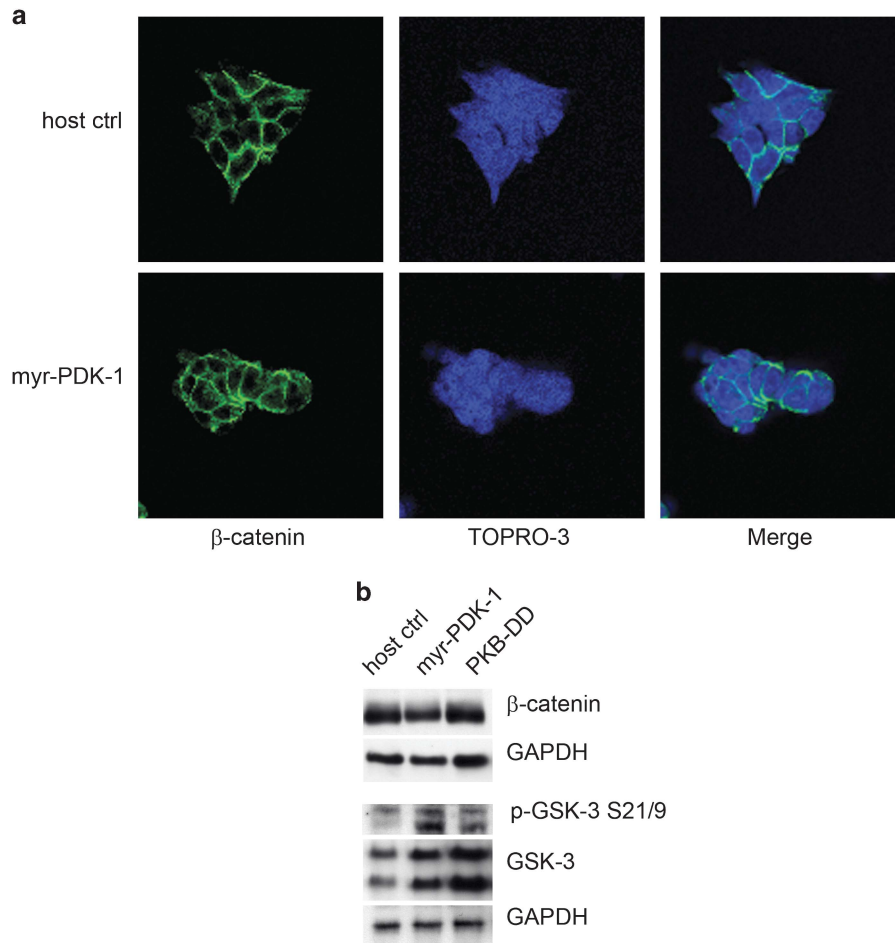


**Figure 4.** Treatment of myr-PDK-1ES cells with rapamycin (rapa). **(a)** Immunofluorescent staining of cells grown in monolayer after 8 h stimulation with rapa; confocal,  $\times 40$  magnification. **(b)** Western blot analysis of whole-cell lysates. Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

conducted with a different, specific anti-PDK-1 antibody showed no change in PDK-1 protein levels (Supplementary Figure 1b). In an experiment where cells were grown in monolayer in the presence of PKB inhibitor for 2–3 weeks before forming EBs and then maintained in the presence of PKB inhibitor, control EBs appeared smaller overall and myr-PDK-1 EBs consistently lost transgene expression (as judged by loss of V5 epitope staining; Supplementary Figure 3). Thus, we investigated the effects of rapamycin treatment on myr-PDK-1 and PKB-DD cells. As the cells were unable to survive long-term exposure to rapamycin, we examined Oct-4 staining in the myr-PDK-1 cells in the presence of rapamycin for 8 h. Rapamycin treatment did not affect Oct-4

expression levels as assessed by immunofluorescent staining (Figure 4a). We further examined the effects of the rapamycin treatment by western blot analysis, and saw that 8 h of treatment resulted in decreased mTOR activity, evident by decreased phosphorylation of p70S6K on threonine 371, and otherwise no effect on p70S6K phospho-T389, endogenous PKB or transgenic PDK-1 expression levels (Figure 4b). Our results indicate that activation of PDK-1 maintains mouse ES cell self-renewal in a PKB-dependent manner in the absence of LIF.

It has previously been shown that PI3K and JAK/STAT pathways act in parallel to maintain pluripotency.<sup>19</sup> To assess roles for the JAK/STAT and other pathways associated with maintaining



**Figure 5.** Analysis of  $\beta$ -catenin expression in myr-PDK-1 and PKB-DD cells. **(a)** Immuno-fluorescent staining of myr-PDK-1 cells with  $\beta$ -catenin. **(b)** Western blot analysis of myr-PDK-1 and PKB-DD cytosolic cell lysates for  $\beta$ -catenin protein expression and whole-cell lysates for phospho-GSK-3 and pan-GSK-3 levels. Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

pluripotent phenotypes, we tested myr-PDK-1 and PKB-DD ES cells for the activation of STAT-3 and ERK in the presence and absence of LIF (Supplementary Figure 1c). No significant differences in either expression or phosphorylation of STAT-3 and ERK were observed, suggesting that the persistence of undifferentiated myr-PDK-1 and PKB-DD cells in the absence of LIF is independent of these pathways.

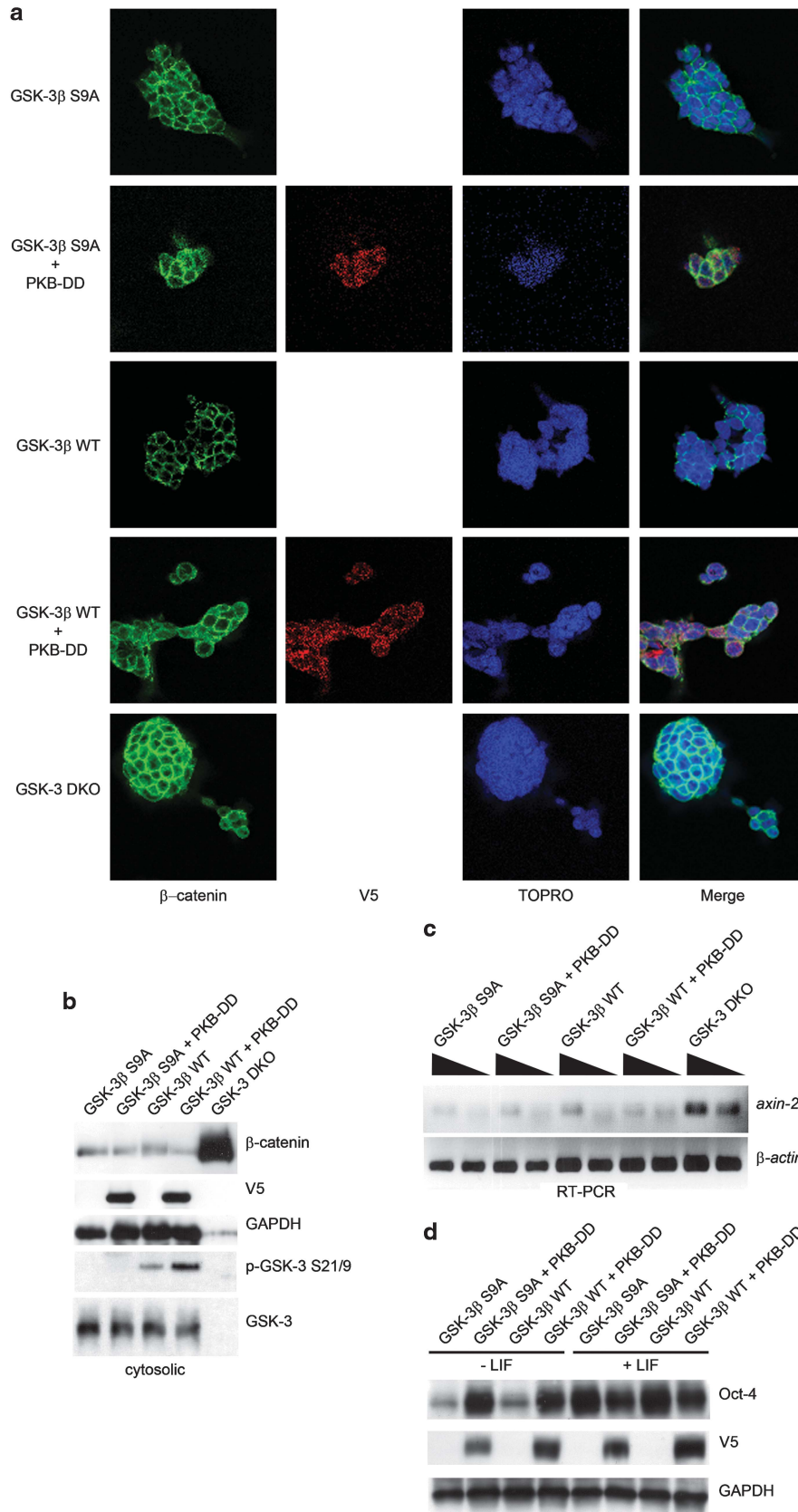
Does PI3K-dependent maintenance of pluripotency require stabilization of  $\beta$ -catenin?

GSK-3 has a key role in promoting the phosphorylation and destabilization of  $\beta$ -catenin in the Wnt pathway. Wnt leads to escape of  $\beta$ -catenin from GSK-3 phosphorylation and allows the protein to accumulate and induce transcription through interaction with TCF/LEF DNA-binding proteins (reviewed in Huang and He,<sup>20</sup> Wu *et al.*,<sup>21</sup> and MacDonald *et al.*<sup>22</sup>). Several studies have suggested that PI3K signaling may regulate  $\beta$ -catenin through inactivation of GSK-3.<sup>23,24</sup> We therefore investigated the phosphorylation state of GSK-3 and expression levels of  $\beta$ -catenin in myr-PDK-1 and PKB-DD cells. Even though GSK-3 $\alpha$  and -3 $\beta$  phosphorylation at Ser 21 and 9, respectively, were increased as expected in these transgenic ES cell lines, we observed no apparent difference in localization (Figure 5a) or in cytosolic protein accumulation of  $\beta$ -catenin (Figure 5b). To further test for PI3K pathway regulation of  $\beta$ -catenin, we used a GSK-3 $\beta$  mutant S9A cell line, which expresses

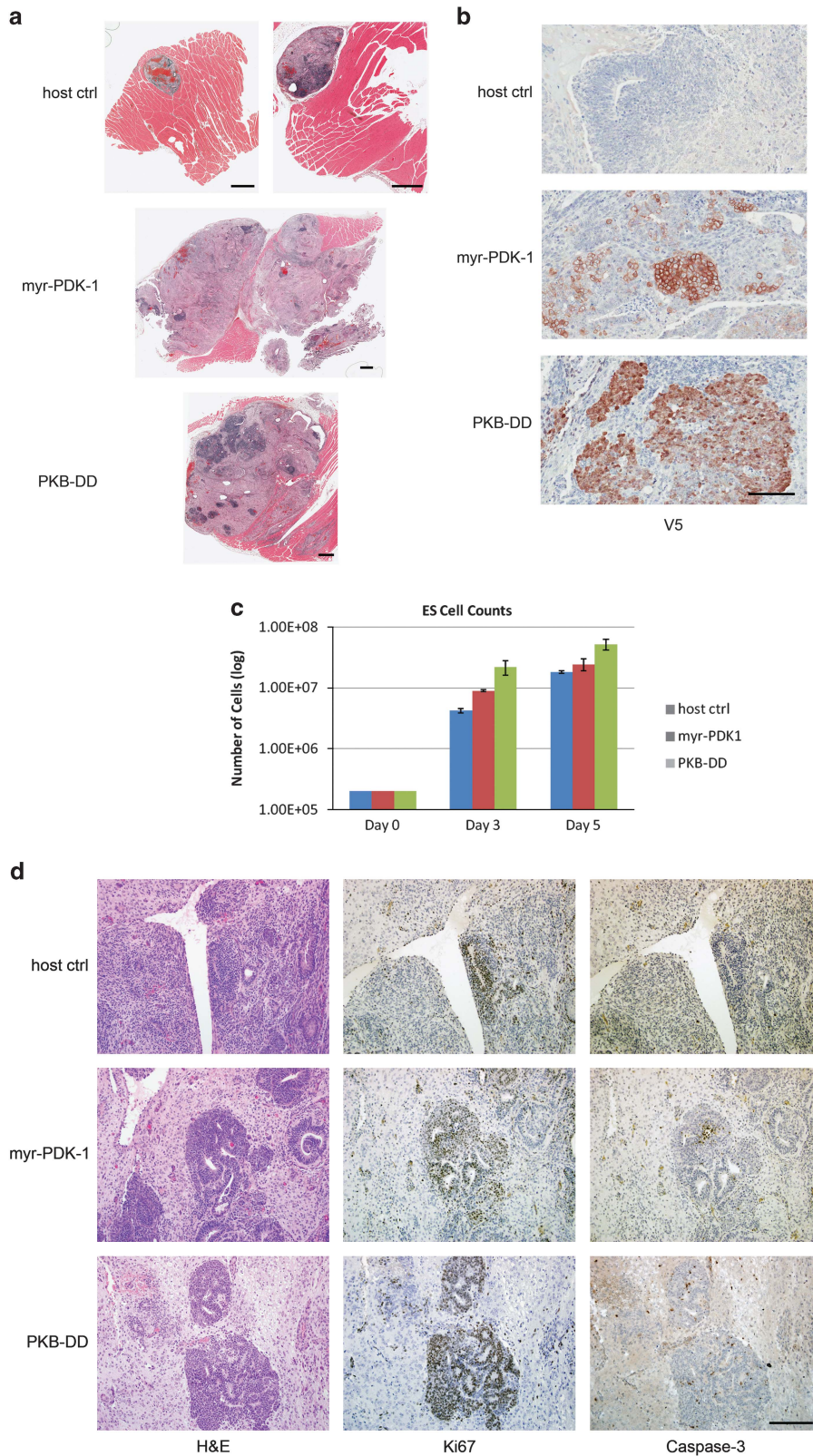
only a mutant of GSK-3 $\beta$  that is unresponsive to PI3K signaling due to mutation of the PKB/Akt phosphorylation site, on a GSK-3 knockout background. These cells were transiently transfected with PKB-DD-V5 and assessed for  $\beta$ -catenin accumulation. We observed no difference in  $\beta$ -catenin localization (Figure 6a) or cytosolic protein levels (Figure 6b) between GSK-3 $\beta$  S9A and GSK-3 $\beta$  WT cells or upon expression of PKB-DD-V5. *Axin-2* transcript levels were similarly unaffected (Figure 6c). Based on these results, we conclude that in our myr-PDK-1 and PKB-DD ES cells, activation of the PI3K pathway does not affect canonical Wnt signaling. We further examined Oct-4 protein expression levels in whole-cell lysates of GSK-3 S9A and GSK-3 $\beta$ -WT cells transiently transfected with myr-PDK-1-V5. Again, GSK-3 does not appear to affect the effects of myr-PDK-1 on Oct-4 expression levels (Figure 6d). Taken together, we conclude that the maintenance of pluripotency by PI3K signaling is independent of  $\beta$ -catenin in our system.

Differentiation and proliferation capacity of transgenic ES cells in teratomas

To further assess the differentiation capacity and proliferation potential of myr-PDK-1 and PKB-DD ES cells, we performed teratoma assays. Equal numbers of host control, myr-PDK1 or PKB-DD ES cells were injected subcutaneously into the hindlimbs of SCID-beige mice and allowed to develop for 3 weeks. Mice

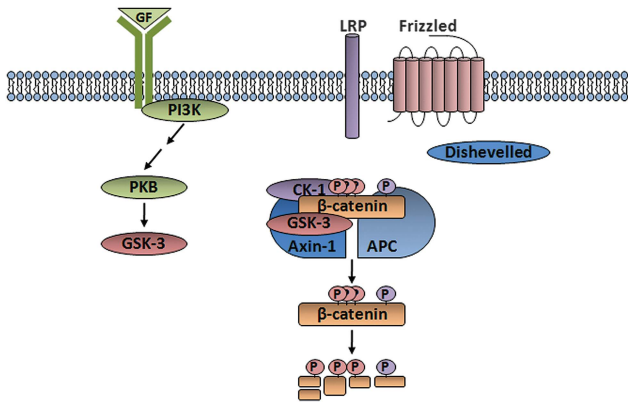


**Figure 6.** Examination of cross talk between PI3K and Wnt signaling via GSK-3. **(a)** Analysis of PKB-mediated regulation of  $\beta$ -catenin expression via GSK-3 by immunofluorescent staining following transient transfection with PKB-DD-V5. **(b)** Western blot analysis of cytosolic cell lysates after transient transfection with PKB-DD-V5. **(c)** Semi-quantitative reverse transcription-PCR for axin-2 (1  $\times$  and 10  $\times$  template dilutions) after transient transfection with PKB-DD. **(d)** Western blot analysis of whole-cell lysates of GSK-3 $\beta$  S9A and GSK-3 $\beta$  WT cells transiently transfected with myr-PDK-1-V5. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



**Figure 7.** Immunohistology of 3-week-old teratomas. **(a)** Teratomas are stained with hematoxylin and eosin stain. Teratomas appear purple and the surrounding muscle fibers pink. Host control (Ctrl) teratomas are small and remain encapsulated, whereas myr-PDK1 teratomas grow into the surrounding muscle tissue. Whole slide scanning, scale bar = 500  $\mu$ m. **(b)** Teratomas were stained for V5 (brownish red) to illustrate areas of transgene expression. myr-PDK1 and PKB-DD expression is patchy throughout the teratoma. Scale bar = 100  $\mu$ m. **(c)** An equal number of cells were plated and allowed to grow in standard tissue culture conditions. Cells were counted on days 3 and 5. **(d)** Immunohistological staining of Ki67 and cleaved Caspase-3 (brown) in teratomas. Scale bar = 100  $\mu$ m.





**Figure 8.** Separate PI3K and Wnt signaling pathways. GSK-3 is a key regulator in both the PI3K and Wnt signal transduction pathways. In the context of the myr-PDK-1 and PKB-DD transgenic ES cell lines, the PI3K signaling pathway does not cross talk with the canonical Wnt signaling pathway through GSK-3. APC, adenomatous polyposis coli; LRP, lipoprotein-related protein; GF, growth factor.

bearing myr-PDK1 and PKB-DD teratomas exhibited very large masses, in some cases encompassing almost the entire hindlimb and impeding mobility. In contrast, mice injected with host control cells appeared unaffected and moved around normally. Upon dissection, a substantial size difference between control and transgenic teratomas was noticeable (Figure 7a). In addition, transgenic teratomas appeared to grow into surrounding muscle tissue, whereas host control teratomas remained encapsulated (Figure 7a). Teratomas were stained for V5 to detect transgene expression. V5 staining was patchy in both myr-PDK1 and PKB-DD teratomas as the transgene did not appear to be expressed in every cell (Figure 7b). Unlike in cell culture where ES cells were maintained in the presence of hygromycin, the loss of transgene expression in the animal was likely due to teratoma growth in the absence of selection.

In tissue culture, myr-PDK1 cells required more frequent passaging than host control and R1 cell lines; PKB-DD cells required even more frequent passaging. When the same number of cells were initially plated and allowed to grow over a period of 5 days, myr-PDK1 cells grew at a faster rate than controls and PKB-DD cells grew most rapidly (Figure 7c). Furthermore, staining for proliferation and apoptosis markers, Ki67 and cleaved Caspase-3, respectively, revealed an increase in Ki67 and a reduction in Caspase-3 intensity in myr-PDK-1 and PKB-DD teratomas compared with host cell controls (Figure 7d). Collectively, these results support roles for the PI3K signaling in enhancing the proliferation and survival of ES cells.

## DISCUSSION

In this study, we generated stable isogenic myr-PDK-1 ES cell lines to examine the role that constitutively active mutants of the PI3K pathway (PDK-1 and PKB/Akt) have in ES cell self-renewal. Several groups have previously demonstrated roles for components of the PI3K signaling cascade in maintaining pluripotency, mostly based on loss-of-function studies.<sup>6,10,12,25</sup> Inhibition of PI3K signaling has additionally been shown to reduce the self-renewal capacity of induced pluripotent stem cells.<sup>26</sup> Consistent with stabilization of pluripotency, we demonstrate here that activated myr-PDK-1 ES cells retain AP activity in the absence of LIF, and EBs appear morphologically undifferentiated and display sustained Oct-4 staining compared with controls.

Although PDK-1 targets multiple downstream substrates, including PKB, S6K, RSK, SGK and atypical PKC isoforms,<sup>27</sup> the

effect of myr-PDK-1 on pluripotency appears to occur via its activation of PKB, as treatment of myr-PDK-1 ES cells and EBs with PKB inhibitors reversed the LIF-independent pluripotent phenotype. Similar to studies demonstrating a role for an activated myristoylated form of PKB in promoting LIF-independent self-renewal,<sup>10,19</sup> we also showed that activated PKB-DD ES cells and EBs exhibit LIF-independent pluripotency. To probe the molecular mechanism(s) by which myr-PDK-1 and PKB-DD may mediate LIF-independent pluripotency, roles for downstream targets of these protein kinases were examined, including mTOR complex-1 (mTORC-1) and GSK-3. Both myr-PDK-1 and PKB-DD ES cells were treated with rapamycin, an inhibitor of mTOR. PDK-1 and PKB activate mTORC-1 signaling to promote protein synthesis and cell growth.<sup>28</sup> Upon treatment with an inhibitor of mTOR, no change was observed in Oct-4 staining or pluripotency, indicating that another pathway downstream of PDK-1 and PKB is responsible for the maintenance of Oct-4 expression and pluripotency in the absence of LIF.

It is well established that PKB/Akt phosphorylates and inhibits GSK-3.<sup>29</sup> Furthermore, inhibition of GSK-3 has been linked to the maintenance of an undifferentiated pluripotent stem cell state.<sup>30,31</sup> Through its association with the destruction complex, GSK-3 targets β-catenin for degradation. The ability for PKB to stabilize β-catenin in the cytoplasm through its inhibition of GSK-3 has been tested by a number of groups.<sup>24,32–35</sup> Here, we investigated whether this mechanism has a role in the maintenance of a PKB-mediated LIF-independent pluripotent stem cell state. Consistent with several other studies,<sup>36–38</sup> neither myr-PDK-1 nor PKB-DD ES cells enhanced β-catenin levels, either in the presence of a wild-type or mutant version of GSK-3 that is unresponsive to PKB. Furthermore, no changes were observed in Oct-4 levels, suggesting that the maintenance of PKB-mediated LIF-independent pluripotency is also GSK-3 independent. This result supports the hypothesis that PI3K and Wnt signaling pathways are insulated and do not cross talk—at least in ES cells (Figure 8).

In mediating ES cell pluripotency, PI3K signaling has previously been implicated downstream of both cytokines and growth factors.<sup>12,19</sup> For example, both LIF and insulin-like growth factor have been shown to activate PKB in ES cells.<sup>13,19</sup> More specifically, LIF-mediated PKB activation positively regulates Tbx-3 and subsequently, Nanog and Oct-4 expression.<sup>13,19</sup> It has been suggested that Jak-STAT and PI3K-PKB signal in parallel pathways downstream of LIF to maintain pluripotency.<sup>19</sup> Recently, Oct-4 has been shown to be phosphorylated by PKB in embryonal carcinoma cells, an event that was associated with increased survival and maintenance of expression of nanog.<sup>39</sup> The ability to generate stable isogenic ES cell lines targeting additional components within the PI3K and other pathways should allow further dissection of the functional hierarchy of signaling proteins that control ES cell pluripotency and provide further insight into appropriate targets for drug-mediated modulation of ES self-renewal.

## MATERIALS AND METHODS

### Cell culture

ES cells were maintained on a mouse embryonic fibroblast (MEF) feeder layer in ES cell culture medium (ES-Dulbecco's modified Eagle's medium) consisting of high glucose Dulbecco's modified Eagle's medium (Invitrogen, Burlington, ON, Canada) supplemented with 15% ES cell-qualified fetal bovine serum (Hyclone, Thermo Fisher Scientific, Ottawa, ON, Canada), 50 U penicillin/streptomycin (Invitrogen), 2 mM GlutaMAX (Invitrogen), 0.1 mM MEM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, Oakville, ON, Canada) and 1000 U/ml LIF (Millipore, Billerica, MA, USA) at 37 °C, 5% CO<sub>2</sub>. Before experiments, feeders were removed and ES cells were grown on plates coated with 0.1% gelatin (Sigma). For treatments, ES cells were grown in 1 μM PKBi (Calbiochem, Billerica, MA, USA) or 100 ng/ml rapamycin (Calbiochem).

### Generation of GSK-3 $\beta$ <sup>+/-</sup> mouse ES cells as host cell line

R1 ES cells, first established by Nagy *et al.*,<sup>40</sup> were provided by the stem cell core facility and used to generate the GSK-3 $\beta$ <sup>+/-</sup> host cell line. The targeting vector, pTVB\_deltaX2\_NR\_FRT/Pur\_AgelBclI (a gift of B. Doble) was linearized with the *NdeI* restriction enzyme (NEB, Whitby, ON, Canada), purified using Qiaxll beads (Qiagen, Mississauga, ON, Canada), and electroporated into approximately  $1 \times 10^6$  R1 ES cells using a BIO-RAD Gene Pulser (Bio-Rad, Mississauga, ON, Canada) with a single pulse of 250 V at 500  $\mu$ F. After 48 h, cells were treated with 1.2  $\mu$ g/ml puromycin (Invitrogen). Colonies were picked and split into two replica flat-bottom 96-well plates. One was used for screening by PCR and the other was allowed to propagate. Approximately 500 colonies were picked and screened for homologous recombination by pooled-PCR using primers that flank the short arm of homology. The forward primer (5'-CACAAATGCGACTGACCACTTCCCTTC-3') is found in pTVB\_deltaX2\_NR\_FRT/Pur\_AgelBclI upstream of the short arm and the reverse primer (5'-CTTAACCTACCGTGGATGTGGAATGTG-3') is in the endogenous GSK-3 $\beta$  locus. Four colonies were found to be positive for homologous recombination by pooled-PCR. These were subsequently confirmed by single PCRs for the short arm, the puromycin gene and the FRT-puro cassette, which replaces exon 2. The primer sequences used for puromycin were forward: 5'-ATCGAGCGGGTCACCGAGCT-3' and reverse: 5'-TTGC GGGTCATGCACCAAGT-3' and those used to flank GSK-3 $\beta$  exon 2 were forward: 5'-GGGGCAACCTTAATTCATT-3' and reverse: 5'-TCTGGCTA TAGTATCTAGTAACG-3'.

### Cloning of Flp-In constructs

In order to clone the genes of interest into the Flp-In vector, pEF5/FRT/V5-D-TOPO (Invitrogen), the forward cloning primer consisted of the TOPO recognition sequence (CACC) followed by a full Kozak sequence and the first 20 to 29 nucleotides of the gene of interest. The design of the reverse primer involved removing the stop codon of the gene so that a fusion could be formed in frame with the V5 epitope.

### Generation of stable Flp-In ES cell lines

Lipofectamine 2000 (Invitrogen) was used to co-transfect 0.2  $\mu$ g of pEF\_myrc-myc-his-PDK-1\_FRT\_V5 or pEF\_HA-PKB-DD\_FRT\_V5 and 1.8  $\mu$ g of the pOG44 construct (Invitrogen), which expresses Flp recombinase, into GSK-3 $\beta$  host cells. Cells were placed in a six-well plate (Corning, Tewksbury, MA, USA) and selected with 250  $\mu$ g/ml hygromycin (InvivoGen, San Diego, CA, USA) after 48 h. Colonies were picked and expanded from 96-well plates to replica 24-well plates, where 1.2  $\mu$ g/ml puromycin selection medium was used in place of hygromycin for one of the replica plates. Proper integration of the transgene into the knockout GSK-3 $\beta$  locus will result in colonies that are hygromycin resistant and puromycin sensitive. Colonies were further expanded into 6 cm dishes and lysed with RIPA buffer for western blot analysis of V5 expression.

### GSK-3 knockout and knock-in ES cell lines

GSK-3 DKO, GSK-3 $\beta$  S9A and GSK-3 $\beta$  WT ES cell lines were previously generated and described by Doble *et al.*<sup>11</sup> Briefly, GSK-3 DKO ES cells lack both GSK-3 $\alpha$  and -3 $\beta$  alleles. GSK-3 $\beta$  S9A and GSK-3 $\beta$  WT ES cells were generated by knocking the S9A or WT form of GSK-3 $\beta$  back into GSK-3 DKO ES cells.

### Cell counting

A total of 200 000 cells were plated in triplicate for each time point and grown under standard ES cell tissue culture conditions. On days 3 and 5, adherent and floating cells from each plate were collected and counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Mississauga, ON, Canada).

### Cytosolic lysate preparation and western blot analysis

Cells were rinsed twice with phosphate-buffered saline (PBS) and scraped into 500  $\mu$ l of cold hypotonic lysis buffer: 50 mM Tris pH 7.4, 1 mM EDTA, complete protease inhibitor tablet (Roche, Mississauga, ON, Canada), phosphatase inhibitor cocktail (Sigma), 1 mM sodium orthovanadate, 10 mM NaF and 10 mM  $\beta$ -glycerophosphate. Cells were incubated on ice for 15–20 min, and then pelleted by centrifuging in an ultra-centrifuge at 80 000 r.p.m. for 30 min at 4 °C. The supernatant containing the cytosolic fraction was transferred into a new Eppendorf tube, and protein levels

were quantified using the BCA Protein Assay (Pierce, Thermo Fisher Scientific, Ottawa, ON, Canada).

Protein lysates containing sample buffer and TECEP (Pierce) were boiled for 10 min and loaded onto an 8% SDS-PAGE gel, then transferred onto polyvinylidene difluoride membrane (Millipore) after electrophoresis via semi-dry transfer. After primary antibody incubation, the membrane was washed with TBST and incubated with either goat-anti-mouse or goat-anti-rabbit (Bio-Rad) secondary antibodies. The membrane was washed again and then exposed to film (Kodak) using ECL reagent (Molecular Probes, Burlington, ON, Canada).

### AP activity

A single-cell suspension of 1000 cells/cm<sup>2</sup> were seeded onto a 0.1% gelatin-coated well of a six-well plate (Corning) and grown in monolayer in differentiation medium. On day 5, cells were washed in PBS, fixed in 2% paraformaldehyde/PBS for 30 min at room temperature, washed again in PBS, and stained in the dark using the Vector Blue Phosphatase Substrate Kit III (Vector Laboratories, Burlington, ON, Canada, SK-5300) according to manufacturer's instructions.

### EB assay

Differentiation medium used to maintain EBs were the same as ES-Dulbecco's modified Eagle's medium but with 5% fetal bovine serum and no LIF supplementation. EBs were generated using the hanging drop method. Thirty-microliter drops containing 800 cells/drop were plated onto the lid of a 10-cm<sup>2</sup> tissue culture dish (Corning) containing 5 ml of PBS to prevent the drops from drying out. EBs were maintained as hanging drops in a tissue culture incubator at 37 °C and 5% CO<sub>2</sub> for 3 days and then transferred to ultra-low-binding 96-well plates (Costar, Tewksbury, MA, USA); differentiation medium was changed every other day.

### Immunofluorescent staining and microscopy

Whole EBs were washed in PBS, fixed with ice-cold 4% paraformaldehyde/PBS overnight at 4 °C, permeabilized twice with 0.5% Triton X-100/PBS (PBT) for 15 min at room temperature, blocked in 2% bovine serum albumin/PBT for 1 h, and incubated with primary antibody overnight at 4 °C. EBs were then washed three times in PBS before adding secondary antibody and TOPRO-3 iodide (Invitrogen) and incubating overnight at 4 °C. Primary and secondary antibodies were diluted to final working concentrations in 2% bovine serum albumin/PBT. EBs were mounted with ProLong gold anti-fade reagent (Invitrogen) after being gently washed five times in PBS. Images were obtained with a Zeiss LSM510 confocal microscope and associated software (Toronto, ON, Canada).

### Teratoma assay

Cells were grown for two passages on 0.1% gelatin-coated dishes to eliminate feeder fibroblasts, trypsinized into single-cell suspensions, and resuspended in PBS to a density of  $10^7$  cells/ml. Using a 25-G needle, these cells (200  $\mu$ l) were injected subcutaneously into the hindlimbs of SCID-beige mice (C.B-*Igh-1*<sup>b</sup>GbmsTac-*Prkd*<sup>scid</sup>-*Lyst*<sup>tgN7</sup>; Jackson Laboratory, Bar Harbor, ME, USA). Teratomas were collected after 3 weeks. The tissues were fixed with 10% neutral-buffered formalin overnight at room temperature then stored in 70% ethanol for subsequent paraffin embedding, sectioning and immunohistological staining.

### Hematoxylin and eosin stain and immunohistological staining and microscopy

Sections from paraffin-embedded teratomas were dewaxed and rehydrated using standard procedures. For hematoxylin and eosin stain staining, sections were treated with iodine for 5 min, washed, treated with 3% sodium thiosulphate for 1 min, and washed under running tap water. Subsequently, slides were stained in Harris' Hematoxylin for 5 min, washed and rinsed in 95% ethanol before staining with eosin for 1 min. Slides were then thoroughly washed in running tap water, dehydrated in a series of alcohol rinses, and cleared in three changes of xylene. For immunohistological staining, antigen retrieval was performed using 10 mM sodium citrate buffer for 20 min at 98 °C. Antigen detection and 3,3'-diaminobenzidine (DAB) color development were performed using kits according to the manufacturers' recommendations (Vector Laboratories and DAKO (Burlington, ON, Canada) respectively). Images were obtained with an Olympus microscope.

## Antibodies

The following primary antibodies were used for western blot analysis, immunofluorescent and/or immunohistological staining: mouse anti-V5 (Invitrogen, R960-25), mouse  $\beta$ -actin (Abcam, ab6276), mouse anti-GAPDH (Abcam, ab8245), mouse anti- $\beta$ -catenin (BD Biosciences, 610154), rabbit anti- $\beta$ -catenin (Cell Signaling Technology, Whitby, ON, Canada, CST 9562), rabbit anti-Oct-4 (Santa Cruz, sc-9081), rabbit anti-PDK-1 (CST 3062, 5662), rabbit anti-PKB (CST 9272), rabbit anti-PKB-S473 (CST 9271, 4060), rabbit anti-PKB-T308 (CST 9275), rabbit anti-p70S6K-T389 (CST 9206), rabbit anti-p70S6K-T371 (CST 9208), rabbit anti-p44/42MAPK (CST 4695), rabbit anti-p44/42MAPK-T202/Y204 (CST 9101), mouse anti-STAT-3 (CST 9139), rabbit anti-STAT-3-Y705 (CST 9145), mouse anti-GSK-3 $\alpha/\beta$  (Millipore 4G1E), rabbit anti-Ki67 (Thermo Scientific, SP6), and rabbit anti-cleaved Caspase-3 (Cell Signaling Technology, 9661).

For western blot analysis, horseradish peroxidase-conjugated secondary antibodies were purchased from Bio-Rad: goat anti-mouse (170–6516) and goat anti-rabbit (170–6515). For immunofluorescent staining, fluorochrome-conjugated secondary antibodies were purchased from Invitrogen: goat anti-mouse Alexa Fluor 488 (A11029) and goat anti-rabbit Alexa Fluor 468 (A11011).

## Reverse transcription-PCR

RNA was extracted from cells using TRIzol Reagent (Invitrogen) followed by cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. The template for each PCR reaction was the cDNA obtained from 50 ng total RNA, and the reactions were incubated in a BIO-RAD DNA Engine Dyad Peltier Thermal Cycler at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 1 min, followed by a final extension at 68 °C for 7 min. Primer sequences were forward: 5'-AAGCCTGGCTCCAGAAGATCACAA-3' and reverse: 5'-TTTGAGCCTTCA GCATCCTCTGT-3' for Axin2<sup>11</sup> and forward: 5'-TAGG CACCAGGTGTGATGG-3' and reverse: 5'-CATGCTGGGGTGTGAAGG-3' for  $\beta$ -actin.<sup>13</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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