Controlling embryonic stem cell proliferation and pluripotency: the role of PI3K- and GSK-3-dependent signalling

Melanie J. Welham[1], Emmajayne Kingham[2], Yolanda Sanchez-Ripoll, Benjamin Kumpfmueller, Michael Storm and Heather Bone

Centre for Regenerative Medicine, Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

Abstract

ESCs (embryonic stem cells) are derived from the inner cell mass of pre-implantation embryos and are pluripotent, meaning they can differentiate into all of the cells that make up the adult organism. This property of pluripotency makes ESCs attractive as a model system for studying early development and for the generation of specific cell types for use in regenerative medicine and drug screening. In order to harness their potential, the molecular mechanisms regulating ESC pluripotency, proliferation and differentiation (i.e. cell fate) need to be understood so that pluripotency can be maintained during expansion, while differentiation to specific lineages can be induced accurately when required. The present review focuses on the potential roles that PI3K (phosphoinositide 3-kinase) and GSK-3 (glycogen synthase kinase 3)-dependent signalling play in the co-ordination and integration of mouse ESC pluripotency and proliferation and contrast this with our understanding of their functions in human ESCs.

Control of ESC (embryonic stem cell) fate: an overview

ESCs are derived from early pre-implantation embryos and, when cultured appropriately, can be maintained in a proliferative, self-renewing and pluripotent state almost indefinitely. Pluripotency is the ability to differentiate into all of the cells found in an adult organism, while self-renewal describes the generation of a daughter stem cell from its mother. In the case of ESCs, self-renewal occurs symmetrically, such that when an undifferentiated ESC divides and pluripotency is maintained, both its progeny will be undifferentiated [1]. Thus, although not commonly described as such, self-renewal of ESCs requires simultaneous control of the cell cycle (proliferation) and maintenance of pluripotency. It is important to emphasize that self-renewal is not the same as proliferation [2,3], because the latter can occur when pluripotency is not maintained.

Over the last 5–10 years, our understanding of the molecular components involved in maintaining pluripotency of mESCs (mouse ESCs) has increased dramatically, from a simple 'prelude' where STAT3 (signal transducer and activator of transcription 3) activation by LIF (leukaemia inhibitory factor) was all that seemed necessary, to a complex 'symphony' where extrinsic factors, intracellular signals, transcription factors, epigenetic regulators and miRNAs (microRNAs) have all been implicated [4]. Three transcription factors, Oct4, Sox2 and Nanog, are considered to be the 'master' regulators of ESC pluripotency and were initially proposed to form a self-sustaining gene-regulatory network (reviewed in [5]). However, revision of this model is now necessary to accommodate recent findings, namely that ESC populations are not homogeneous [6,7] and, indeed, within populations of ESCs, expression of Nanog [8], Esrrb (oestrogen-related receptor β) [9] and Rex1 [10] have all been shown to be both heterogeneous and dynamic. Despite our progress in understanding the factors that influence pluripotency, there remains a significant gap in our knowledge regarding the molecular mechanisms responsible for the co-ordination and integration of ESC proliferation and pluripotency [2,3].

The ESC cell cycle

mESCs proliferate rapidly in culture and display unique cell-cycle kinetics, distinct from those of somatic cells, dividing approximately every 11–16 h and exhibiting a shortened G_1-phase [11,12]. Throughout the mESC cell cycle, it has been reported that cyclin levels remain relatively stable [12], cyclin A/E–CDK2 (cyclin-dependent kinase 2) complexes are active and the retinoblastoma protein remains hyperphosphorylated [11,12]. In addition, cell-cycle progression appears to be insensitive to inhibition by $p16^{INK4a}$ [13] and neither do mESCs arrest following DNA damage

Key words: cell cycle, embryonic stem cell, glycogen synthase kinase 3 (GSK-3), phosphoinositide 3-kinase (PI3K), pluripotency, proliferation, self-renewal.

Abbreviations used: CDK, cyclin-dependent kinase; ESC, embryonic stem cell; Esrrb, oestrogen-related receptor β; GSK-3, glycogen synthase kinase-3; hESC, human ESC; LIF, leukaemia inhibitory factor; MEK, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase; mESC, mouse ESC; miRNA, microRNA; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; siRNA, short interfering RNA.

[1]**To whom correspondence should be addressed (email prsmjw@bath.ac.uk).**

[2] Present address: Bone and Joint Research Group, Centre for Human Development, Stem Cells and Regeneration, Developmental Origins of Health and Disease, Institute of Developmental Sciences, University of Southampton, Southampton SO16 6YD, U.K.

[14]. In contrast, it has been reported that levels of cyclins D1, D2 and D3 do vary throughout the cell cycle of hESCs (human ESCs), and knockdown of CDK2 leads to G_1-phase arrest and differentiation to extra-embryonic lineages [15]. Recently, in hESCs, pluripotency regulators have been linked directly with control of the cell cycle. It has been demonstrated that *miR-302* miRNAs are regulated by Oct3/4 and Sox2, that *miR-302a* targets cell-cycle regulators and that inhibition of *miR-302a* results in increased cyclin D1 levels and increased numbers of hESCs in G_1-phase [16]. In mESCs, it has been reported that the *miR-290* cluster of miRNAs regulate G_1/S-phase transition by controlling levels of $p21^{Cip}$ and $p27^{Kip1}$ and are important for promoting rapid proliferation [17]. These results are somewhat at odds with those of earlier studies that had suggested little role for these CDK inhibitors in the control of the mESC cell cycle. Thus, whereas control of the cell cycle may vary between mESCs and hESCs, a number of important questions relating to this regulation remain unanswered.

PI3K (phosphoinositide 3-kinase) signalling and ESC biology

PI3Ks are a family of lipid kinases comprising three subclasses [18,19]. Class IA PI3Ks are heterodimers comprising a regulatory subunit (three genes and five isoforms: p85α, p55α, p50α, p85β and p55γ) and a catalytic subunit (three genes and three isoforms: p110α, p110β and p110δ). The primary product of class I PI3K activity is the phosphoinositide PtdIns(3,4,5)P_3, which recruits pleckstrin homology domain-containing proteins to the plasma membrane, facilitating activation of a range of downstream signalling cascades [20]. Functionally, PI3Ks have been implicated in a wide array of physiological processes, including proliferation, development, growth and migration [20]. Of particular interest in relation to ESCs is the report that ablation of p110β leads to lethality at the pre-implantation stage, suggesting a key role for PI3Ks during early mouse embryogenesis [21]. Studies conducted over the last few years have suggested roles for PI3Ks in both control of the ESC cell cycle and proliferation, and also in maintenance of pluripotency.

Involvement of PI3Ks in the control of ESC proliferation

The first suggestion that PI3Ks were involved in the control of ESC proliferation came from the observation that mESCs lacking the PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour-suppressor gene (a negative regulator of PI3K signalling) exhibited enhanced proliferation and diminished dependence on serum [22]. Subsequently, incubation of mESCs with high doses of the broad-selectivity PI3K inhibitor LY294002 decreased proliferation and led to cells accumulating in G_1-phase [23]. ERas (ESC-expressed Ras), a constitutively active member of the Ras family of small GTPases, is expressed specifically in mESCs and its deletion also leads to decreased proliferation [24]. Interestingly, this proliferative defect could be rescued by overexpression of a membrane-bound form of the catalytic PI3K subunit p110α, providing a further link between PI3Ks and mESC proliferation [24]. Additional evidence to support a role for PI3K signalling in ESC proliferation comes from the finding that deletion of mTOR (mammalian target of rapamycin), a downstream target of the PI3K signalling pathway, leads to an early embryonic lethality in mice and an inability of mTOR-null mESCs to proliferate; similar results are observed upon inhibition of mTOR with rapamycin [25]. Despite these reports, the molecular mechanisms that underpin the involvement of PI3Ks in regulation of ESC proliferation are unresolved. One possibility is that PI3K-dependent signals contribute to promoting the constitutive activity of cyclin E–CDK2 complexes in mESCs. In somatic cells, PI3K signalling inhibits the activity of the transcription factor FOXO3 (forkhead box O3). In ESCs, this could limit expression of $p27^{Kip1}$, meaning that cyclin E–CDK2 activity would be largely unchecked. In addition, PI3K-mediated inhibition of GSK-3 (glycogen synthase kinase 3) activity would prevent the phosphorylation-dependent ubiquitination and degradation of cyclin E, which again could contribute to the maintenance of high constitutive levels of cyclin E–CDK2 complexes that play a role in driving ESC proliferation.

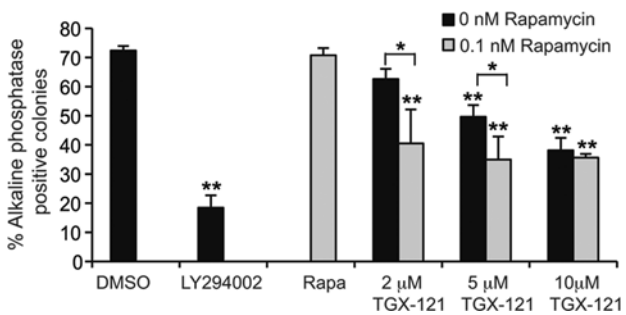
PI3Ks and regulation of mESC fate

Our work, alongside that of others, has also implicated PI3K-dependent signalling in the control of mESC self-renewal. We were the first to suggest a role for PI3K signalling in maintenance of mESC pluripotency, demonstrating that doses of LY294002 close to its IC_{50} value of 5 μM reduced the ability of mESCs to retain pluripotency [26]. Other groups have demonstrated that an activated form of Akt, one of the key downstream targets of PI3K signalling, promotes self-renewal independently of LIF [27]. Akt was also identified in an RNAi (RNA interference) screen as a regulator of mESC pluripotency [28]. Relatively few studies have examined the role of PI3Ks in the regulation of hESC fate. PI3K signalling has been implicated in neurotrophin-mediated survival of hESCs [29] as well as maintenance of pluripotency [30], although these studies were limited to the use of LY294002.

We have identified genes regulated by PI3K-dependent signalling in mESCs, among which are a number of known regulators of pluripotency including Nanog, Klf4, Tbx3, Tcl1 and Esrrb [31,32]. It has subsequently been suggested that PI3K signalling controls Nanog expression via effects on Tbx3 [33], results entirely consistent with our own observations. Our transcriptome analyses also revealed potential novel regulators of pluripotency, including the tyrosine phosphatase Shp1 (Src homology 2 domain-containing protein tyrosine phosphatase 1) and members of Zscan4 family of zinc-finger proteins [32]. We demonstrated that Zscan4c plays a role in maintaining ESC pluripotency [32], whereas, more recently, it has been reported that the Zscan4 family plays a key role in telomere elongation and genome stability in mESCs [34]. These two findings are not

Figure 1 | Partial inhibition of mTOR potentiates the loss in self-renewal observed upon inhibition of p110β

mESCs were incubated with vehicle (DMSO), LY294002 or the p110β-selective inhibitor TGX-121 at the concentrations indicated in the presence or absence of 0.1 nM rapamycin (Rapa). After 4 days, colonies were fixed and stained for alkaline phosphatase activity. Results are mean+S.E.M. percentages of alkaline phosphatase-positive colonies ($n = 3$). Statistical analysis was conducted using ANOVA and Fisher's post-hoc test to compare each treatment; $^{*}P<0.05$, $^{**}P<0.01$. The number of colonies formed in each condition was not significantly different.



incompatible, as failure to maintain a stable genome would probably result either in differentiation or apoptosis.

Given the fact that PI3Ks have been implicated in regulation of both ESC proliferation and pluripotency, we hypothesized that particular isoforms of class IA PI3Ks may couple to distinct functional responses in mESCs. siRNA (short interfering RNA)-mediated knockdown or small-molecule-based inhibition of the p110β PI3K isoform (with TGX-121 or -221) led to a decrease in mESC self-renewal, suggesting that p110β couples primarily to maintenance of pluripotency. In contrast, siRNA-induced knockdown or inhibition of the p110α catalytic isoform (with PIK-75 or -15e) reduced proliferation, without significantly affecting pluripotency [35]. Interestingly, we observed that simultaneous inhibition of p110α and p110β reduced self-renewal further, leading us to propose that there may be cross-talk between PI3K isoforms in mESCs [35]. These observations led us to test whether inhibition of other signalling molecules, also implicated in control of the proliferation of mESCs, could also potentiate the effects p110β inhibition. We found that suboptimal doses of rapamycin, used in conjunction with TGX-121, resulted in a greater loss of self-renewal than inhibition of p110β alone (Figure 1). On the basis of our observations, it is tempting to propose that PI3K signalling plays a role in integrating the signals that maintain pluripotency and those that regulate mESC proliferation (for a model, see Figure 2). Clearly, further studies will be required to rigorously test this hypothesis.

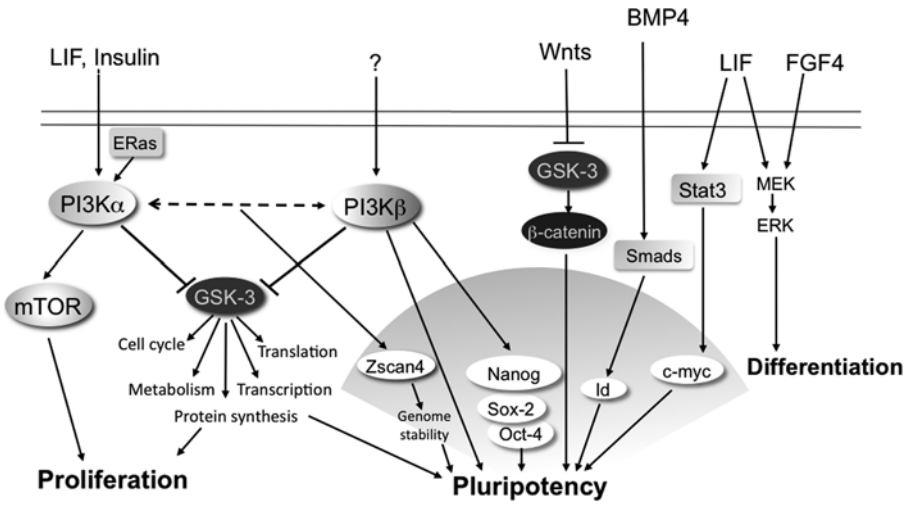
GSK-3 and regulation of ESC fate

GSK-3 is involved in a wide range of physiological processes, including development, proliferation and metabolism [36]. In mice and humans, two isoforms of GSK-3 exist (α and β), which, although highly homologous, play non-redundant roles [37]. Initial reports relating to the role of GSK-3 in ESCs were conflicting. Ding et al. [38] reported that a kinase inhibitor, termed TWS119, enhanced neuronal differentiation of mESCs and mouse embryonal carcinoma cells, the primary target of which was reported to be GSK-3β. In contrast, Sato et al. [39], using a chemically distinct GSK-3 inhibitor termed BIO, reported that treatment of mouse or hESCs with BIO enhanced self-renewal and pluripotency [39]. This latter report proposed that BIO was acting as a Wnt mimetic, via inhibition of GSK-3 and led to the suggestion that Wnt signalling played a role in maintenance of ESC pluripotency, a proposal generating much discussion and still not completely resolved [40]. One aspect not considered by Sato et al. was the fact that GSK-3 can be regulated by many different signalling pathways [36]. In particular, in response to growth factor stimulation, PI3K signalling inactivates GSK-3 via Akt-mediated phosphorylation of Ser^{9}/Ser^{20} [36]. We sought to clarify the role of GSK-3 in ESC self-renewal and used a chemical genetic approach to achieve this [41]. Our results demonstrated that, in the presence of LIF and serum, inhibition of GSK-3 activity (with a series of bisindolylmaleimides) enhanced mESC self-renewal and, in comparison with BIO and TWS119, our GSK-3 inhibitors showed greater selectivity [41]. Interestingly, deletion of all four GSK-3 alleles from mESCs [42] phenocopied the effect of our GSK-3 inhibitors, providing clear evidence that inhibition of GSK-3 enhances mESC self-renewal. While our studies were nearing completion, Austin Smith's team from Cambridge described the 'ground state' of mESC pluripotency [40,43]. Their studies demonstrated that pluripotency of mESCs could be maintained in the absence of exogenous growth factors using a minimal medium as long as inhibitors of MEK (mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase) and GSK-3 were present ('2i' conditions). Inhibition of MEK signalling blocks the differentiation-inducing effects of autonomously secreted FGF4 (fibroblast growth factor 4) [44,45], but cells cultured with MEK inhibitors alone grew poorly. Addition of GSK-3 inhibitors improved clonal propagation and cell growth, leading to the suggestion that GSK-3 inhibition was required under these very defined conditions to promote cell metabolism and proliferation [40,43], which in turn is consistent with a role for PI3K signalling in controlling mESC proliferation. In contrast, we had conducted our studies into GSK-3 action in ESCs in a complete medium containing serum, and, under these conditions, inhibition of GSK-3 does not consistently alter ESC proliferation (Y. Sanchez-Ripoll and M.J. Welham, unpublished work). Therefore it may be that the differential roles attributed to GSK-3 in defined compared with serum-containing medium reflect the involvement of GSK-3 in a wide array of cellular responses [36] (see Figure 2), and, depending on the cellular context, i.e. the composition of the extracellular environment, particular roles take on greater importance.

More recently we have exploited our newly developed selective GSK-3 inhibitors to re-evaluate the role of GSK-3

Figure 2 | Model showing the involvement of PI3K and GSK-3-dependent signals in control of mESC behaviour

The key extracellular, intracellular and nuclear factors that regulate mESC proliferation, pluripotency and promotion of differentiation are depicted. PI3K isoforms play a role in regulation of both proliferation and maintenance of pluripotency, whereas involvement of GSK-3 signalling in these processes may be dependent on the extracellular environment. See the text for discussion. BMP4, bone morphogenetic protein 4; ERK, extracellular-signal-regulated kinase; FGF4, fibroblast growth factor 4; Stat3, signal transducer and activator of transcription 3.



in hESCs. Despite the previous report that BIO can maintain hESCs in a pluripotent state [39], we have found that our GSK-3 inhibitors do just the opposite and induce differentiation towards mesendodermal lineages (H. Bone, D. Tosh and M.J. Welham, unpublished work). These observations reflect the findings that treatment of hESCs with Wnts also promotes differentiation [46–48]. Our recent data highlight further the different responses of mESCs and hESCs to the same factors. The notion that mESCs and hESCs are derived from different developmental stages of embryogenesis has been gaining acceptance within the stem cell community and the differential role of GSK-3 signalling that we have demonstrated adds to the growing list of differences documented between mESCs and hESCs.

Summary

It is clear from recent studies that great progress has been made in our understanding of the range of molecular signals regulating pluripotency, proliferation and self-renewal of ESCs. The challenge now is to decipher how these pathways are integrated such that maintenance of pluripotency and proliferation are co-ordinately regulated, a challenge that would surely benefit from a systems biology approach?

Funding

Work in the Welham group has been supported by funding from Biotechnology and Biological Sciences Research Council, European Union Framework Programme 6, Medical Research Council, Stem Cells for Safer Medicines and Wellcome Trust.

References

1 Smith, A.G. (2001) Embryo-derived stem cells: of mice and men. Annu. Rev. Cell Dev. Biol. **17**, 435–462
2 He, S.H., Nakada, D. and Morrison, S.J. (2009) Mechanisms of stem cell self-renewal. Annu. Rev. Cell Dev. Biol. **25**, 377–406
3 Orford, K.W. and Scadden, D.T. (2008) Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nat. Rev. Genet. **9**, 115–128
4 Boiani, M. and Schoeler, H.R. (2005) Regulatory networks in embryo-derived pluripotent stem cells. Nat. Rev. Mol. Cell Biol. **6**, 872–881
5 Niwa, H. (2007) How is pluripotency determined and maintained? Development **134**, 635–646
6 Silva, J. and Smith, A. (2008) Capturing pluripotency. Cell **132**, 532–536
7 Canham, M., Sharov, A., Ko, M. and Brickman, J.M. (2010) Functional heterogeneity of embryonic stem cells revealed through translational amplification of an early endodermal transcript. PLoS Biol. **8**, e1000379
8 Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L. and Smith, A. (2007) Nanog safeguards pluripotency and mediates germline development. Nature **450**, 1230–1238
9 Van Den Berg, D.L.C., Zhang, W., Yates, A., Engelen, E., Takacs, K., Bezstarosti, K., Demmers, J., Chambers, I. and Poot, R.A. (2008) Estrogen-related receptor β interacts with Oct4 to positively regulate Nanog gene expression. Mol. Biol. Cell **28**, 5986–5995
10 Toyooka, Y., Shimosato, D., Murakami, K., Takahashi, K. and Niwa, H. (2008) Identification and characterisation of subpopulations in undifferentiated ES cell culture. Development **135**, 909–918
11 Savatier, P., Huang, S., Szekely, L., Wiman, K.G. and Samarut, J. (1994) Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. Oncogene **9**, 809–818
12 Stead, E., White, J., Faast, R., Conn, S., Goldstone, S., Rathjen, J., Dhingra, U., Rathjen, P., Walker, D. and Dalton, S. (2002) Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. Oncogene **21**, 8320–8333
13 Savatier, P., Lapillonne, H., vanGrunsven, L.A., Rudkin, B. and Samarut, J. (1996) Withdrawal of differentiation inhibitory activity leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. Oncogene **12**, 309–322
14 Aladjem, M.I., Spike, B.T., Rodewald, L.W., Hope, T.J., Klemm, M., Jaenisch, R. and Wahl, G.M. (1998) ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. Curr. Biol. **8**, 145–155

15 Neganova, I, Zhang, X, Atkinson, S and M, L. (2009) Expression and functional analysis of G_1 to S regulatory components reveals an important role for CDK2 in cell cycle regulation in human embryonic stem cells. Oncogene **28**, 20–30
16 Card, D.A.G., Hebbar, P.B., Li, L., Trotter, K.W., Komatsu, Y., Mishina, Y. and Archer, T.K. (2008) Oct4/Sox2-regulated *miR-302* targets cyclin D1 in human embryonic stem cells. Mol. Biol. Cell **28**, 6426–6438
17 Wang, Y., Baskerville, S., Shenoy, A., Babiariz, J.E., Baehner, L. and Blelloch, R. (2008) Embryonic stem cell-specific microRNAs regulate the G_1–S transition and promote rapid proliferation. Nat. Genet. **40**, 1478–1483
18 Vanhaesebroeck, B., Ali, K., Bilancio, A., Geering, B. and Foukas, L.C. (2005) Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem. Sci. **30**, 194–204
19 Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M. and Bilanges, B. (2010) The emerging mechanisms of isoform-specific PI3K signalling. Nat. Rev. Mol. Cell Biol. **11**, 329–341
20 Cantley, L.C. (2002) The phosphoinositide 3-kinase pathway. Science **296**, 1655–1657
21 Bi, L., Okabe, I., Bernard, D.J. and Nussbaum, R.L. (2002) Early embryonic lethality in mice deficient in the p110β catalytic subunit of PI 3-kinase. Mamm. Genome **13**, 169–172
22 Sun, H., Lesche, R., Li, D.M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999) PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc. Natl. Acad. Sci. U.S.A. **96**, 6199–6204
23 Jirmanova, L., Afanassieff, M., Gobert-Gosse, S., Markossian, S. and Savatier, P. (2002) Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G_1/S transition in mouse embryonic stem cells. Oncogene **21**, 5515–5528
24 Takahashi, K., Mitsui, K. and Yamanaka, S. (2003) Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature **423**, 541–545
25 Murakami, M., Ichisaka, T., Maeda, M., Oshiro, N., Hara, K., Edenhofer, F., Kiyama, H., Yonezawa, K. and Yamanaka, S. (2004) mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol. Cell. Biol. **24**, 6710–6718
26 Paling, N.R., Wheadon, H., Bone, H.K. and Welham, M.J. (2004) Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. J. Biol. Chem. **279**, 48063–48070
27 Watanabe, S., Umehara, H., Murayama, K., Okabe, M., Kimura, T. and Nakano, T. (2006) Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene **25**, 2697–2707
28 Pritsker, M., Ford, N.R., Jenq, H.T. and Lemischka, I.R. (2006) Genomewide gain-of-function genetic screen identifies functionally active genes in mouse embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. **103**, 6946–6951
29 Pyle, A., Lock, L. and Donovan, P. (2006) Neurotrophins mediate human embryonic stem cell survival. Nat. Biotechnol. **24**, 344–350
30 Armstrong, L., Hughes, O., Yung, S., Hyslop, L., Stewart, R., Wappler, I., Peters, H., Walter, T., Stojkovic, P., Evans, J. et al. (2006) The role of PI3K/AKT, MAPK/ERK and NFκB signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. Hum. Mol. Genet. **15**, 1894–1913
31 Storm, M.P., Bone, H.K., Beck, C.G., Bourillot, P.Y., Schreiber, V., Damiano, T., Nelson, A., Savatier, P. and Welham, M.J. (2007) Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. J. Biol. Chem. **282**, 6265–6273
32 Storm, M.P., Kumpfmueller, B., Thompson, B., Kolde, R., Vilo, J., Hummel, O., Schulz, H. and Welham, M.J. (2009) Characterisation of the PI3K-dependent transcriptome in murine ES cells: identification of novel regulators of pluripotency. Stem Cells **27**, 764–775
33 Niwa, H., Ogawa, K., Shimosato, D. and Adachi, K. (2009) A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. Nature **460**, 118–122
34 Zalzman, M., Falco, G., Sharova, L.V., Nishiyama, A., Thomas, M., Lee, S.-L., Stagg, C.A., Hoang, G.H., Yang, H.-T., Indig, F.E. et al. (2010) Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature **464**, 858–863
35 Kingham, E. and Welham, M.J. (2009) Distinct roles for class IA PI3K catalytic subunit isoforms in the regulation of murine embryonic stem cell behaviour. J. Cell Sci. **122**, 2311–2321
36 Doble, B.W. and Woodgett, J.R. (2003) GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci. **116**, 1175–1186
37 Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.-S., Jin, O. and Woodgett, J.R. (2000) Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation. Nature **406**, 86–90
38 Ding, S., Wu, T.Y., Brinker, A., Peters, E.C., Hur, W., Gray, N.S. and Schultz, P.G. (2003) Synthetic small molecules that control stem cell fate. Proc. Natl. Acad. Sci. U.S.A. **100**, 7632–7637
39 Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. and Brivanlou, A.H. (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat. Med. **10**, 55–63
40 Wray, J., Kalkan, T. and Smith, A.G. (2010) The ground state of pluripotency. Biochem. Soc. Trans. **38**, 1027–1032
41 Bone, H.K., Damiano, T., Bartlett, S., Perry, A., Letchford, J., Sanchez Ripoll, Y., Nelson, A.S. and Welham, M.J. (2009) Involvement of glycogen synthase kinase-3 in regulation of murine embryonic stem cell self-renewal revealed by a series of bisindolylmaleimides. Chem. Biol. **16**, 15–27
42 Doble, B., Patel, S., Wood, G., Kockeritz, L. and Woodgett, J. (2007) Functional redundancy of GSK-3α and GSK-3β in Wnt/β-catenin signalling shown by using an allelic series of embryonic stem cell lines. Dev. Cell **12**, 957–971
43 Ying, Q.-L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and Smith, A. (2008) The ground state of embryonic stem cell self-renewal. Nature **453**, 519–523
44 Kunath, T., Saba-El-Leil, M.K., Almousailleakh, M., Wray, J., Meloche, S. and Smith, A. (2007) FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development **134**, 2895–2902
45 Stavridis, M., Lunn, J.S., Collins, B.J. and Storey, K.G. (2007) A discrete period of FGF-induced Erk1/2 signalling is required fro vertebrate neural specification. Development **134**, 2889–2894
46 D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K. and Baetge, E.E. (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat. Biotechnol. **24**, 1392–1401
47 Hay, D.C., Fletcher, J., Payne, C., Terrace, J.D., Gallagher, R.C., Snoeys, J., Black, J.R., Wojtacha, D., Samuel, K., Hannoun, Z. et al. (2008) Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. Proc. Natl. Acad. Sci. U.S.A. **105**, 12301–12306
48 Sumi, T., Tsuneyoshi, N., Nakatsuji, N. and Suemori, H. (2008) Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/β-catenin, Activin/Nodal and BMP signaling. Development **135**, 2969–2979

Received 22 December 2010
doi:10.1042/BST0390674