# Investigation of phosphoinositide 3-kinase dependent signalling in the regulation of embryonic stem cell fate

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*Award date:*
2009

*Awarding institution:*
University of Bath

# Investigation of phosphoinositide 3-kinase dependent signalling in the regulation of embryonic stem cell fate

Submitted by

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For the degree of Doctor of Philosophy (PhD)

University of Bath
Department of Pharmacy and Pharmacology

February 2009

# Acknowledgements

Throughout my PhD journey of scientific and personal discovery, I have had the pleasure of meeting and befriending a number of people at Bath University. Namely, Prof Melanie Welham, whose endless enthusiasm for research, kind support and advice is uplifting and motive. Working with Melanie has been both a pleasure and a valuable experience that I have enjoyed very much. Accordingly, I thank Melanie for this experience and both Melanie and members of her research group for all the good times and memories, scientific and extra-curricular, that I will take with me. In particular, I thank, Dr Heather Bone for imparting her knowledge and experience, instilling lab citizenship and our early morning chats, and Dr Mike Storm for his patient genetics guidance. I thank past PhD students of the research group Dr Lisa McRae and Dr Belinda Thompson for their support and friendship and wish the best of luck to current and future PhD students.

My parents, Alan and Joyce Kingham, have always supported me throughout my education. From the very start they have enabled me in any way they could to realise my vision and achieve my goals. The time during my PhD has been no exception to that dedication. I can not thank them enough for their sustained devotion, belief in me and love, all of which I am eternally grateful for.

Captain Gerald Johnston, my loving Fiancé, has shown persevering commitment to our relationship, even during my darkest PhD days and his infinite encouragement was felt from as far as Afghanistan. His lack of understanding of science but aspirations for our future, made time together the perfect escape from lab work, and allowed me to return to the lab with renewed enthusiasm.

Excerpt from ‘If’ by Rudyard Kipling

If you can dream - and not make dreams your master;
If you can think - and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;

# Abstract

In order to harness the therapeutic potential of stem cells, a clear understanding of the factors and mechanisms governing their fate is required. Self-renewal, pluripotency and proliferation are important cellular functions for maintaining the stem cell state. The class $I_A$ phosphoinositide 3-kinase (PI3K) family of lipid kinases regulate a variety of physiological responses including cell migration, proliferation and survival. Moreover, this class of PI3Ks were previously reported to play a role in proliferation and maintenance of self-renewal in murine embryonic stem cells (mESCs). Here the activation of PI3Ks, the intracellular signalling, including cross-talk between other important pathways, and the roles of specific catalytic subunits of the PI3K class I family have been investigated.

Despite inhibition of PI3Ks giving rise to differentiated cell types, early lineage commitment of mESCs was shown to be regulated by pathways not involving PI3Ks. Differentiation towards mesoderm, endoderm and ectoderm were detected upon broad selectivity inhibition of PI3Ks with LY294002. Cross-talk between PI3K and MAPK pathway signalling was highlighted as a possible mechanism for PI3Ks to regulate self-renewal. Inhibition of PI3Ks with LY294002 led to an enhancement in MAPK pathway activation. On further investigation, activation of MAPK pathway signalling by inducible expression of constitutively active Mek brought forth a minimal reduction in self-renewal. Furthermore, inhibition of p110β induced an enhancement in Erk phosphorylation akin to that induced by LY294002, implicating this isoform in regulating MAPK signalling under normal mESC culture conditions.

Insulin was shown to activate PI3Ks in mESCs and could be inhibited by treatment with pharmacological inhibitors of the p110α catalytic subunit isoform. Further investigation into the role of p110α in mESCs revealed a role in cell proliferation and metabolic activity. However, pharmacological or siRNA-mediated interference of this isoform did not perturb self-renewal. In contrast, p110β was identified as having a predominant role in the maintenance of self-renewal of mESCs. Both specific pharmacological inhibition and siRNA targeted knockdown of p110β led to a marked loss in alkaline phosphatase staining and a reduction in Nanog and Rex1 expression, indicating a loss of self-renewal.

Thus, independent roles for p110α and p110β in regulating mESC proliferation and self-renewal were found to be the result of coupling to different PI3K catalytic subunit isoforms. Interestingly, reducing proliferation by inhibition of p110α or mTOR led to a greater decline in mESC self-renewal when induced by inhibition of p110β. This demonstration of cross talk between the pathways that regulate proliferation and self-renewal suggests a priming effect where the rate of proliferation could sensitise mESCs to levels of p110β activation in order to regulate self-renewal and ultimately cell fate.

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# Abbreviations

| | |
|---|---|
| APS | Ammonium Persulphate |
| ADP | Adenosine Diphosphate |
| APC | Adenomatous polyposis coil |
| ATP | Adenosine Triphosphate |
| BIO | 6-bromoindirubin 3'-oxime |
| BMM | Bone marrow-derived macrophage |
| BMP | Bone morphogenic protein |
| BSA | Bovine Serum Albumen |
| CCCP | Carbonyl Cyanide *m*-Chlorophenyl Hydrazone |
| CDK | Cyclin-dependent kinase |
| cDNA | Complementary DNA |
| CHO | Chinese hamster ovary |
| CHO-IR | Chinese hamster ovary cells expressing human insulin receptor |
| CKIα | Casein kinase Iα |
| $DiOC_6$ | 3,3' –dihexyloxacarbocyanide iodide |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic Acid |
| dsRNA | Double stranded Ribonucleic acid |
| E | Stage of embryonic development, indicating days post coitum |
| ECC | Embryonal carcinoma cell |
| ECL | Enhanced Chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid discodium salt |
| EGF | Epidermal Growth Factor |
| Erk | Extracellular regulated kinase |
| ESC | Embryonic Stem Cell |
| FACS | Fluorescence Activated Cell Sorter |
| FBS | Foetal bovine serum |
| FCS | Foetal Calf Serum |
| FGF | Fibroblast Growth Factor |
| fMLP | N-formyl-methyl-leucyl-phenylalanine |
| Fox | Forkhead box |
| FYVE | Fab1p YOPB Vps27p EEA1 |
| Gab1 | Grb-2-associated binder |
| GDP | Guanosine Diphosphate |
| GEF | GTP/GDP exchange factor |
| GMEM | Glasgow Modified Eagle Medium |
| GPCR | G Protein Coupled Receptor |
| G Protein | GTP binding Protein |
| Grb2 | Growth Factor Receptor bound protein 2 |
| Gsk-3 | Glycogen synthase kinase 3 |
| GS | Glycogen synthase |
| GTP | Guanosine Triphosphate |
| HBSS | Hank's Balanced Salt Solution |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hESC | Human Embryonic Stem Cell |
| HRP | Horse Radish Peroxidase |
| ICM | Inner Cell Mass |
| Id | Inhibitor of differentiation |
| IGF | Insulin-like growth factor |
| IL | Interleukin |
| iPSC | Induced pluripotent stem cells |
| IRS | Insulin receptor substrate |

| | |
|---|---|
| Jak | Janus Kinase |
| kDa | Kilo Daltons |
| Klf4 | Krupple-like factor 4 |
| KO SR | Knockout Serum Replacement |
| LEF | Lymphoid enhancer factor |
| LIF | Leukemia inhibitory factor |
| LIFR | LIF Receptor |
| LPA | Lysophosphatidic Acid |
| LRP | Low density lipoprotein |
| MAPK | Mitogen activated protein kinase |
| MEF | Murine Embryonic Fibroblasts |
| Mek | Mitogen-activated extracellular signal-regulated kinase |
| mRNA | Messenger Ribonucleic Acid |
| MTG | Monothioglycerol |
| mTOR | Mammalian target of rapamycin |
| mESC | Murine Embryonic Stem Cell |
| NEAA | Non-essential amino acids |
| NT | Non-targeting |
| p70S6K | p70 ribosomal S6 kinase (S6K) |
| $p87^{PIKAP}$ | PI3K adaptor protein of 87kDa |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PDGF | Platelet Derived Growth Factor |
| PDK1 | 3-phosphoinositide-dependent protein kinase 1 |
| PH | Pleckstrin Homology |
| PI | Phosphatidylinositol |
| PIA | $N^6$-(2-phenylisopropyl)adenosine |
| PCR | Polymerase chain reaction |
| PIF | PDK1-interacting fragment |
| PI3K | Phosphoinositide 3-kinase |
| PIK3IP1 | PI3K interacting protein |
| PI(3)P | Phosphatidylinositol-3-phosphate |
| PKB (A/C/G) | Protein kinase B (A/C/G) |
| PMSF | Phenylmethylsulphonylfluoride |
| POU | Pit Oct Unc |
| PRK2 | PKC-related kinase |
| PTEN | Phosphatase and tensin homologue |
| PTX | Pertussis toxin |
| PX | Phox Homology |
| QPCR | Quantitative PCR |
| RISC | RNA induced silencing complex |
| RNAi | Ribonucleic Acid interference |
| RP | Retinoblastoma Protein |
| RT-PCR | Reverse Transcription PCR |
| S1P | Sphingosine-1 phosphate |
| SDS-PAGE | Sodium Dodecyl Sulphate-Poly acrylamide gel electrophoresis |
| S.E.M. | Standard Error of the Mean |
| SGK | Serum and glucocorticoid-induced protein kinase |
| SH2/3 | Src-homology domain 2/3 |
| SHIP | SH2-containing inositol 5-phosphatase |
| Shp2 | Src-homology 2 containing phosphatase 1 |
| shRNA | Short-hairpin Ribonucleic acid |
| siRNA | Short interfering Ribonucleic acid |
| Smad | *Caenorhabditis elegans* protein Sma, *Drosophila* mothers against decapentaplegic (mad) |
| SOCS | Supressor of cyctokine signalling |
| Sox | SRY-related HMG box |

| | |
|---|---|
| SRF | Serum response factor |
| SSEA | Stage specific embryonic antigen |
| Stat3 | Signal Transducer and Activator of Transcription 3 |
| TAE | Tris-acetate EDTA |
| TBS | Tris Buffered Saline |
| TBST | TBS plus 0.05% |
| TCF | T-cell factor |
| TEMED | Tetramethylethylenediamine |
| Tet | Tetracycline |
| TGF | Transforming Growth Factor |
| TRK | Tropomyosin-related kinase |
| tTA | Tetracycline-sensitive transactivator |
| XTT | Sodium 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methox-6-nitro) benzene sulfonic acid hydrate |

# 1. INTRODUCTION

## 1.1 Intracellular signalling pathways

Cell signalling is the perception of an extrinsic signal, supplied by the environment of a cell, and its interpretation into a cellular response. The underlying mechanism involves the detection of an extrinsic signal, often by a cell surface receptor, followed by the activation of signalling pathways within the cell. Intracellular signalling comprises a complex system of signalling molecules that sequentially activate or inhibit other signalling molecules in specific pathways. However, specific pathways are often part of multifaceted networks with many divergent branches that exhibit a high level of cross-talk, as well as positive and negative feedback. A single extrinsic signal or ligand, such as a growth factor, hormone or cytokine, may activate a single pathway leading to an appropriate cellular response. However, more often a number of pathways are activated providing detailed information regarding the environment of a cell. Such complicated intracellular signalling appears to be required in order to regulate cellular activities such as cell division, migration and apoptosis. Further diversity is provided by multiple isoforms of the same protein, arising from different genes or alternative splicing of the same gene, which are categorised into families of signalling molecules that display similar enzymatic or functional activity. The phosphoinositide 3-kinase family of enzymes were discovered in the 1980s and have gained the attention of many research groups owing to the increasingly diverse cellular responses that are mediated by signalling pathways involving different members of this superfamily.

## 1.2 Phosphoinositide 3-Kinase

### *1.2.1 Signalling by intracellular messengers*

The actions of phosphoinositides as second messengers were first reported in the early 1980s. Inositol lipids were described as signal transducers, linking growth factor receptors to the activation of oncogenes (Berridge and Irvine, 1984). A few years later it was reported that stable complexes of middle T antigen and $pp60^{c\text{-}src}$ existed in association with an 81kDa protein (Courtneidge and Heber, 1987). This protein was reported to be phosphorylated at tyrosine residues and its presence in kinase assays correlated with phosphatidylinositol (PI) kinase activity (Courtneidge and Heber, 1987). Indeed two PI kinases were identified in fibroblasts, one of which was reported to associate with tyrosine kinases (Whitman et al., 1987), consistent with the tyrosine phosphorylation previously described (Courtneidge and Heber, 1987). Upon further

characterisation, the site of phosphorylation mediated by this kinase was described as the D3 hydroxyl group of the inositol headgroup leading to the production of the phospholipid, phosphatidylinositol-3-phosphate (PI(3)P) (Whitman et al., 1988). It is now widely known that the phosphoinositide 3-kinase (PI3K) family of kinases catalyse the transfer of the γ-phosphate of adenosine triphosphate (ATP) to phosphoinositides at the D3 position of the inositol ring (Figure 1.1**A**). Different PI3Ks respond to different stimuli and exhibit a preference over certain inositol substrates. Thus a variety of second messenger products can be produced by PI3Ks (Figure 1.1**B**), which leads to activation of diverse downstream pathways and effects on a variety of cellular responses.

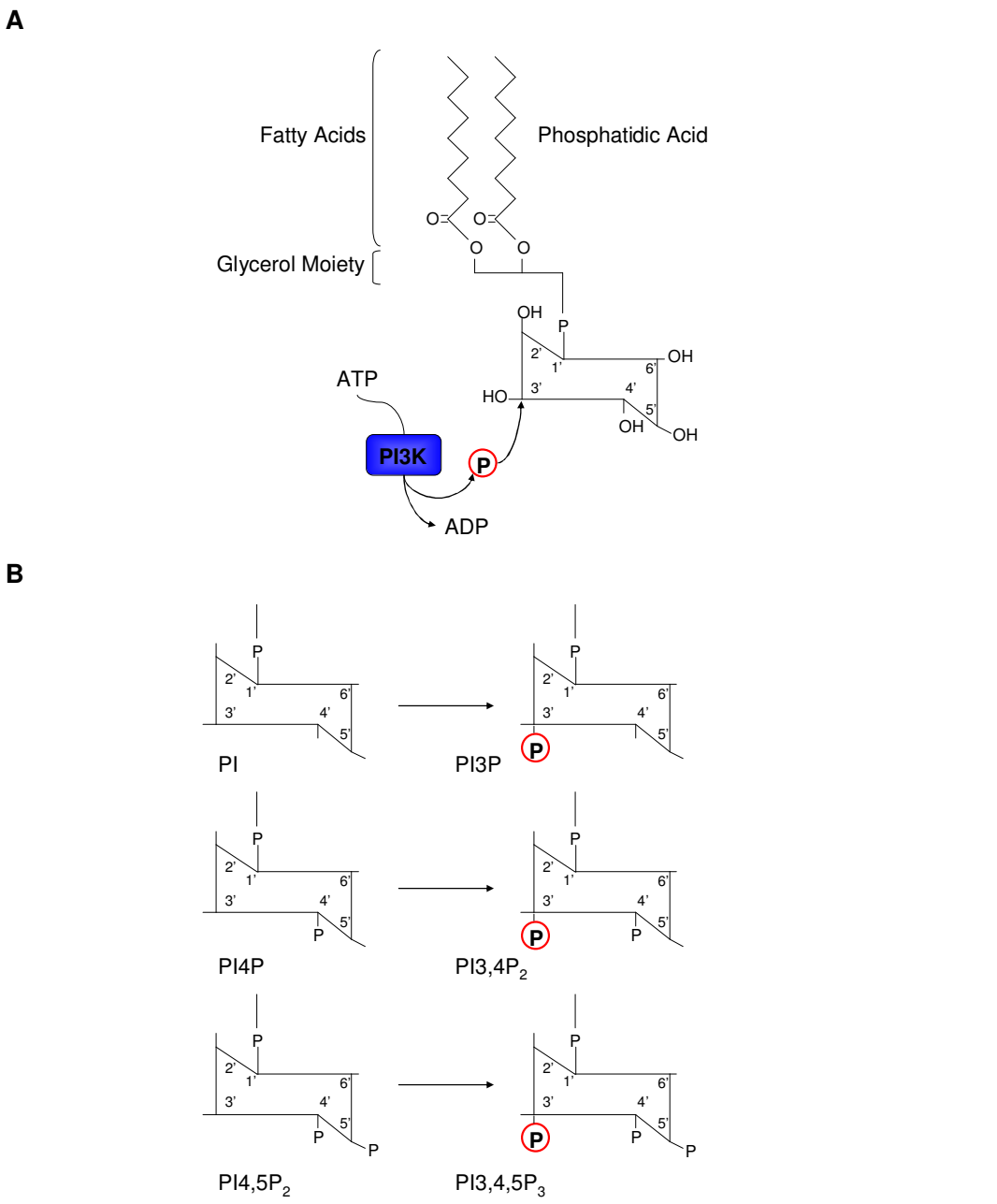


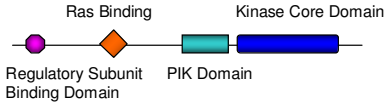
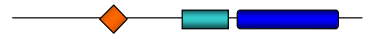
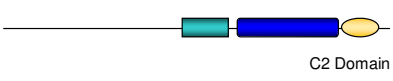
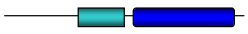

**Figure 1.1 PI3K catalyses the addition of a phosphate group to the D3 position of the inositol ring of phosphatidyl inositols.**

**A** The fatty acid side chains of phospholipids localise within the lipid bilayer of the cell membrane leaving the hydrophilic inositol ring (head group) accessible in the cytosol. All kinases catalyse phosphotransferase reactions, typically transferring the γ-phosphate of ATP to a substrate to produce a phosphorylated substrate and adenosine diphosphate (ADP). PI3K, in particular, catalyses the transfer of phosphate to the D3 hydroxyl position of the inositol ring of phospholipids, to produce phosphoinositide 3-phosphate (PIP). **B** Structures of phosphoinositides produced by the actions of PI3Ks. Adapted from Vanhaesebroeck and Waterfield 1999, Hawkins et al., 2006.

### *1.2.2 Phosphoinositide 3-kinases: structure and function*

Mammalian PI3Ks are divided into three classes (summarised in Table 1.1). PI3Ks are categorised according to their catalytic and regulatory subunits, receptor activation and lipid substrates. The role of class I PI3Ks are investigated in the present study and will therefore be described in more detail (Section 1.2.2.1). Class II PI3Ks contain a C2 domain for calcium-dependent binding to phospholipids (MacDougall et al., 1985) and are typically activated by tyrosine kinases and heterotrimeric GTP-binding proteins (G proteins) (reviewed by Vanhaesebroeck and Waterfield, 1999). Class III PI3Ks are homologues of the yeast vesicular protein-sorting protein Vps34p. Reports suggest that class III PI3Ks may not require activation by extracellular stimuli but may contribute to cellular levels of phosphoinositide 3-phosphate (reviewed by Herman et al., 1992). Cloning of the catalytic domain of PI3Ks and Vps34p revealed high sequence homology (Schu et al., 1993). Indeed sequence alignment of kinase domains has furthered the discovery of more PI3K family members, each having a conserved kinase core domain (Table 1.1).

**Table 1.1 Three classes of PI3K have been described in mammalian cells.**
Compiled from Vanhaesebroeck and Waterfield 1999, Wymann and Pirola 1998, Domin and Waterfield 1998.

| Class | Catalytic Subunit Structure | Catalytic Subunit | Regulatory Subunit | Activation | Lipid Substrates |
|---|---|---|---|---|---|
| $I_A$ |  | p110α<br>p110β<br>p110δ | p85α<br>p85β<br>p55γ | Tyrosine Kinases<br>Ras | *In vivo:*<br>$PI4,5P_2$<br>*In vitro:*<br>PI<br>PI4P<br>$PI4,5P_2$ |
| $I_B$ |  | p110γ | p101 | Heterotrimeric G proteins<br>Ras | *In vivo:*<br>$PI4,5P_2$<br>*In vitro:*<br>PI<br>PI4P<br>$PI4,5P_2$ |
| II |  | PI3K-C2α<br>PI3K-C2β<br>PI3K-C2δ | | Tyrosine Kinases<br>Heterotrimeric G proteins | *In vitro:*<br>PI<br>PI4P<br>$PI4,5P_2$ |
| III |  | Vps34p analogues | p150 | Not activated by extracellular stimuli | *In vivo:*<br>PI<br>*In vitro:*<br>PI |

#### *1.2.2.1 Class I PI3Ks*

Class I PI3Ks are subdivided into two groups; Class $I_A$ and Class $I_B$ (Table 1.1). Class $I_A$ PI3Ks consist of a 110kDa catalytic subunit of which there are three isoforms p110α, p110β and p110δ encoded by the genes *Pik3ca, Pik3cb* and *Pik3cd* respectively (Hiles et al., 1992, Hu et al., 1993, Vanhaesebroeck et al., 1997). Following on from earlier immunoprecipitation studies, the catalytic subunit was found to be associated with an 85kDa regulatory subunit, often referred to as an adaptor protein. In fact there are several regulatory subunits expressed in mammalian cells; p85α, p85β, p50α, p55α and p55γ (Domin and Waterfield, 1997). *Pik3r1, Pik3r2,* and *Pik3r3* encode p85α, p85β and p55γ respectively, while alternative splicing of *Pik3r1* gives rise to p50α and p55α.

The regulatory subunit consists of an N terminal Src homology 3 (SH3) domain and two C terminal Src homology 2 (SH2) domains (Figure 1.2). SH3 domains bind proline rich regions of proteins and therefore serve to regulate PI3K activity by protein-protein interactions. Additionally, the SH3 domain of p85 can interact with its own proline rich region (Kapeller et al., 1994). However, the shorter p55 isoform lacks SH3 domains (Antonetti et al., 1996). SH2 domains bind to phosphorylated tyrosine residues within consensus motifs on activated receptors or adaptor proteins (Songyang et al., 1993). The region between the two SH2 domains of p85 subunits is highly conserved and provides a site for phosphorylation by p110α or p110β. Phosphorylation of a conserved serine residue within the inter-SH2 domain coincides with an increase in PI3K activity (Dhand et al., 1994). Antibodies against the p85 inter-SH2 domain region block the binding of p110 to p85 *in vitro* (Dhand et al., 1994). Thus, interaction of p85 and p110 subunits and intrinsic protein-serine kinase activity may act as an autoregulatory mechanism for PI3K activity. The interaction of p85 with p110 and other proteins is shown in Figure 1.2.

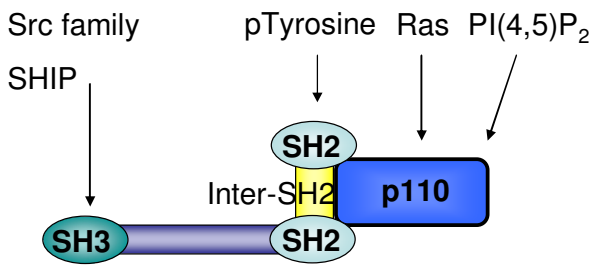



**Figure 1.2 Interaction of p85 and p110 is required for catalytic activity**
The inter-SH2 domain of p85 binds p110 and also provides a site for intrinsic serine kinase-mediated phosphorylation for autoregulation. The SH2 domains of p85 have affinity for phosphorylated tyrosine residues on the intracellular domains of receptors, recruiting the p110/p85 heterodimer to the plasma membrane. At the membrane, p85/p110 heterodimers can interact with various proteins to form a protein complex capable of kinase activity and production of second messenger phosphatidylinositol lipids. Adapted from Vanhaesebroeck et al., 2005.

Prior to stimulation, p85 may act to inhibit catalytic activity of p110 since monomers of p110α or p110β were shown to exhibit kinase activity, which was reduced by co-expression with p85 (Yu et al. 1998). Activation of class $I_A$ PI3Ks occurs by a number of overlapping mechanisms but typically involves initial extracellular stimuli such as growth factors, hormones, neurotransmitters or antigens (Wymann and Pirola 1998). Activation of receptors following ligand stimulation results in autophosphorylation of the intracellular portion of the receptors either by dimerisation or ligand-induced conformation change. SH2 domains of p85α bind phosphorylated tyrosine residues within the receptor consensus motif pYXXM (where X denotes any amino acid) (Carpenter et al., 1993, Rordorf-Nikolic et al., 1995). In insect cells, it was reported that the monomeric activity of p110α could be decreased by coexpression of p85 and that addition of phosphotyrosyl peptides restored activity to that of monomeric p110α (Yu et al., 1998). However, in HEK293 cells, the low monomeric activity of p110 was enhanced by coexpression of p85. Therefore, a model was proposed where by, p85 both stabilises and inhibits p110 by conformational change, and upon binding of p85 to phosphorylated tyrosine consensus motifs inhibition of p110 is reversed (Yu et al., 1998). Nevertheless, SH2 domain binding to phosphotyrosine recruits p85/p110 complexes to the membrane allowing association with adaptor proteins and receptor protein tyrosine kinases, typically growth factor receptors (van der Geer et al., 1994).

Class I PI3Ks share Ras as a common binding partner. Ras is a small GTPase, which is reported to bind Class I PI3Ks when associated with GTP but not when bound to GDP (Rodriguez-Viciana et al., 1994, Rubio et al., 1997). Furthermore, expression of constitutively active Ras results in an enhancement of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ levels (Rodriguez-Viciana et al., 1994) indicating that PI3Ks are activated downstream of Ras. In contrast, others report that Ras is activated by PI3Ks. Expression of mutant PDGF receptor lacking PI3K docking sites failed to activate Ras (Satoh et al., 1993) while expression of PDGF receptor containing only PI3K docking sites was sufficient to mediate Ras activation (Valius and Kazlauskas, 1993). In fact, both receptor-PI3K and Ras-PI3K interactions occur to form a ternary complex with cross-talk. In this model, p85 recruitment to phosphotyrosine residues and p110 interaction with Ras allows the formation of a ternary complex at the plasma membrane. This complex aids allosteric cooperativity thereby enhancing the activity of both p110 and Ras (Kaur et al., 2006).

Following recruitment to the receptor signalling complex, class I PI3Ks preferentially utilise $PI(4,5)P_2$ as a substrate, by transferring the terminal phosphate of ATP, to yield $PI(3,4,5)P_3$. PI3Ks have also been shown to phosphorylate PI, PI(4)P and $PI(4,5)P_2$ *in vitro* (Irvine, 1992). These lipid second messengers serve as an amplification step in PI3K signal transduction since once formed, the activated PI3K-receptor complex continues to phosphorylate PI substrates until activation ceases. Additionally the second messenger products of PI3K are capable of activating multiple effector proteins. PI3K effector proteins contain conserved Pleckstrin Homology (PH) domains that specifically bind PI3K lipid products inducing recruitment to the plasma membrane (Katan and Allen, 1999). For example, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ recruit Protein Kinase B (PKB, also referred to as Akt) to the plasma membrane by binding the PH domain of PKB (Burgering and Coffer, 1995). PKB is a serine/threonine protein kinase which is activated by a number of extrinsic stimuli including insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (reviewed by Hanada et al., 2004). Activation of PKB by these factors can be prevented by pharmacological inhibition of PI3Ks or by the expression of a dominant negative form of PI3K, either of which reduce levels of $PIP_3$ and therefore reduce PKB activation (Burgering and Coffer, 1995). In addition to PH domains, other domains that bind phosphoinositides include phox homology (PX) and Fab1p YOPB Vps27p EEA1 (FYVE) domains, which typically bind the phosphoinositide products of class II and III PI3Ks (reviewed by Sasaki et al., 2007).

At the membrane, PKB is brought into close proximity with phosphoinositide-dependent protein kinase (PDK1). PDK1 also contains a PH domain at the C terminal end of the protein, which anchors PDK1 to the plasma membrane allowing interaction with other proteins (Komander et al., 1994). For example, PDK1 acts to phosphorylate Thr308 of PKB (Alessi et al., 1997) (Figure 1.3). Additionally, phosphorylation at Ser473 of PKB occurs when PDK1 is bound to Protein Kinase C-related kinase-2 (PRK2) (also referred to as PDK1-interacting fragment (PIF)) in the presence of $PI(3,4,5)P_3$ (Balendran et al., 1999). Others report that PDK1 is not necessary for Ser473 phosphorylation of PKB, but that the presence of PDK1 may enhance phosphorylation at this site (Williams et al., 2000). Once activated, by phosphorylation at both Ser473 and Thr308, PKB can signal downstream to mediate a number of cellular functions including metabolism, cell growth and apoptosis. In addition, activity of PI3K and PKB resulting from stimulation of cells with insulin, Wnt or growth factors such as PDGF or EGF can inhibit glycogen synthase kinase-3 (Gsk-3) (Bardy et al., 1998, Cross et al., 1995, Doble and Woodgett, 2003, van Noot et al., 2002).

PDK1 has also been implicated in the activation of AGC family kinases, named after the well characterised protein kinases A, G and C (PKA, PKG and PKC) but also including p70 ribosomal S6 Kinase (p70S6K), serum and glucocorticoid-induced protein kinase (SGK) and PKB (Mora et al., 2004, Williams et al., 2000). Unlike PKB, S6K and SGK do not interact with $PI(3,4)P_2$ or $PI(3,4,5)P_3$. Activation by PDK1 occurs when S6K or SGK directly bind to a hydrophobic pocket adjacent to the kinase domain of PDK1, termed the PIF pocket (Biondi, 2001). In PDK1 knock-out cells activation of PKB and p70S6K is inhibited and PDK1 substrates lack phosphorylation at the conserved threonine residue (Williams et al., 2000). PDK1 orchestrates a variety of cellular functions including regulation of metabolism, cell cycle, apoptosis and transcription factors (Hanada et al., 2004, Hennessy et al., 2005).

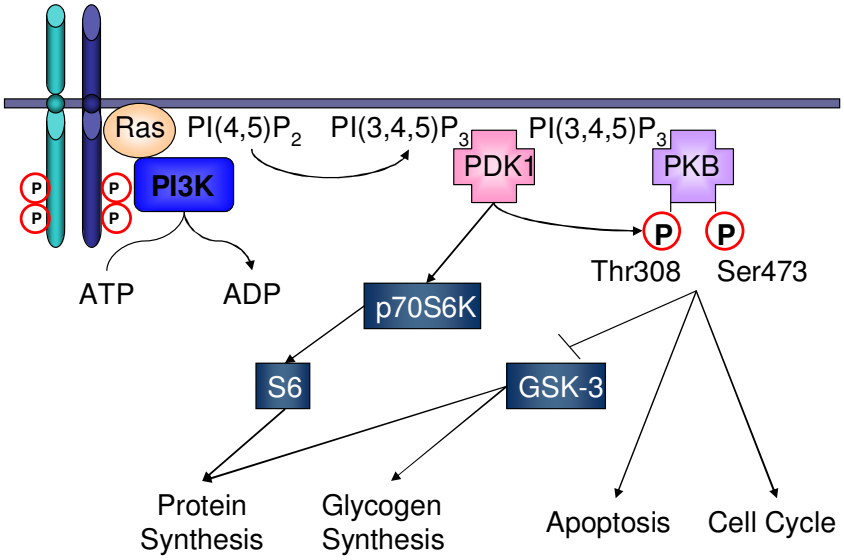



**Figure 1.3 Activation of PI3K and downstream signalling**

Activation of PI3K occurs via interaction of p85/p110 dimers with phosphorylated tyrosine residues of receptors or adaptor molecules and interaction with Ras. PI3Ks catalyse the addition of a phosphate group from ATP to $PI(4,5)P_2$ to form $PI(3,4,5)P_3$ and ADP. Activation of downstream effectors occurs by recruitment of effector PH domains to $PI(3,4,5)P_3$ in close proximity to PDK1. PKB is activated by phosphorylation at two key sites, threonine 308 (Thr308) and serine 473 (Ser473). Downstream effectors of PI3K mediate an array of cellular events.

Currently there is only one member of the Class $I_B$ PI3Ks, PI3Kγ. The p110γ catalytic subunit, encoded by the gene *Pik3cg*, associates with a 101kDa regulatory protein (p101) encoded by the gene *Pik3r5*. Incidentally, p101 has little sequence homology to the class $I_A$ regulatory subunits but $p87^{PIKAP}$ (PI3K adaptor protein of 87kDa), a recently described novel regulatory subunit of p110γ shared sequence homology with p101 (Voigt et al., 2006). High sequence homology was found between $p87^{PIKAP}$ and p101 particularly in regions known to participate in p110γ binding. Expression of $p87^{PIKAP}$ was detectable in dendritic cells, macrophages, and neutrophils but expression was notably high in the heart (Voigt et al., 2006).

The p110/p101 heterodimer is activated by G protein interaction following stimulation of G protein coupled receptors (GPCRs) (Figure 1.4) (Stephens et al., 1997). More specifically, the $G_{\beta\gamma}$ subunit was reported to mediate the activation of p110/p101 via the N terminus of p110γ (Krugmann et al., 1999). In an alternative study, p101 was reportedly not required for $G_{\beta\gamma}$-mediated activation of p110γ kinase activity but found to

sensitise p110γ for $G_{\beta\gamma}$ activation in the presence of $PI(4,5)P_2$ thereby determining substrate specificity (Maier et al., 1999). However, *in vivo*, p101 is required for $G_{\beta\gamma}$-mediated activation of p110γ since p101 acts to recruit the p110/p101 heterodimer to the plasma membrane via interaction of p101 and $G_{\beta\gamma}$ (Brock et al., 2003). Additionally, $p87^{PIKAP}$ was reported to have sequence similarity to p101 in the same region used for $G_{\beta\gamma}$ association (Voigt et al., 2006).

A number of research papers have been published reporting that p110β of the class $I_A$ PI3Ks can be alternatively activated by G proteins (Figure 1.4). In an *in vitro* kinase assay, purified $G_{\beta\gamma}$ subunits were shown to activate p110β leading to an increase in $PI(3,4,5)P_3$. This activation was substantially enhanced in the presence of tyrosine phosphorylated peptides (Maier et al., 1999). In immunoprecipitates, lysophosphatidic acid (LPA), a GPCR ligand that utilises $G_{i,}$ was shown to activate PI3Kβ (Roche et al., 1998). However, in an NIH3T3-derived cell line lacking p110β expression, LPA stimulation failed to activate PKB (Murga et al., 2000). Furthermore, in Chinese hamster ovary (CHO) cells expressing the GPCR adenosine receptor ($A_1R$), stimulation with the $A_1R$ ligand PIA [$N^6$-(2-phenylisopropyl)adenosine] induced PKB phosphorylation, which could be inhibited by the introduction of a vector producing p110β-targeting short hairpin RNA (shRNA) (Kubo et al., 2005). Recently a mouse line was created with a conditional genetic inactivation of p110β that induces the expression of a truncated, catalytically inactive form of p110β (Guillermet-Guibert et al., 2008). Crossed heterozygous mice produced fewer viable mice than predicted however, mouse embryonic fibroblasts (MEFs) and bone marrow-derived macrophages (BMMs) could be isolated from viable mice. These cells were used to demonstrate G protein activation of p110β. In BMMs, stimulation with complement 5a, a GPCR agonist, resulted in PKB phosphorylation by both p110β and the class $I_B$ catalytic isoform p110γ. In MEFs, which express p110β but not p110γ, p110β-mediated activation of PKB was induced following stimulation with the GPCR agonists LPA and sphingosine-1-phosphate (S1P) (Guillermet-Guibert et al., 2008). Thus, depending on cell type or stimulating factor, activation of GPCRs may result in activation of either p110γ or p110β.

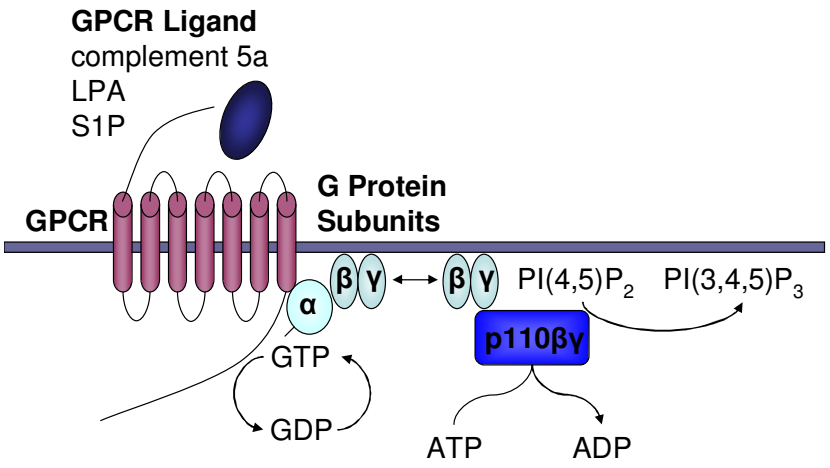



**Figure 1.4 GPCR-mediated activation of p110β and p110γ**

The G protein coupled receptors (GPCRs) are 7 transmembrane domain containing receptors which can mediate the activation of p110γ, and more recently reported p110β, via a heterotrimeric G protein subunit complex. Activated G proteins are guanosine triphosphate (GTP) coupled. In general, dissociation of GTP-bound $G_{\alpha}$ from the heterotrimeric complex results in activation of the remaining dimer $G_{\beta\gamma}$, of the other two G proteins, $G_{\beta}$ and $G_{\gamma}$. $G_{\beta\gamma}$ is reported to activate p110β and p110γ in certain cell types given the appropriate extracellular stimuli. When $G_{\alpha}$ hydrolyses GTP to guanosine diphosphate (GDP), $G_{\alpha}$ regains its affinity for $G_{\beta\gamma}$ and the heterotrimeric complex re-associates. Adapted from Yart et al., 2002.

#### *1.2.2.2 Regulation of PI3K activity*

With a variety of receptors capable of activating PI3Ks, it follows that strict regulation of activity is required. This is primarily mediated by the actions of two phosphatases; SH2-containing inositol 5-phosphatase (SHIP) and phosphatase and tensin homolog (PTEN). SHIP dephosphorylates the D5 position of phosphoinositol lipids (Rohrschneider et al., 2000) and PTEN dephosphorylates the D3 position (Cantley and Neel, 1999, Stambolic et al., 1998) (Figure 1.5). The actions of either of these phosphatases reduce the levels of $PI(3,4,5)P_3$ present and therefore reduce the recruitment of PH domain containing effectors to the plasma membrane. However, since PTEN removes the phosphate group at the D3 position it reverts PI3K products to PI3K substrates. PI3Ks are known to promote growth due to activation by growth factor receptors. Thus, PTEN is a tumour suppressor since it reduces levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in cells and therefore reduces growth promoting signals

(Cantley and Neel, 1999). Indeed PTEN-null cells exhibit constitutively active PKB due to high levels of $PI(3,4,5)P_3$ (Stambolic et al., 1998).

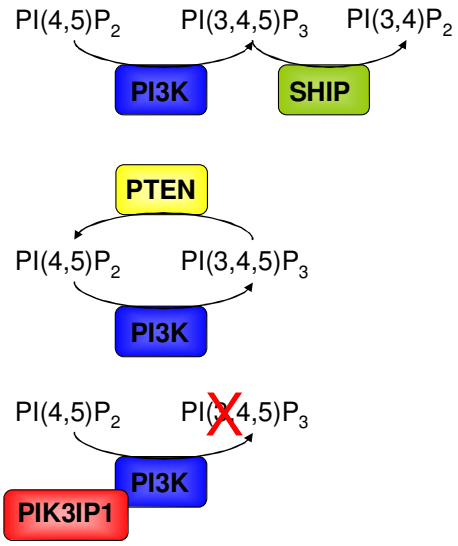



**Figure 1.5 Regulation of PI3K activity**

SHIP removes the D5 phosphate from inositol phosphate products of PI3K. PTEN removes the D3 phosphate from inositol phosphate products of PI3Ks. Therefore, the activity of either SHIP or PTEN reduces the amount of $PI(3,4,5)P_3$ in cells and counteracts the activity of PI3Ks. PI3K-interacting protein, PIK3IP1 is a novel regulator of PI3K activity with similarities to p85. However, instead of aiding activation and recruitment of PI3Ks to the plasma membrane, PIK3IP1 negatively regulates PI3K activity.

More recently, a novel PI3K interacting protein (PIK3IP1) has been reported. PIK3IP1 is a transmembrane protein and shares sequence homology with the p85 regulatory subunit. It is this homologous region of PIK3IP1 that binds to p110 resulting in reduced PI3K activity and limiting PKB activation (Zhu et al., 2007) (Figure 1.5). Indeed, further investigation has highlighted a role for this protein in preventing the development of hepatocellular carcinoma in both human disease and in a mouse model. Isolated hepatic cells from PIK3IP1 transgenic mice exhibited reduced PI3K signalling and suppressed hepatic cell growth (He et al., 2008). Furthermore, these mice had a diminished rate of spontaneous hepatocellular carcinoma development. Consistent with this, tissue from human liver tumours displayed reduced expression of PIK3IP1 (He et al., 2008).

Classically, p85 is reported to regulate the activation of p110 isoforms with associated interaction of receptor-tyrosine kinases (Dhand et al., 1994). However, it became apparent that monomeric p85 subunits could act as inhibitors of PI3K activation. In this model, free p85, not bound to p110, competes with heterodimeric p85-p110 for phosphorylated tyrosine residues. Binding of free p85 to phosphorylated tyrosine residues of activated receptors prevents the binding of p85-p110, thereby reducing PI3K activity (Geering et al., 2007). Thus, p85 not only positively regulates p110 catalytic activity via p110 interaction at the plasma membrane but also negatively regulates kinase activity by competitive binding depending on the balance of p85 and p110 subunits (Brachmann et al., 2005). In addition, the expression of p85 and p110 subunits can be interlinked. Given these two factors, targeted deletion of individual regulatory subunits could lead to altered expression of other subunits and unpredictable signalling outcomes. For example, in cells isolated from p85α knock-out mice, the expression of p85β is elevated, while the expression of p110α, p110β and p110δ is reduced compared to cells from wild-type mice (Fruman et al, 1999,Suzuki et al., 1999, Lu-Kuo et al., 2000). Knock-out mice lacking the p85 regulatory subunit tend to have increased insulin sensitivity and accordingly, cells isolated from these mice show increased $PI(3,4,5)P_3$ levels and enhanced PKB phosphorylation at Ser473 (Vanhaesebroeck et al., 2005, Foukas et al., 2006). A comprehensive study compared the kinase activity and insulin signal transduction of various PI3K regulatory subunits with the p110α catalytic subunit. Increasing the expression of p85α had a negative effect on PI3K activity. Furthermore, expression of p85α lacking the p110 binding domain also inhibited phosphotyrosine associated PI3K activity (Ueki et al., 2000).

#### *1.2.2.3 Class I PI3Ks: Functional roles and disease states*

The variety and combination of regulatory and catalytic subunits of PI3Ks allows cells to mediate cellular functions in an isoform selective manner, thus roles for individual isoforms are emerging.

A major role for p110α in regulating growth and metabolic regulation has been reported. Growth and metabolic activity via p110α appears to be induced following insulin or growth factor stimulation (Roche et al., 1998). Knock in of kinase dead p110α produced viable heterozygous mice with stunted growth and hyperinsulinaemia (Foukas et al., 2006) where as homozygous p110α knock out mice were embryonic lethal, reportedly due to proliferative defects (Bi et al., 1999). Mouse PI3K catalytic

gene knock out and knock in studies are compared and discussed in further detail in Section 1.3.6.3. Of relevance, p110α was shown to be selectively recruited to the insulin receptor substrate (IRS) complex for signal transduction following insulin or insulin-like growth factor stimulation (Foukas et al., 2006). Furthermore, selective inhibitors of p110α blocked insulin-stimulated $PI(3,4,5)P_3$ production, inhibited insulin-induced PKB phosphorylation and reduced glucose uptake in adipocytes and myotubes (Knight et al., 2006). Perhaps unsurprisingly, given the regulation of cell growth by p110α, activating mutations of p110α or enhancements in copy number of *PIK3CA*, which encodes human p110α, are found in cancerous tissues (Shayesteh et al., 1999, Levine et al., 2005, Wu et al., 2005) highlighting an oncogenic role for p110α. Although p110α is implicated in many cancerous tissue types mutations and increased copy numbers of p110α are commonly found in ovarian and breast tumours (Shayesteh et al., 1999, Bachman et al., 2004, Levine et al., 2005) and colorectal tumours (Samuels et al., 2004). Furthermore, mutations of p110α are reportedly located in the kinase and helical domains inducing enhanced activity (Samuels et al., 2004). Additionally, amplifications or mutations of the genes encoding PKB isoforms, a major effector of PI3Kα, are also reported in human tumours in particular ovarian cancer (Cheng et al., 1992, Liu et al., 1998). Consistent with this, inhibitory mutations of the phosphatases, PTEN and SHIP, endogenous antagonists of PI3K activity, result in excess cellular $PI(3,4,5)P_3$ which can lead to cancerous phenotypes (Hennessy et al., 2005). Additionally a germline mutation of PTEN has been implicated in Cowden's Disease, an inheritable syndrome that enhances the risk of breast, thyroid and skin tumour development (Liaw et al., 1997).

Despite both p110α and p110β being ubiquitously expressed, much less is known about the functional role of p110β. Initially, p110β was implicated in stabilising platelet integrin $\alpha_{IIb}\beta_3$ and subsequent formation of adhesion bonds, a step in platelet activation and thrombus formation. Selective pharmacological inhibition of p110β prevented sustained platelet aggregation in response to weak agonist stimulation but was not necessary for initial platelet aggregation (Jackson et al., 2005). Other reports point towards a functionally redundant role for p110β in cell growth. LPA or insulin were demonstrated to stimulate p110β-mediated signalling (Roche et al., 1998). In most cell types, p110α is the only mediator of insulin signalling. However, in certain cell types, for example HepG2 cells, inhibition of p110β alone did not alter insulin-induced PKB phosphorylation whereas inhibition of p110β in combination with inhibition of p110α reduced PKB phosphorylation. Additionally in J774.2 macrophages, p110α, p110β and p110δ all contribute to insulin signalling in a functionally redundant manner (Chaussade et al., 2007). Additionally, both p110β and p110γ contribute to GPCR-

mediated signalling in macrophages but p110β was reported to be dispensable (Guillermet-Guibert et al., 2008).

The broad expression profiles of p110α and p110β are in stark contrast to the largely restricted expression of p110δ to cells of the immune system (Vanhaesebroeck et al., 1997). This is exemplified by a major role for p110δ in the lymphohaemopoietic system. Gene knock in mice expressing catalytically inactive p110δ are reported to have impaired B and T cell immune responses (Okkenhaug et al., 2002). Furthermore, p110δ was found to be essential for B cell development, activation and function (Clayton et al., 2002, Jou et al., 2002). Selective inhibition of p110δ in neutrophils prevented *N*-formyl-methyl-leucyl-phenylalanine (fMLP)–induced PI(3,4,5)$P_3$ production and chemotaxis, an essential stage in innate immunity (Sadhu et al., 2003). However, a redundant role for p110δ in insulin signalling in HepG2 cells and J774.2 macrophages was identified. Although p110α is the major PI3K involved in insulin signal transduction, co-inhibition of p110α and p110δ further reduced insulin-stimulated PKB phosphorylation compared to inhibition of p110α alone in HepG2 cells (Chaussade et al., 2007).

The class $I_B$ PI3K p110γ exhibits expression restricted to the haematopoietic system, endothelium, heart and brain. Accordingly, knock out and catalytically inactive knock in studies in mice report a viable and fertile phenotype with normal growth rates (Crackower et al., 2002, Hirsch et al., 2000, Patrucco et al., 2004). However, p110γ has been implicated in mediating inflammatory processes and in the cardiovascular system using anti-p110γ antibodies and selective pharmacological inhibitors (reviewed by Rückle et al., 2006).

#### *1.2.2.4 Functional roles of other classes of PI3K*

A great deal of focus has been placed on defining the physiological roles of class I PI3Ks, however, the importance of other PI3K classes are beginning to emerge. The class II PI3K PI3K-C2β has been implicated in the migration of ovarian and cervical cancer cells (Maffucci et al., 2005) and recombinant expression of this isoform in HEK293 cells enhanced migration and decreased cell adhesion (Domin et al., 2005). In contrast, PI3K-C2α has been implicated in both the formation of clathrin-coated structures and clathrin-mediated trafficking (Gaidarov et al., 2001, Gaidarov et al., 2005). Consistent with this PI3K-C2α was found to be involved in an ATP-dependent

priming phase of regulated exocytosis in neuroendocrine cells (Meunier et al., 2005). In contrast, down-regulation of PI3K-C2α expression is reported to inhibit noradrenaline-induced contraction, defining a role for this isoform in vascular smooth muscle contraction (Wang et al., 2006).

The class III PI3K is primarily involved in trafficking of protein and vesicles. Within the endocytic system, hVps34, the mammalian homologue of Vps34 in *Saccharomyces cerevisiae*, mediates the internalisation of PDGF receptors (Siddhanta et al., 1998) and is required for the formation of internal vesicles within multivesicular endosomes (Futter et al., 2001).

### *1.2.3 Pharmacological inhibitors of PI3Ks*

Dysregulation of PI3Ks or proteins that regulate PI3Ks often leads to inflammatory disorders, autoimmune diseases and, most widely reported, cancer development and progression (Rommel et al., 2007, Hennessy et al., 2005). Therefore, inhibition of PI3Ks became a desirable target for therapeutic intervention of such disease states.

Early pharmacological inhibitors of PI3Ks exhibited broad selectivity. The *Penicillium funiculosum* metabolite, wortmannin, was first described for its anti-inflammatory actions on acute oedema and inhibition of the respiratory burst in neutrophils and mononuclear phagocytes (Wiesinger et al., 1974, Baggiolini et al., 1987). Authors proposed that this compound interfered in some way with the signal transduction system mediating the activation of the respiratory burst oxidase response to a stimulus. Indeed, PI3K was later identified as a target of wortmannin. In neutrophils, treatment with wortmannin prevented fMLP-induced $PI(3,4,5)P_3$ production and direct inhibition of PI3Ks was confirmed *in vitro* (Arcaro and Wymann, 1993). The irreversible actions of wortmannin are the result of covalent bonding to a catalytic lysine residue within the ATP-binding pocket of PI3Ks. Mutation of this amino acid residue to an arginine residue impairs wortmannin binding to PI3Ks (Wymann et al., 1996).

LY294002, another early PI3K inhibitor, also targets the ATP-binding pocket of PI3Ks but, in contrast to wortmannin, LY294002 is a reversible competitive inhibitor of PI3Ks (Vlahos et al., 1994). With the exception of Class II PI3K-C2α that is wortmannin-resistant (Domin et al., 1997), both wortmannin and LY294002 exhibit no selectivity for individual classes or isoforms of the PI3K family, what is more, their therapeutic use is

limited due to toxic effects *in vivo* (Hennessy et al., 2005). Unwanted side-effects are often the result of inhibition of PI3K-related kinases which share sequence similarity and structurally comparable kinase and ATP-binding domains, leaving them open targets for wortmannin and LY294002 (Abraham et al., 1996).

Two newer, Class I PI3K inhibitors have been described. ZSTK474 is an s-triazine derivative. ZSTK474 was identified in a screen for potential compounds exhibiting strong inhibition of tumour cell proliferation (Yaguchi et al., 2006). Further investigation revealed that ZSTK474 was more potent than wortmannin or LY294002 at inhibiting growth and an effective anti-tumour compound. Indeed, ZSTK474 was shown to induce a reduced level of cell proliferation by cell cycle arrest at $G_0/G_1$, without indication of enhanced apoptosis (Dan et al., 2008). Inhibition of PI3Ks by ZSTK474 is achieved by binding the ATP-binding site in a similar manner to that previously described for LY294002 and wortmannin. Reversible inhibition of class $I_A$ PI3Ks was in the nM range when tested using a recombinant protein assay, while other protein kinases tested, including MEK, p70S6K, Pim1, protein kinases A, B and C, were not appreciably inhibited by ZSTK474 at μM concentrations (Yaguchi et al., 2006). In a more detailed study on individual class I PI3K isoforms, ZSTK474 was found to inhibit all isoforms of this class with similar selectivity (Kong and Yamori, 2007). The mammalian target of rapamycin (mTOR), a serine/threonine kinase and member of the PIKK subfamily of kinases, contains a conserved kinase domain and acts downstream of PI3Ks to regulate cell growth via p70S6K (Abraham et al., 1996). Both LY294002 and wortmannin exhibit inhibition of mTOR which contributes to the toxicity of these inhibitors and makes them unlikely therapeutic agents (Knight et al., 2006, Brunn et al., 1996). ZSTK474 is reported to weakly inhibit mTOR, but at concentrations higher than those needed to inhibit class I PI3Ks (Kong and Yamori, 2007).

PI-103 is described as a dual inhibitor of p110α and mTOR. Unlike other PI3K inhibitors, PI-103 was shown to inhibit both rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 (Knight et al., 2006). Effective inhibition of glioma cell proliferation without inducing DNA damage was achieved by treatment with PI-103 (Fan et al., 2006). In addition, an *in vivo* mouse model indicated that PI-103 could reduce xenograft size without the complications of toxicity associated with other PI3K inhibitors (Fan et al., 2006). Furthermore, the usefulness of PI-103 as a therapeutic agent was compounded by a report describing an enhanced sensitivity of tumour cells to radiotherapy following incubation with PI-103 (Prevo et al., 2008). However, in an *in vitro* screen of small molecule inhibitors of PI3Ks, PI-103 was only slightly more selective for p110α than for other p110 isoforms, limiting its use for the investigation of

the specific physiological roles mediated by individual PI3K isoforms (Knight et al., 2006).

#### *1.2.3.1 Selective class I PI3K inhibitors*

Class I PI3Ks have been implicated in cellular processes such as migration, survival and proliferation and their deregulation results in diseases such as cancer and inflammatory disorders (discussed in section 1.2.2.3). Subsequently, the therapeutic potential of inhibiting PI3Ks was realised but such broad inhibition of the PI3K family and PI3K-related kinases by LY294002 or wortmannin contributed to unwanted side-effects during drug discovery screens, restricting their use medicinally. Furthermore, a lack of selectivity for individual kinases impedes the investigation of the role of particular isoforms in cellular functions. The crystallisation of broad selectivity inhibitors bound to the ATP-binding site of PI3K catalytic subunits and the elucidation of crystal structures led to the possibility of developing isoform selective inhibitors by exploiting subtle differences in ATP-binding pockets (Knight et al., 2006, Walker et al., 2000). Therefore, these selective isoform inhibitors provide new hope for targeting isoforms implicated in specific diseases whilst limiting toxic effects and have aided investigation into the functional and physiological roles of individual isoforms in specific systems (reviewed by Crabbe et al., 2007).

The ATP binding pockets of PI3Ks have similar amino acid sequences in each isoform, thus selective inhibitors must exploit subtle conformational differences in order to achieve selectivity (Knight et al., 2004). Modelling based on the structure of LY294002 and the crystal structure of p110γ indicated that selectivity arises due to an induced conformational change resulting from inhibitor occupancy of the entrance to the ATP binding pocket (Walker et al., 2000, Knight et al., 2006). At this site, hydrogen bonds form between PI3K and the inhibitor in much the same orientation as hydrogen bonds formed with ATP. Furthermore, affinity arises by accessing deeper within the ATP binding pocket; co-crystallisation of p110γ and highly selective inhibitors confirmed that this deep hydrophobic pocket was occupied (Knight et al., 2006). The chemical structures of all PI3K inhibitors used in this study are shown in Table 2.3.

The synthesis of IC87114 (Sadhu et al., 2001), one of the earliest isoform selective inhibitors, led to the confirmation that p110δ has an essential role in neutrophil polarisation and specific directional movement (Sadhu et al., 2003). In the same screen used to assess PI-103 selectivity, the $IC_{50}$ value for IC87114 against p110δ was

0.13μM while the $IC_{50}$ values for p110β and p110γ were 16μM and 61μM respectively (Knight et al., 2006). Of the Thrombogenix series of compounds, TGX-121 (Robertson et al., 2001) and TGX-221 (Jackson et al., 2004) preferentially inhibit p110β. TGX-221 is more potent than TGX-121, and aided the investigation of the role of p110β in platelet activation in response to fluid sheer stress (Jackson et al., 2005). Two structurally distinct p110α inhibitors have been used in the present study, PIK-75 and compound 15e. PIK-75 was also screened for kinase selectivity and found to inhibit p110α with an $IC_{50}$ of 5.8nM, approximately 10 fold, 100 fold and 200 fold more potent at inhibiting p110α than p110γ, p110δ and p110β respectively (Knight et al., 2006). However, PIK-75 displays inhibition of DNA-PK in the nM range and inhibition of mTORC1 and mTORC2 in the μM range (Knight et al., 2006). In comparison, little information is available regarding compound 15e other than synthesis information and preliminary studies indicating that compound 15e is effective at inhibiting A375 cell proliferation with an $IC_{50}$ of 580nM (Hayakawa et al., 2006). As yet, no reports exist which assess the effects of compound 15e on mTOR or other PI3K related kinases. The class $I_B$ PI3K inhibitor AS605240 has been used to effectively inhibit joint inflammation in a mouse model of rheumatoid arthritis (Camps et al., 2005) and to prolong survival in a mouse model of systemic lupus erythematosus (Barber et al., 2005). Thus, both broad selectivity and class I PI3K selective inhibitors have contributed to investigations into the roles of PI3K class I isoforms in healthy and disease states, and may provide future therapies (Crabbe et al., 2007).

## 1.3 Stem Cells

### *1.3.1 Stem cells: A brief history*

In the 1960s researchers discovered that cells of the bone marrow were capable of forming more than one type of specialised blood cell (Till and McCullock, 1961). Consequently the idea of a common, 'stem' cell population giving rise to other cell types came to be. The first known stem cells to be isolated and grown in culture were those from mouse teratocarcinomas. These gonadal tumours comprised both undifferentiated cells and differentiated cells from each of the three embryonic germ layers; endoderm, mesoderm and ectoderm (Martin and Evans, 1975). The undifferentiated cells, named Embryonic Carcinoma Cells (ECCs), were shown to be capable of differentiation into any cell type of the three germ layers when co-cultured with mitotically inactivated embryonic fibroblast cells. This feeder layer of cells provided ECCs with factors thought to be important for growth and differentiation (Martin, 1980). Investigation into ECC culture requirements and characterisation of these cells led to the subsequent isolation of embryonic stem cells (Evans and Kaufman, 1981).

#### *1.3.1.1 Fertilisation and pre-implantation development*

Following fertilisation, the pre-implantation embryo is dependent on autocrine factors for survival and growth. The embryo undergoes sequential mitotic cell divisions, establishes polarity and undergoes compaction, where cell outlines cease to be visible, to form the morula. The blastocyst stage of embryonic development starts at three to four days post-fertilisation in mice. At this stage, the earliest differentiation steps have already taken place. The blastocyst comprises of an outer trophectoderm layer, a cavity, known as the blastocoel, and an inner cell mass (ICM) that gives rise to the embryo proper (Figure 1.6). Isolated pluripotent cells of the ICM of blastocyst stage embryos generate all cell types of the adult organism *in vitro* but do not generate extraembryonic lineage cells of the trophoblast or primitive endoderm (Rossant, 2008).

During the late blastocyst stage, the outer trophectoderm attaches to the uterine lining to commence implantation. Additionally, trophectoderm develops into extraembryonic ectoderm followed by the placenta and chorion or terminal differentiation into primary trophoblast cells (Rossant and Cross, 2001). At the blastocyst stage the ICM delaminates and gives rise to primitive ectoderm and a layer of primitive endoderm

(Figure 1.7). Primitive ectoderm develops into the epiblast, from which all of the embryo cell types (three germ layers) are derived as well as extraembryonic mesoderm. Primitive endoderm, at the surface of the ICM, forms parietal endoderm and visceral endoderm, contributing to the yolk sac (Lu et al., 2001). Development of the implanted embryo ensues. At embryonic day 10.5 (E10.5, days post-fertilisation) in mice, the embryo is termed a foetus and the gestation period for mice is generally 19-21 days. (Boiani and Scholer, 2005, Wang and Dey, 2006).

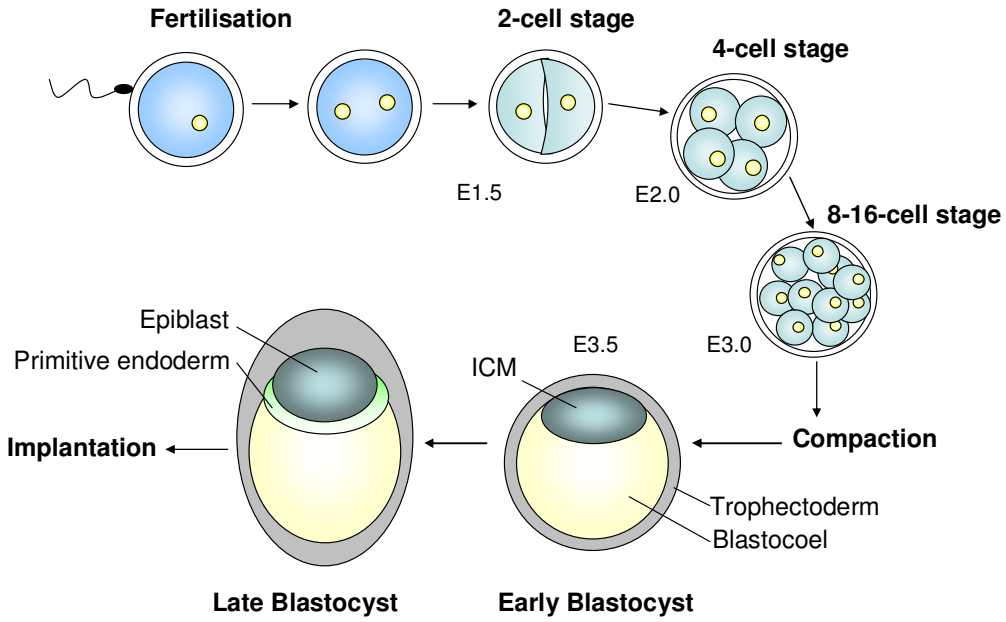



**Figure 1.6 Pre-implantation development in the mouse**

Following fertilisation, the embryo undergoes multiple rounds of mitotic cell division. Cells flatten and their outlines are not easily distinguishable at compaction. On embryonic day 3.5 (E3.5) a blastocyst forms consisting of an inner cell mass (ICM) and a blastocoel cavity. The blastocyst is surrounded by trophectoderm cells. When the embryo implants, cells of the trophectoderm develop into the trophoblast cells, while the cells of the ICM contribute to the epiblast and primitive ectoderm. Adapted from Boiani and Scholer, 2005, Wang and Dey, 2006.

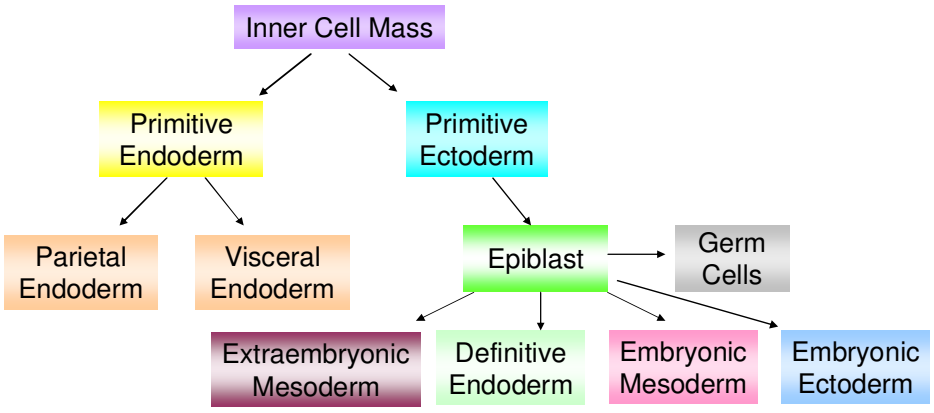



**Figure 1.7 Early lineage commitment**

Cells of the ICM differentiate into two distinct lineages; Primitive endoderm and Primitive ectoderm. Primitive ectoderm gives rise to the epiblast, from which all cell types of the embryo are derived by differentiation into cells representing the three germ layers. Additionally, the epiblast forms extraembryonic mesoderm and germ cells.

### *1.3.2 Derivation, sources and properties of stem cells*

Pluripotency describes the ability of stem cells to form any differentiated cell type of the adult organism. The inner cell mass contains pluripotent cells from which murine embryonic stem cell (mESC) lines can be derived (Evans and Kaufman, 1981). Isolation of these cells from blastocyst stage embryos can be achieved at E3.5 (Figure 1.8).

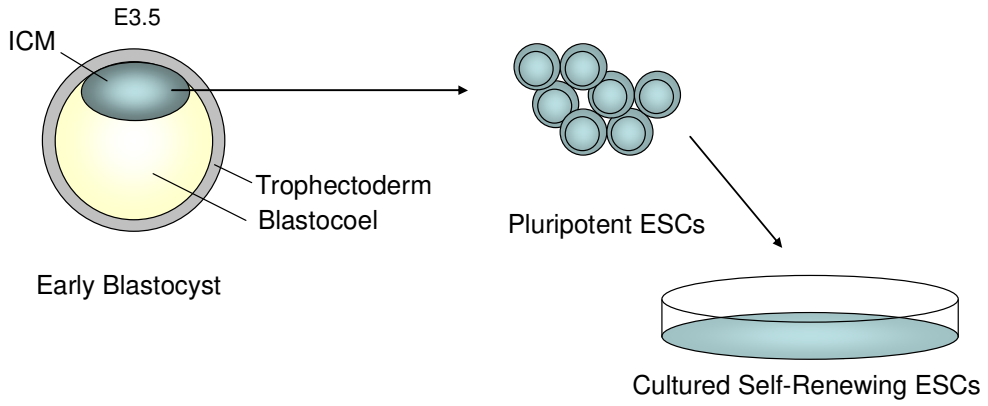



**Figure 1.8 Isolation of murine ESC lines**

At E3.5 cells of the ICM are pluripotent. ESC lines can be isolated from blastocyst stage embryos and grown in culture in a pluripotent, self-renewing manner. ESCs characteristically have large nuclei compared to cytosol volume. Given the right growth conditions, these ESC lines can be cultured long-term whilst retaining their pluripotency.

#### *1.3.2.1 The properties of self-renewal and pluripotency*

Stem cells have the unique properties of self-renewal and pluripotency. The term self-renewal encompasses a number of cellular events; maintenance of pluripotency, continuous proliferation, inhibition of apoptosis and prevention of differentiation. Thus, self-renewal is defined as proliferation with the suppression of differentiation (Smith, 2001, Burdon et al., 2002). Differentiation describes the process of a cell, such as a stem cell, becoming a more specialised cell type whereas self-renewal maintains the less specialised cell type but retains the ability to differentiate. Therefore self-renewal is necessary to maintain pluripotency (Figure 1.9).

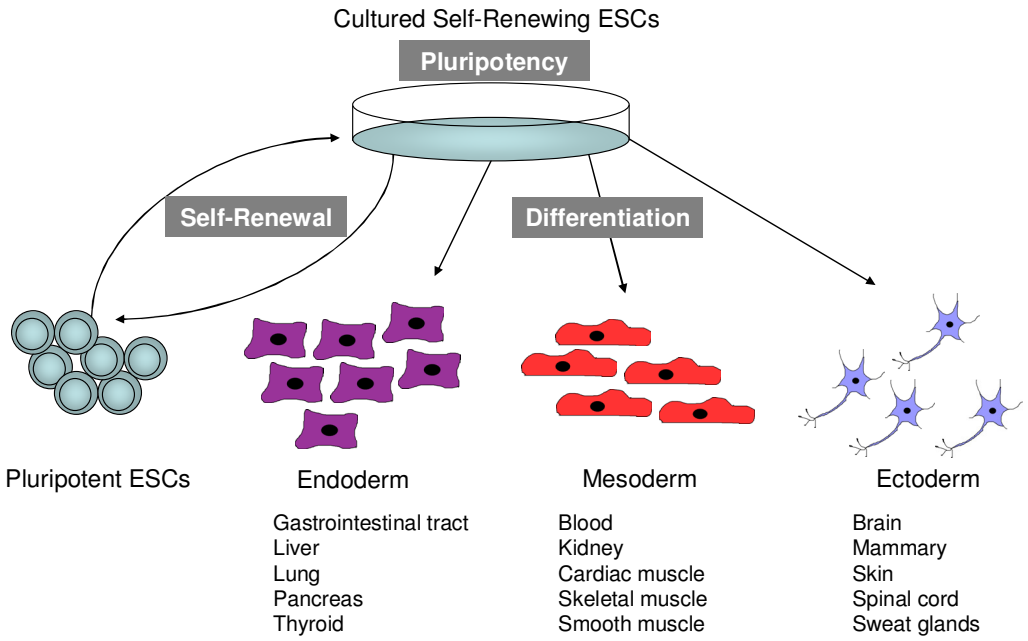



**Figure 1.9 Self-renewal and pluripotency**

Self-renewal of ESCs maintains a population of undifferentiated, pluripotent, proliferating cells. However, spontaneous differentiation is a feature of ESCs in culture. Differentiation into any cell type is a unique property of embryonic stem cells and demonstrates their pluripotency. Embryonic stem cells form progenitor cells that undergo further differentiation along a particular lineage toward specialised cell types (Evans and Kaufman, 1981, Smith, 2001, Keller, 2005). Embryonic stem cells can give rise to any cell type of the adult organism by differentiation to any of the three germ layers (Evans and Kaufman, 1981). Embryonic stem cells *in vivo* and *in vitro* primarily differentiate into multipotent progenitor cells. Unlike embryonic stem cells, progenitor cells are already specialised to a certain degree, thus further differentiation and specialisation is limited to a particular lineage.

#### *1.3.2.2 Human embryonic stem cells*

Human ESC (hESC) lines were first established in 1998 (Thomson et al., 1998) following previous success in the isolation of ESC from other primate species (Thomson et al., 1995, Thomson and Marshall, 1998). Despite ethical concerns and controversy, the isolation of hESCs was conducted with a view to researching potential cell-based therapies and for the study of early human embryo development. *In vivo* hESCs can divide indefinitely maintaining an undifferentiated, pluripotent state (Hwang et al., 2004, Park et al., 2003a) much the same as mESCs. Additionally the pluripotent

nature of hESCs allows differentiation to produce cells representative of all lineages of the adult organism (Amit et al., 2000, Itskovitz-Eldor et al., 2000, Reubinoff et al., 2000, Schuldiner et al., 2000, Pera et al., 2004, Draper and Fox, 2003). The similarities and differences in the underlying mechanisms that regulate maintenance of undifferentiated, pluripotent ESCs from humans and mice are described in section 1.3.7.

#### *1.3.2.3 Other sources of stem cells*

Unlike embryonic stems cells which are pluripotent, the ability of adult stem cells to differentiate is restricted. Thus, adult stem cells are described as multipotent or unipotent since their differentiation potential is limited to several distinct specialised cell types or a single mature cell type. Adult stem cells also have limited self-renewal potential compared to ESCs but serve to generate new tissue to replace lost or damaged tissue through homeostasis or injury. Examples of adult stem cells include haematopoietic stem cells which give rise to all myeloid and lymphoid blood lineages, mesenchymal stem cells, derived from bone marrow that produce bone, cartilage and tendons, and gut crypt cells that form enterocytes, enteroendocrine cells and Paneth cells (Ogawa, 1993, Eckfeldt et al., 2005). Aside from ESCs and adult stem cell populations, other stem cell sources include fetal and umbilical cord stem cells. These are reported to be multipotent but researchers hold hopes that these may also be used to derive specialised cell types for transplantation (Mimeault et al., 2007).

### *1.3.3 Potential uses of ESCs*

ESCs are a good model system for early embryonic development. Much research has involved gene targeting in ESCs, or the isolation of ESCs from embryos following gene targeting, providing evidence for the role of particular genes in development and differentiation (Williams et al., 2000, Winnier et al., 1995, Anton et al., 2007, Lin et al., 2005, Sun et al., 1999, Stiles et al., 2002, Stambolic et al., 1998, Suzuki et al., 1998). In addition ESCs undergo both spontaneous and induced differentiation when cultured *in vitro* allowing research into the field of lineage commitment (Cheng et al., 1998, Ying et al., 2003a, Ying et al., 2003b). Using ESCs as a model of early embryo development, populations of cells have been identified that are examples of early progenitors gaining insights into lineage commitment and the induction of differentiation. For example, the culture conditions necessary to induce mESCs to differentiate into early primitive ectoderm-like cells, similar to primitive ectoderm cells of

an E4.75 mouse embryo, have been described (Rathjen et al., 1999). The proliferation capacity and cell cycle characteristics of ESCs resembles that of cancerous cells which has furthered knowledge of tumour growth and allowed more specific treatments to be investigated (Cheng et al., 1998, Sun et al., 1999, Savatier et al., 1994).

The limited supply of adult stem cells, the isolation of which is often difficult and painful for the donor, is compounded by their limited capacity for self-renewal, reducing the ability of adult stem cells to be expanded in culture *in vitro* for investigation. The pluripotent nature of ESCs means that these cells have the ability to become a broad array of cell types, while adult stem cell differentiation is somewhat restricted. Therefore, ESCs having undergone expansion and directed differentiation have the potential to treat more diverse pathological disorders. The use of ESCs in the field of regenerative medicine, the use of live cells or tissue structures to repair or replace cells or tissue that is damaged or dysfunctional, is rapidly expanding. Much research focuses on both the expansion of ESCs and directed differentiation into homogenous cultures *ex vivo*, followed by transplantation (Figure 1.10). Notably, the formation of insulin-secreting cells derived from ESCs provides new hope for diabetes treatment (Soria et al., 2000, Segev et al., 2004). Myocardial infarction causes ischemic damage to cardiac cells which could be replaced with cardiomyocytes derived from ESCs (Kolossov et al., 2006). In the field of neurodegeneration research, ESC-derived neural precursor cells have already been described (Lang et al., 2004) and used to effectively relieve motor dysfunction in a rat model of Parkinson's disease (Bjorklund et al., 2002). Furthermore, the concept of regenerative medicine has been expanded beyond purely the use of ESC-derived cell types and tissue structures. Genetic manipulation prior to transplantation may not only reduce the risk of rejection but ESC-derived cells may also be a novel vehicle for gene therapy to treat genetic diseases and cancer (Mimeault et al., 2007).

An important area of investigation regarding the use of ESC-derived cells for transplantation is how to avoid the transplantation of undifferentiated ESCs, which has been shown to produce teratocarcinomas (Thomson et al., 1998, Fujikawa et al., 2005). Specific lineage selection, elimination of undifferentiated cells and transplantation of precursor cells versus mature, terminally differentiated cells may all avoid unwanted teratocarcinoma formation and enhance the establishment of transplanted cells (Keller, 2005). Additionally, the intraspecies and interspecies transfer of pathogens, a risk of growing human stem cells on feeder layers prior to transplantation, might be overcome by using ESC-derived feeder cells too. Both mouse and human ESC-derived embryonic fibroblast cells have been isolated and

characterised for use as feeder cells for culturing undifferentiated, pluripotent ESCs (Shi et al., 2006b, Wang et al., 2005).

ESCs and ESC-derived cell types may provide researchers with an unlimited supply of cells on which to test new candidate drugs. The area of drug discovery and development currently relies on animal models, limited human cell lines and human volunteers. It is hoped that drug toxicity testing on ESC-derived cell types will benefit this area of research (Keller, 2005). Furthermore, given that ESCs are, in themselves, a model of early embryo development, they could be used to predict the toxicological effects of new drugs on embryos in a more ethically sound manner (Figure 1.10).

Recent research has led to a unique method of inducing differentiated cells towards a pluripotent state. Mouse skin cells were reverted to ESC-like cells by the expression of four mESC-specific genes (Takahashi and Yamanaka, 2006, Okita et al., 2007). More recently, adult human fibroblasts have been genetically manipulated to form these induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007) and although less efficient, only three genes were required to create iPSCs from both mouse and human fibroblasts (Nakagawa et al., 2008) (iPSCs are described in more detail in Section 1.3.4.8). Indeed a number of strategies have been taken to achieve reprogramming of somatic cells including nuclear transfer, cell fusion, cell explantation and virus infection (Jaenisch and Young, 2008). Currently, the efficiency of generating iPSCs is extremely low due to multiple transfection requirements, the slow formation of iPSCs from somatic cell types and subsequent selection of iPSCs from the population. It is therefore important that iPSCs continue to be investigated alongside ESCs since if the differences between iPSCs and ESCs are not detrimental to their therapeutic potential, iPSCs could be used to generate patient-specific regenerated tissues for therapy (Condic and Rao, 2008). Furthermore, the use of patient-specific iPSCs would negate immune-rejection issues that would be associated by using even best-match ESC-derived cells (Figure 1.10). Avoiding the use of immunosuppressants would also overcome a challenge in the fields of cell replacement therapy and regenerative medicine.

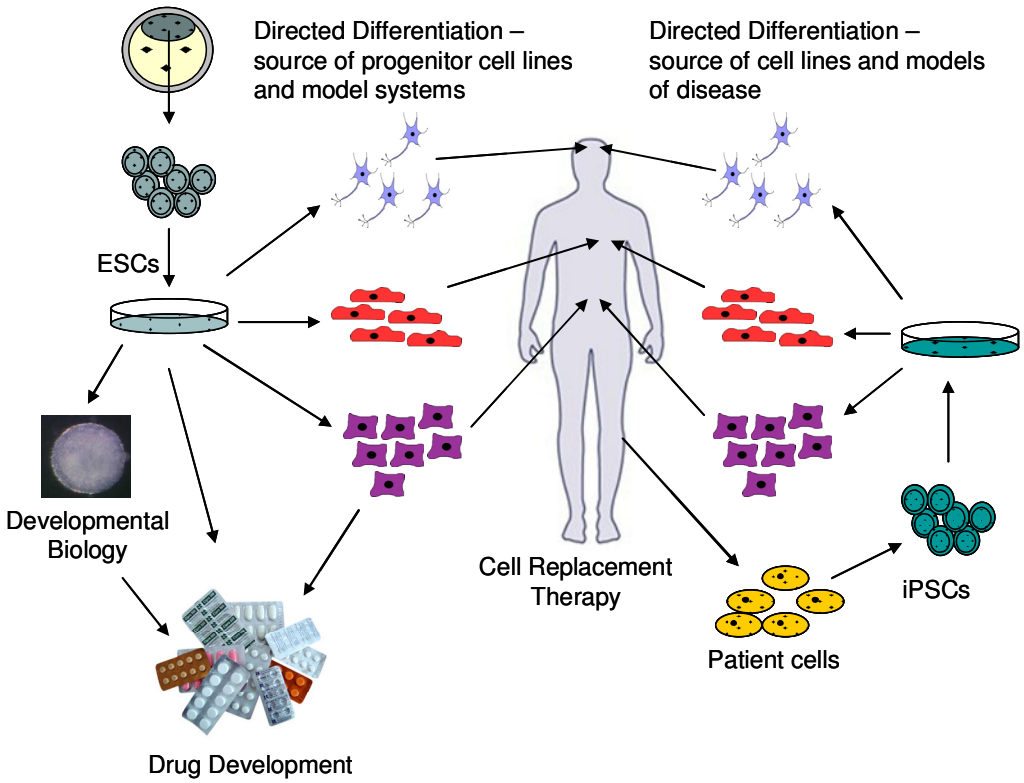



**Figure 1.10 Potential and current uses of ESCs and iPSCs**

ESCs not only have great potential for use in the fields of regenerative medicine and cell replacement therapy but also provide a model system for the study of developmental biology and for drug development (Keller, 2005). In addition, ESC-derived cells might be used for cell replacement therapy, drug development and as a source of progenitor or differentiated cell lines for research. More recently, iPSCs, which are derived from differentiated cell types and forced towards a pluripotent state, exhibiting properties similar to ESCs have been described (Takahashi et al., 2007, Nakagawa et al., 2008). Investigations into iPSCs are still in the early stages but they might provide patient-specific cells for replacement therapy or be used as disease model cell lines.

In order to realise the therapeutic potential of ESCs or iPSCs, a thorough understanding of the underlying mechanisms regulating self-renewal and differentiation is required. Murine ESCs were the first to be isolated and show some similarities to human ESCs, thus make an ideal replacement with fewer ethical concerns (Koestenbauer et al., 2006, Evans and Kaufman, 1981). Much research has focused on identifying master regulators of self-renewal but as with other important cellular functions, multiple pathways exist to maintain, regulate, direct and modify cell fate.

### *1.3.4 Murine embryonic stem cell self-renewal*

#### *1.3.4.1 Markers of mESC self-renewal*

In order to effectively research and assess self-renewal of mESCs, a number of markers are used. It is important to note that the presence of self-renewal markers indicates that ESCs are in an undifferentiated state but does not indicate that ESCs are pluripotent. To assess pluripotency, cells must be induced to differentiate or allowed to freely differentiate before detection of a panel of markers representing early lineages are used to confirm pluripotency.

Alkaline phosphatase is a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions. It is present within all tissues of the body but is elevated in cells under specific diseased states and in cells of the liver, kidney, bone, placenta and embryo (Scutt and Bertram, 1999). Dense staining of alkaline phosphatase was demonstrated in the ICM of murine blastocysts (Johnson et al., 1977). Consistent with this, alkaline phosphatase activity is detectable on the cell surface of undifferentiated, self-renewing ESCs but expression is lost upon differentiation (Pease et al., 1990). Enzymatic assay for alkaline phosphatase activity is frequently used as a measure of self-renewal and, due to cell surface expression in undifferentiated cells, positive staining correlates with alkaline phosphatase activity at the cell surface of self-renewing ESCs (Pease et al., 1990).

A number of transcription factors are known to have higher expression in self-renewing ESCs compared to differentiated cell types. Oct4 was first identified as an ESC and germ line specific transcription factor (Okamoto et al., 1990). Oct4 is encoded by the gene *Pou5f1* and is a member of the Octamer transcription factor family, which contain a POU (Pit Oct Unc) domain. POU domains share sequence homology with the mammalian genes *Oct1*, *Oct2* and *Pit1*, and the *Caenorhabditis elegans* gene *Unc-86* (Ryan and Rosenfeld, 1997). Oct4 interacts with the octamer DNA motif (5'-ATGCAAAT-3') via its POU domain to regulate transcription. Oct4 is expressed in pre-implantation embryos, undifferentiated mESCs, epiblast cells and germ cells and Oct4 expression is essential for the formation of pluripotent founder cells within the mammalian embryo (Okamoto et al., 1990, Schöler et al., 1990). Consistent with this, cells isolated from blastocyst stage embryos lacking Oct4 expression are not pluripotent (Nichols et al., 1998) and induction of mESC differentiation with retinoic acid results in a reduction in Oct4 expression (Okamoto et al., 1990). Oct4 regulates mESC fate by the level of Oct4 expression in that a less than two-fold increase in Oct4

expression was reported to result in differentiation towards primitive endodermal and mesodermal lineages (Niwa et al., 2000) Conversely, the same study reported that reduced expression of Oct4, to levels less than half that of normal expression, led to a loss of pluripotency and the expression of trophectodermal markers (Niwa et al., 2000). Therefore, Oct4 is thought to maintain self-renewal at a critical level of expression, deviations from this level of expression determine both cell fate and lineage commitment (Niwa, 2001).

Another important regulator of self-renewal is Sox2, a Sox (SRY-related HMG box) gene family transcription factor with notable expression in pluripotent and multipotent cell types such as the ICM, epiblast cells, germ cells and extraembryonic ectoderm. In the absence of Sox2, blastocysts form, but embryos fail to survive after implantation (Avilion et al., 2003). Around the same time, a report was published detailing an *in silico* screen for genes that maintain pluripotency and are expressed exclusively in the ICM and in ESCs. This screen highlighted nine genes but for one particular gene, expression was not detected in twelve somatic tissues (Mitsui et al., 2003). Furthermore, expression of this gene in ESCs was reportedly reduced by retinoic acid-induced differentiation (Mitsui et al., 2003). Following functional expression cloning, this gene was demonstrated to express a transcription factor, which was subsequently named Nanog after Tír na nÓg (an island in Celtic mythology which, translated to English, means 'land of the ever young') (Chambers et al., 2003). Nanog is a homeodomain-containing transcription factor and is necessary for pluripotency of the ICM and ESCs (Mitsui et al., 2003), thus it is often referred to as a master regulator of self-renewal, although Oct4 and Sox2 are also required for maintaining self-renewal. A 5 fold enhancement in the expression of Nanog is reported to maintain mESC self-renewal even in the absence of LIF, a major promoter of mESC self-renewal, whereas reduced Nanog expression leads to extraembryonic endoderm differentiation (Mitsui et al., 2003, Chambers et al., 2003).

The zinc finger protein Reduced Expression-1, Rex1 (also referred to as *Zfp42*) was initially identified in ECCs and noted for its expression, which was down-regulated following retinoic acid-induced differentiation (Hosler et al., 1989). Rex1 was demonstrated to be specifically expressed in ESCs, pre-implantation embryos, trophoblast and spermatocytes. Furthermore, it it a suitable marker for self-renewing mESCs since expression is reduced following induction of differentiation (Rogers et al., 1991). Although Rex1 is used as a marker of self-renewing mESCs, unlike Nanog and Oct4, Rex1 expression is dispensable for the maintenance of self-renewal of mESCs and is not required for the normal development of mouse embryos (Masui et al., 2008).

Detection of one or more of these markers is frequently used to assess self-renewal of ESCs and forms the basis of investigations into the signalling underlying the maintenance of self-renewal.

#### *1.3.4.2 Leukaemia inhibitory factor-mediated intracellular signalling*

Murine ESCs can be cultured on a feeder layer of mouse embryonic fibroblast cells (MEFs) which produce the cytokine Leukaemia Inhibitory Factor (LIF). LIF supports mESC growth and maintenance of self-renewal (Smith et al., 1988, Bard and Ross, 1991, Gough et al., 1989, Williams et al., 1988) and must be supplemented in the medium for mESCs to be grown in feeder-free conditions.

LIF binds to a heteromeric complex of the LIF recetor (LIFR) and the common cytokine receptor gp130 (Ernst and Jenkins, 2004). Janus-associated tyrosine kinase (JAK) remains associated with gp130 but phosphorylates tyrosine residues of gp130 and LIFR upon LIF binding. This phosphorylation prompts the recruitment of signal transducer and activator of transcription 3 (Stat3), the SH2 domains of which bind tyrosine phosphorylated residues on the intracellular regions of these receptors (Burdon et al., 1999, Stahl et al., 1995). Recruitment allows the phosphorylation of Stat3 by JAK. Phosphorylated Stat3 homodimerises and translocates to the nucleus where it regulates transcription of target genes (Figure 1.11).

LIF-induced activation of the Jak/Stat3 pathway is important for the maintenance of pluripotency and self-renewal of mESCs (Boeuf et al., 1997, Burdon et al., 2002, Niwa et al., 1998, Matsuda et al., 1999, Smith, 2001). In fact, LIF withdrawal causes a reduction in Nanog protein expression, coinciding with a loss of self-renewal (Storm et al., 2007). Artificial induction of Stat3 activation was sufficient to maintain self-renewal without the requirement for LIF (Matsuda et al., 1999). Inhibition of Stat3 activation, by the expression of a mutated form of Stat3 lacking the ability to dimerise, induced differentiation of mESCs (Niwa et al., 1998). It was observed by Raz et al., 1999 that different mESC lines exhibit varying dependency on LIF but a strong correlation existed between nuclear Stat3 binding activity and the formation of pluripotent mESC colonies of cells plated at clonal density (Raz et al., 1999). Moreover, over-expression of Stat3 removes the requirement for LIF in order to support self-renewal in the presence of serum (Boeuf et al., 1997). The key effector and transcriptional target of the LIF/Stat3 pathway has been reported to be the proto-oncogene c-myc, which, in other cell types

regulates cell proliferation, growth and apoptosis. The activity c-myc in mESCs has been reported to be necessary for self-renewal maintenance (Cartwright et al., 2005).

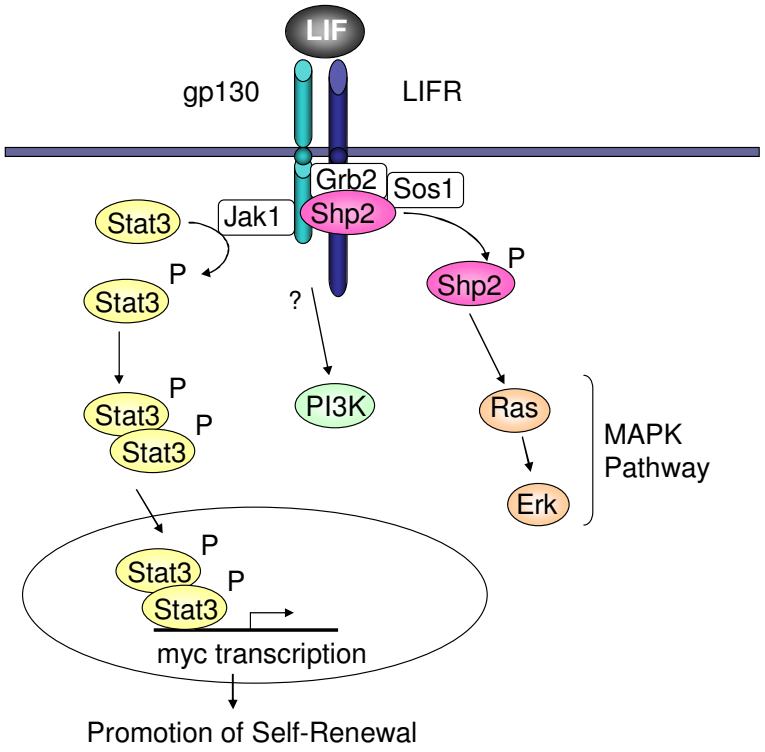



**Figure 1.11 LIF-dependent regulation of self-renewal**

LIF promotes the self-renewal of mESCs via the activation of Stat3. Phosphorylated Stat3 dimerises and translocates to the nucleus where it regulates the expression of c-myc. LIF can also activate PI3K and Ras/Erk signalling by a Stat3-independent route.

The Mitogen Activated Protein Kinase (MAPK) and PI3K signalling pathways are also reported to be activated by LIF (Figure 1.11) indicating that LIF is capable of regulating Stat3-independent pathways in mESCs (Burdon et al., 1999, Qu and Feng, 1998). The mechanism of activation of the MAPK pathway by LIF is thought to be via SH2 domain-containing tyrosine phosphatase-2 (Shp-2). Shp-2 is recruited to the plasma membrane, via its SH2 domains binding to phosphorylated tyrosine residues, where it binds the adaptor protein growth factor receptor binding protein (Grb2). By binding Grb2, which is itself bound to the guanine nucleotide exchange factor for Ras (Sos1), Shp-2 is capable of activating MAPK signalling via Ras following LIF receptor stimulation (Figure 1.11) (Fukada et al., 1996, Ernst et al., 1996). The mechanism of LIF-induced activation of the PI3K pathway in mESCs is yet to be defined although, in Hep-G2 cells, Shp-2 was reported to associate with Grb2-associated binder (Gab), to

activate the PI3K pathway following activation of the cytokine receptor gp130 (Takahashi-Tezuka et al., 1998), which is a component of the LIF receptor heterodimer (Ernst and Jenkins, 2004). The activation of MAPK and PI3K signalling and their role in regulating mESC fate are described in detail in Sections 1.3.4.6 and 1.3.6 respectively.

With regards to the maintenance of self-renewal, LIF activation of Stat3 is reported to prevent mesoderm and endoderm differentiation (Ying et al., 2003a) while in serum-free conditions LIF induces neural differentiation (Ying et al., 2003a, Ying et al., 2003b). In such serum-free culture conditions, BMP4 is required in addition to LIF, in order to maintain self-renewal of mESCs (see Sections 1.3.4.3 and 1.3.4.4).

LIF has also been reported to activate Src family non-receptor tyrosine kinases. In particular c-Yes and Hck were shown to be activated by LIF in mESCs (Annerén et al., 2004, Meyn et al., 2005). Introduction of an activated mutant of Hck into mESCs reduced the concentration of LIF required to maintain self-renewal (Annerén et al., 2004). Furthermore, Hck was found to be associated with gp130 of the LIF receptor heterodimer (Ernst et al., 1994). Activity of c-Yes could be stimulated by LIF or by factors present in serum and activity was down-regulated upon LIF withdrawal or following the induction of differentiation, although protein levels remained the same. Differentiation of mESCs was induced following treatment with a selective Src family kinase inhibitor or expression of c-Yes short interfering RNA (siRNA). Furthermore, c-Yes activity was found to be independent of the Jak/Stat and MAPK pathways and represents an alternative pathway for LIF to maintain self-renewal (Annerén et al., 2004). To date seven of the eight known Src family kinases have been shown to be expressed in self-renewing mESCs (Thomas and Brugge, 1997). Expression of Lyn, Fyn, Src and Yes remains constant during embryoid body differentiation but Fgr, Hck and Lck expression decreases upon differentiation. Intriguingly, partial inhibition with low doses of inhibitors or inhibitors that target only selective Src family kinases induced differentiation even in the presence of LIF, while higher doses or more potent inhibitors maintained the undifferentiated state even in the absence of LIF. In-depth analysis of the expression profiles of Src family kinase members in mESCs alongside the inhibition profiles of the inhibitors used led to a model of two opposing pathways for Src family members. The authors suggested Hck and Yes promote self-renewal in the presence of LIF. Upon LIF removal when Hck expression is decreased, or in the presence of Hck and Yes selective inhibition, this pro-self-renewal signal is reduced allowing Src and Fyn to promote differentiation (Figure 1.12) (Meyn et al., 2005).

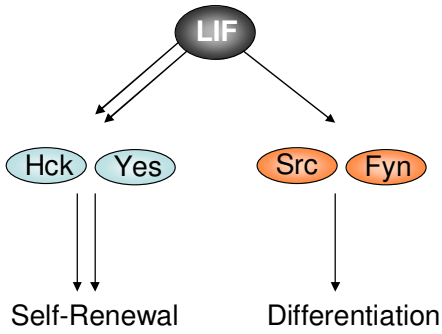



**Figure 1.12 Non-receptor tyrosine kinase regulation of self-renewal**
The Src family of non-receptor tyrosine kinases have been shown to be activated by LIF, independent of the Jak/Stat pathway. Src and Fyn are expressed in self-renewing mESCs and throughout differentiation. Hck and Yes promote self-renewal via a predominant pathway. When Hck expression is reduced upon the initiation of differentiation, Src and Fyn act to promote differentiation. Adapted from Meyn et al., 2005.

#### *1.3.4.3 Bone morphogenic proteins contribute to mESC self-renewal maintenance*

Bone morphogenic proteins (BMPs) are growth factors belonging to the transforming growth factor beta (TGFβ) superfamiy. BMPs have two types of receptors, all of which are transmembrane serine/threonine kinases; Type I receptors, Alk2, Alk3 (BmprIa) and Alk6 (BmprIb) and type II receptor, BmprII. Undifferentiated mESCs express BmprIa and BmprII (Qi et al., 2004). Binding of BMP to a dimer of type I and type II receptors initiates the canonical BMP signalling pathway (Figure 1.13). Ligand binding activates intracellular mediators, Smad proteins. The nomenclature of Smad proteins is derived from BMP-like signalling pathways in *Caenorhabditis elegans* (Sma) and *Drosophila* mothers against decapentaplegic (Mad) (Attisano and Wrana, 2002). In vertebrates there are eight members of the Smad family which are divided into three subgroups; receptor R-Smads, inhibitory I-Smads and a common-partner co-Smad. R-Smads Smad2 and Smad3 are activated via TGFβ and activin receptors, where as R-Smads Smad1, Smad5 and Smad8 are phosphorylated by type I BMP receptors (Itoh et al., 2000). Activation of Smad1, Smad5 or Smad8 results in homodimerisation followed by the formation of a heterotrimeric complex with co-Smad, Smad4 (Figure 1.13). In contrast, I-Smads are inhibitory, thus Smad6 and Smad7 inhibit TGFβ family

signalling and prevent the activation of R-Smads and co-Smads (Hata et al., 1998, Nakao et al., 1997). TGFβ signalling can induce the expression of Smad6 and Smad7 thereby initiating a negative feedback loop in order to regulate R-Smad and co-Smad signalling (Whitman, 1997). In the absence of I-Smads, the heterotrimeric complexes of R-Smads and co-Smads translocate and accumulate in the nucleus where, in conjunction with other transcription factors, they function to regulate the expression of target genes dependant on cell type (Itoh et al., 2000, Derynck and Zhang, 2003). In mESCs, Smad complexes are reported to regulate the transcription of members of the *Id* (inhibitor of differentiation) gene family, expression of which inhibits basic helix-loop-helix transcription factors required for differentiation (Ruzinova and Benezra, 2003).

Cross-talk exists between BMP-induced signalling and that mediated by other growth factors and cytokines. For example, reciprocal inhibition exists between Smad activity and the MAPK pathway (Figure 1.13). FGF or Ras activated Erk inhibits Smad translocation to the nucleus via phosphorylation of a linker region between the two globular domains of Smad1 (Kretzschmar et al., 1997). Further to the prevention of nuclear accumulation, phosphorylation by Erk allows additional phosphorylation of the linker region by Gsk-3 (Fuentealba et al., 2007). This double phosphorylation results in selective binding of Smurf1, an E3 ubiquitin ligase, followed by polyubquitinisation of phosphorylated Smad1 and subsequent degradation (Sapkota et al., 2007). Thus BMP-induced Smad signalling and degradation is regulated by other pathways which are of importance in stem cell fate.

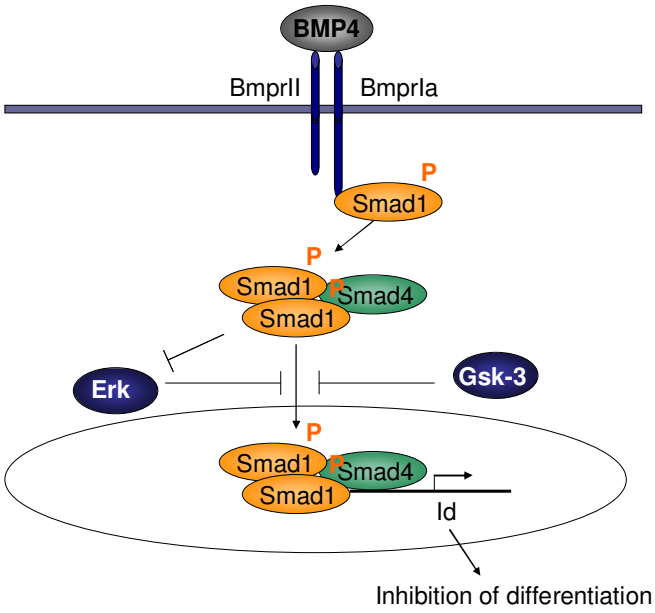



**Figure 1.13 The canonical BMP signalling pathway**

Following the binding of BMP4 to BMP receptors type I and type II, type I receptor-mediated Smad1/5/8 phosphorylation occurs. Phosphorylated Smad1 dimerises and binds to co-Smad, Smad4. This heterotrimeric complex translocates to the nucleus where it regulates transcription of Id genes. Expression of particular Id genes inhibits differentiation of mESCs. Phosphorylation of Smad1 at specific sites by FGF-induced Erk activity or inhibition of Gsk-3 by Wnt signalling prevents nuclear accumulation of the Smad1-Smad4 complex.

Over-expression of Smad1/4 or expression of constitutively active BMP receptor in mESCs relieves the requirement for LIF (Ying et al., 2003b). Moreover, mESCs can not be derived from blastocysts lacking Bmpr1a or Smad4 (Qi et al., 2004). Thus BMP signalling appears to be an important contributor, not only to the maintenance of self-renewal of mESCs in culture, but also for the establishment of ESC lines.

#### *1.3.4.4 Serum-free chemically defined culture of mESCs*

LIF can only maintain self-renewal in the presence of serum-containing media. In serum-free culture conditions, LIF alone induces neural differentiation (Wilson and Edlund, 2001, Ying et al., 2003a, Ying et al., 2003b). In conjunction with LIF, BMP4 is required to completely eliminate the requirement for serum. Such serum-free media is supplemented with N2 (Bottenstein and Sato, 1979) and B27 (Brewer and Cotman,

1989, Brewer et al., 1993) supplements of known components (listed in Table 2.1), thus can also be described as chemically-defined media. In the absence of LIF, BMP was reported to prevent the differentiation of mESCs and embryoid bodies towards neural lineages (Ying et al., 2003b, Wiles and Johansson, 1999) and to promote mesodermal differentiation (Nakayama et al., 2000). Consistent with this, treatment of hESCs with BMP alone was reported to promote differentiation towards mesoderm, endoderm and trophoblast cell types (Schuldiner et al., 2000, Xu et al., 2002, Pera et al., 2004). In mESCs, BMP is known to mediate its effects on maintaining a balance of self-renewal via Smad-mediated transcriptional regulation of Id2 gene expression (Hollnagel et al., 1999, Ying et al., 2003b). Moreover, Id2 over-expression can suppress neural differentiation in the absence of BMP (Ying et al., 2003b). Additionally, Nanog was reported to bind to Smad1 to prevent the binding to co-Smad (Smad4). This negative regulation of the BMP/Smad pathway by Nanog was suggested by authors to be a control mechanism for inhibiting BMP-induced mesoderm differentiation (Suzuki et al., 2006). Further to this, embryos with a homozygous *Bmp4*-null mutation fail to undergo mesoderm differentiation (Winnier et al., 1995). Thus, BMP4 and LIF act in synergy in serum-free media to maintain mESC pluripotency by balanced inhibition of lineage commitment towards neural and non-neural fates respectively (Figure 1.14).

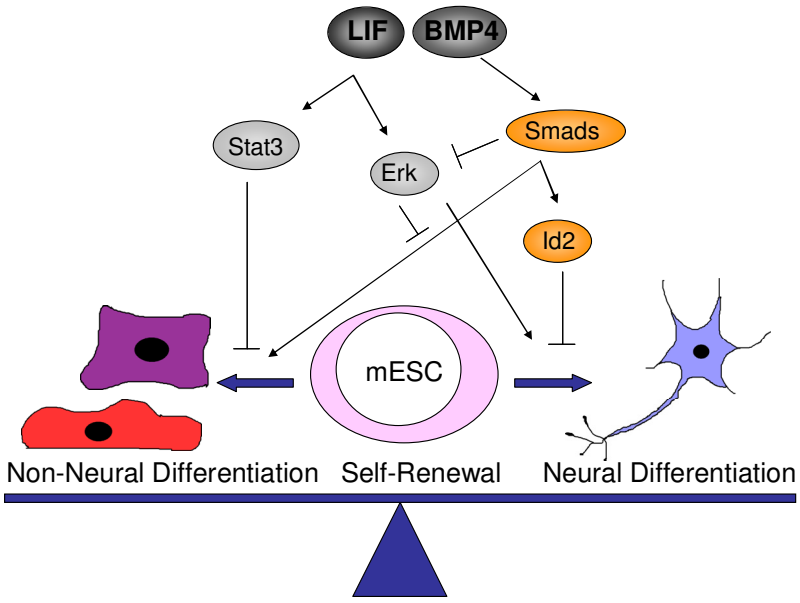



**Figure 1.14 Cross-talk of BMP4 and LIF signalling maintains pluripotency**

A balance of LIF and BMP-induced signalling is required to maintain self-renewing pluripotent mESCs in serum-free culture conditions. LIF promotes neural differentiation via the activation of Erk and inhibits non-neural differentiation via Stat3. Conversely BMP4 inhibits neural differentiation via the expression of Id2 and promotes non-neural differentiation.

#### *1.3.4.5 Autocrine and paracrine signalling in ESCs*

Although chemically defined media allows the effects of individual factors to be assessed, ESCs are known to secrete their own survival enhancing factors into culture media. In a screen for proteins secreted by undifferentiated ESCs, a variety of chemokines, growth factors and colony stimulating factors were detected at higher levels in ESC-conditioned media compared to background levels in serum-containing media (Guo et al., 2006). Conditioned media from mESCs was used to demonstrate an anti-apoptotic effect on H9c2 cardiac myoblast cells (Singla et al., 2008). An increase in cell survival was observed for H9c2 cells cultured in mESC conditioned media following $H_2O_2$ treatment to induce apoptosis. Furthermore, this reduction in apoptosis was found to be mediated by PI3K/PKB signalling (Singla et al., 2008). Additionally, mESC-conditioned media is reported to enhance the survival and colony formation of murine bone marrow myeloid progenitor cells (Guo et al., 2006). Murine ESC-conditioned media containing LIF, when applied to fresh mESCs, induces enhanced Nanog mRNA expression compared to expression levels found in mESCs cultured in unconditioned media and LIF (Welham et al., 2007). Even conditioned media made in the absence of LIF, leads to a small increase in Nanog expression when used to culture fresh mESCs and a significant increase when LIF is added after conditioning (Welham et al., 2007).

Human ESCs have also been reported to exhibit paracrine signalling, necessary for survival and maintenance of self-renewal (reviewed by Stewart et al., 2008). FGF2, known to promote self-renewal of hESCs, was reported to induce the expression of TGFβ family members, notably BMP4, by MEFs (Greber et al., 2007). This report suggests that MEF's support hESC self-renewal by expressing self-renewal promoting factors in response to FGF2. Furthermore, hESC-derived fibroblast-like cells were demonstrated to express insulin-like growth factor 2 (IGF-2), to maintain the survival of hESCs, in response to FGF (Bendall et al., 2007). This reciprocal paracrine signalling of IGF-2 and FGF between hESCs and hESC-derived fibroblast-like cells was reported to maintain hESCs in an undifferentiated state (Bendall et al., 2007). Therefore, it is likely that ESCs secrete factors that aid survival and maintain self-renewal via autocrine and paracrine signalling.

#### *1.3.4.6 MAPK pathway signalling promotes differentiation*

Six distinct classes of mitogen activated protein kinases (MAPKs) exist and all respond to extracellular stimuli, mitogens. In mESC signalling, the classical MAPKs, Extracellular signal-regulated kinases Erk1 and Erk2 along with p38 isoforms have been reported to be active. Erk and p38 MAPK signalling is important for proliferation in many other cell types (reviewed by Roux and Blenis, 2004). However, Burdon et al., demonstrated that pharmacological inhibition of Mek (MAPK Erk Kinase) with PD098059 in mESCs did not affect the propagation of cells, in fact, inhibition of Erk activation enhanced self-renewal (Burdon et al., 1999).

Erk1/2 activity can be stimulated by fibroblast growth factor 4 (FGF4) in mESCs (Kunath et al., 2007) and, interestingly, FGF4 is secreted by undifferentiated cells (Ma et al., 1992, Rathjen et al., 1999). However, mESCs lacking FGF4 do not form neural or mesodermal lineages, thus FGF4 and Erk signalling is required for complete lineage commitment (Kunath et al., 2007). In agreement with this research, Stavridis et al., 2007 demonstrated that FGF-induced Erk1/2 signalling is required during neural specification, albeit for a discrete stage-dependent period. Activation of Ras/Erk signalling via Grb2 and SHP-2 is also necessary for lineage commitment (Fukada et al., 1996, Ernst et al., 1996). Grb2-null ESCs lack the ability to differentiate into endoderm (Cheng et al., 1998). Consistent with this, transfection with constitutively active Mek induced primitive endoderm differentiation. What is more, the Grb2/Mek pathway was shown to repress Nanog expression following induction of MAPK signalling by sodium vanadate or by cell aggregation (Hamazaki et al., 2006).

Early embryogenesis can be modelled using pluripotent, self-renewing ESCs. To form embroyid bodies, ESCs are allowed to aggregate and are provided with vital differentiation inducing factors. Embryoid bodies have significant morphological and gene expression pattern similarities with early embryos (Desbaillets et al., 2000). Retrospectively, the formation of embryoid bodies and the expression of marker genes from each of the three primitive layers can be used to confirm the pluripotency of ESCs. Embryoid body formation using ESCs expressing a truncated FGF receptor 2 mutant failed to yield epithelial differentiation (Chen et al., 2006). In accordance, the inhibition of Mek following embryoid body formation was shown to prevent differentiation (Burdon et al., 1999). Expression of a mutant activated form of Ras in mESCs results in reduced self-renewal marker expression. Consistent with this, Ras was found to be activated in embryoid body differentiation. The Raf/Mek/Erk pathway

was shown to mediate Ras-activated differentiation towards extraembryonic endoderm (Yoshida-Koide et al., 2004).

A peak in p38 MAPK activity was noted between 3 and 4 days after LIF withdrawal and at day 5 of embryoid body formation (Aouadi et al., 2006). Additionally, ESCs lacking p38α, a member of the p38 MAPK family, exhibited decreased cardiomyogenesis but enhanced differentiation towards neural cell types *in vitro*. *In vivo*, deletion of p38α was embryonic lethal at E11.5-12.5 (Tamura et al., 2000) indicating that p38α is required for normal embryo development and maintenance of pluripotency. Consistent with p38 activity being associated with a loss of self-renewal, inhibition of p38 MAPK allowed an ESC line to be derived from blastocysts lacking the type I BMP receptor BmprIa (Qi et al., 2004). Thus, activation of MAPKs promotes differentiation by a number of mechanisms, including cross-talk with signalling pathways that promote self-renewal. MAPK signalling in mESCs is summarised in (Figure 1.15).

Given that MAPK signalling is activated by secreted factors and even by LIF, the major promoter of mESC self-renewal, and activation of MAPK signalling promotes differentiation, it follows that this pathway is tightly regulated by cross-talk with self-renewal promoting pathways. Consistent with this notion, it was proposed that mESC fate is determined by a fine balance between Stat3 and Erk signalling, positive and negative regulators of self-renewal respectively (Burdon et al., 1999, Niwa et al., 1998). BMP4 exerts its self-renewal-promoting effects in part by inhibiting both Erk and p38 MAPK via XIAP (X-linked inhibitor of apoptosis) (Figure 1.15) (Qi et al., 2004). XIAP expression is enhanced when BMP4 is added to culture media and links BMP type 1 receptors to p38 MAPK (Yamaguchi et al., 1999). Additionally inhibition of PI3Ks enhances basal Erk activity suggesting a dampening effect on the MAPK pathway by PI3K activity may exist in mESCs (Paling et al., 2004). Therefore, LIF and cytokine-induced activation of Erk is regulated by other signalling pathways in order to maintain a balance of self-renewal promoting signalling.

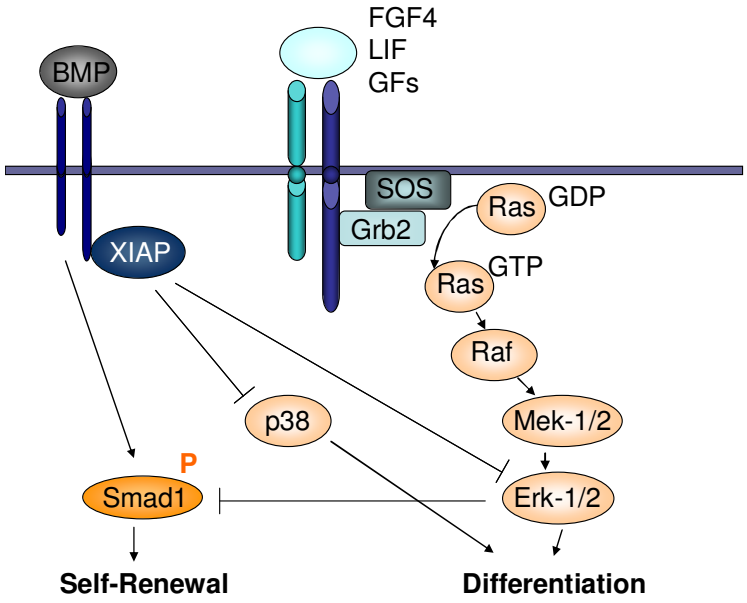



**Figure 1.15 MAPK signalling and regulation in mESCs**

MAPK signalling via the classical Erk1 and Erk2 MAPKs or via p38, promotes differentiation in a number of ways. BMP mediates its effects on self-renewal via Smad activation (described fully in the text, Section 1.3.4.3 and Figure 1.13). Smad pathway activity is inhibited by Erk, and p38 may also exhibit cross talk with the BMP/Smad pathway. Reciprocally, MAPKs can be inhibited by XIAP (X-linked inhibitor of apoptosis) following stimulation of the BMP receptor.

### *1.3.4.7 Glycogen synthase kinase-3β and mESC fate*

Glycogen Synthase Kinase-3β (Gsk-3β) is part of the canonical Wnt pathway, in which the transcription factor β-catenin regulates the expression of Wnt target genes (Figure 1.16). Briefly, the canonical Wnt pathway involves the stabilisation of β-catenin present in the cytoplasm. β-catenin uses the scaffold proteins adenomatous polyposis coli (APC) and Axin to complex with casein kinase Iα (CKIα) and Gsk-3β. These kinases phosphorylate β-catenin, marking it for ubiquitination and proteasomal degradation. When Wnt ligands bind to a Frizzled family receptor and low-density lipoprotein receptor-related protein LRP-5/6/arrow family co-receptor, a signalling cascade involving over 50 known components is initiated. Of these known components, the functions of those contributing to the canonical Wnt signalling pathway are best characterised. LRP is phosphorylated by CKIγ and Gsk-3β at the membrane. Phosphorylation of LRP provides a binding site for Axin which subsequently docks at

the membrane. Dishevelled protein associates with the Frizzled receptor and Axin, increasing the affinity of Dishevelled for Gsk-3β. Thus, the APC/Axin/CKIα/Gsk-3β destruction complex dissociates leading to the accumulation of cytoplasmic β-Catenin. Interaction with T-cell factor and Lymphoid enhancer factor (TCF/LEF) family of transcription factors allows β-catenin to bind to Wnt-responsive genes. Interaction with β-catenin removes transcriptional repression applied by TCF/LEF transcription factors and allows the transcription of genes such as c-Myc and CyclinD1 (Eisenmann, 2005, Kestler and Kühl, 2008).

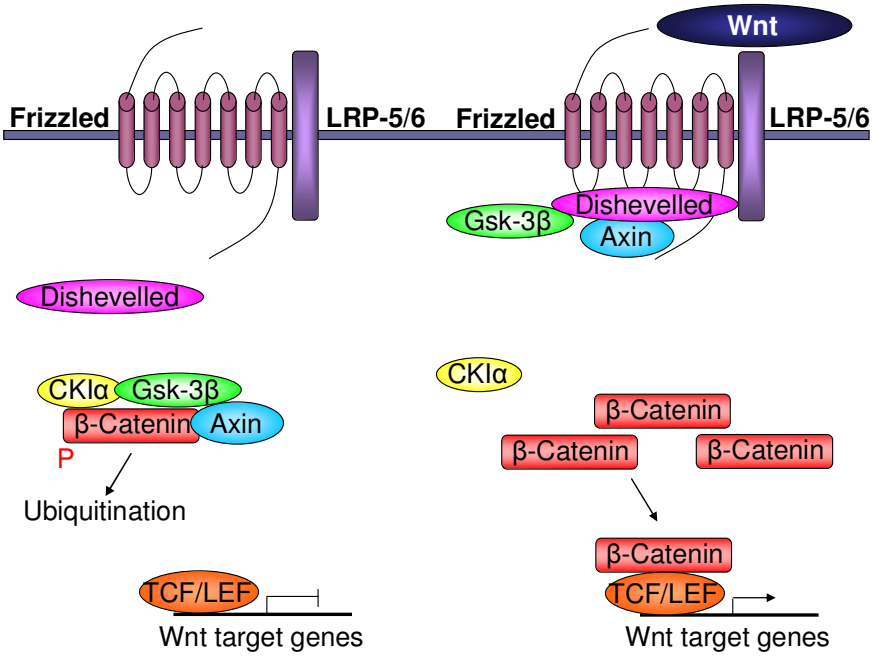



**Figure 1.16 The cannonical Wnt pathway**

Prior to a Wnt signal (left panel), a destruction complex of APC, Axin, CKIα, Gsk-3β forms and phosphorylates β-catenin for ubiquitination. TCF and LEF family transcription factors inhibit the transcription of Wnt target genes. Upon Wnt ligand binding (right panel) to the Frizzled receptor, the destruction complex dissociates and components associate with Dishevelled and Frizzled at the plasma membrane. Accumulation of β-catenin induces translocation to the nucleus, where β-catenin interacts with target genes to allow transcription. Adapted from Eisenmann, 2005, Kestler and Kühl, 2008.

Conditioned medium from cells expressing Wnt3a applied to mESCs supported the growth, maintenance of pluripotency and maintenance of self-renewal in the absence of LIF (Singla et al., 2006). Wnt5a and Wnt6 are also reported to promote self-renewal of cultured mESCs (Hao et al., 2006). Additionally, the canonical Wnt pathway was reported to be fully functional in mESCs and found to induce the expression of the self-renewal marker Rex1 (Sato et al., 2004). In mESCs deficient for β-catenin, the expression of Rex1 is reduced but negligible changes in the expression of Nanog, Sox2 and Id genes were reported (Anton et al., 2007). Consistent with a lack of change in the expression of transcription factors important for maintaining the undifferentiated state, β-catenin-null mESCs maintained self-renewal and pluripotency in culture (Anton et al., 2007). However, a conditional activation mutant of β-catenin, that allows translocation to the nucleus, was sufficient to support mESC self-renewal in the absence of LIF (Hao et al., 2006). In the same investigation, Wnt3a treatment of mESCs induced enhanced Stat3 mRNA and protein levels, which were shown to be the result of activated β-catenin. These results suggest that Wnt and LIF-induced signalling promote self-renewal and converge on Stat3 in order to do so (Hao et al., 2006). Withdrawal of LIF induces a down-regulation of Nanog and β-catenin within 3 days, however Oct-4 down-regulation does not occur until day 6. Oct4 and β-catenin have been shown to associate and sustain Nanog expression in order to contribute to the maintenance of self-renewal (Takao et al., 2007).

β-catenin is not the only substrate of Gsk-3, others have been identified such as glycogen synthase (GS) and eukaryotic protein synthesis initiation factor-2B (eIF-2B). Stimulation of cells with growth factors results in the phosphorylation and inactivation of Gsk-3 via PI3K/PKB (Cross et al., 1995). In the absence of growth factor stimulation, when Gsk-3 is active, Gsk-3 phosphorylates and inactivates GS and eIF-2B to inhibit glycogen and protein synthesis (Cohen et al., 1997, Doble and Woodgett, 2003). Gsk-3 has been shown to phosphorylate Smad1 in mESCs thereby preventing the nuclear accumulation of Smad complexes (Fuentealba et al., 2007). Further to this, others have reported Wnt signalling in differentiation processes. APC-deficient mESCs, or expression of a dominant negative form of β-catenin in mESCs, prevents differentiation towards neural lineages. Consistent with this, a reduction in the expression of BMP-4 in APC-deficient mESCs compared to wild-type mESCs is observed (Haegele et al., 2003). An endogenous antagonist of Wnt signalling, Dickkopf-1, was shown to be necessary for the induction of neural differentiation. Dickkopf-1 is a secreted glycoprotein that interacts with LRP-5/6 inducing endocytosis of the co-receptor of Wnt signalling (Mao et al., 2002). Dickkopf-1 is induced in embryoid body formation

following exposure of embryoid bodies to retinoic acid, a method of inducing neural differentiation (Verani et al., 2000).

In the regulation of mESCs, both the canonical and alternative pathways involving Gsk-3 and Wnt signalling have been identified. Alternative pathways involving Gsk-3 in mESCs are discussed in Section 1.3.6.1.

It is widely accepted in the stem cell field that multiple pathways contribute to the maintenance of self-renewal and pluripotency of ESCs. Much emphasis is placed on balancing signalling promoting self-renewal with signalling promoting differentiation in order that self-renewal is maintained. Consequently, a “ground state” of pluripotency was hypothesised (Ying et al., 2008). Smith and co workers sought to investigate the default fate of mESCs if self-renewal promoting signals (FGF4, Stat3 and Gsk3) and differentiation promoting signals (Erk) were removed. In a serum and LIF-free media containing the FGF receptor inhibitors PD184352 and SU5402, plus the Gsk-3 inhibitor, CHIR99021, it was reported that undifferentiated mESCs could be continually cultured and expanded. Additionally, this media was reported to sufficiently support the derivation of mESCs from blastocyst stage embryos (Ying et al., 2008). Thus, authors demonstrated that in the absence of Stat3, Erk and Gsk-3 signalling and in the absence of autocrine FGF4 signalling, mESCs adopt a self-renewing, pluripotent ground state. This observation was suggested to be an intrinsic self-maintaining mechanism and a feature of mESCs in culture. Furthermore, the same group reported that the dual inhibition of Mek, upstream of Erk, and Gsk-3 with PD0325901 and CHIR99021 respectively supported efficient reprogramming of brain-derived neural stem cells into iPSCs following transfection with Klf4 and Oct4 (Silva et al., 2008). However, LIF was required, in conjunction with these two inhibitors, in order to establish fully reprogrammed iPCSs, suggesting that Stat3 signalling is not entirely dispensable particularly for reprogramming. These observations indicate that the pluripotent ground state, observed in ESCs in the absence of fate-determining signalling, can be recaptured following induction of differentiated cells towards induced pluripotent cells.

#### *1.3.4.8 Transcriptional regulation of self-renewal*

Extrinsic factors present in culture media and secreted by ESCs activate an array of intracellular signalling pathways. These pathways have been shown to maintain self-renewal by; balancing signals that promote differentiation to particular lineages, inhibition of differentiation, promotion of proliferation and promotion of cell survival. Together these signalling pathways regulate a transcriptional network of core regulators, namely transcription factors, some of which are used as markers of self-renewal (discussed in Section 1.3.4.1).

In ESCs, the level of Oct4 expression controls lineage commitment rather than self-renewal directly. Cells isolated from the blastocyst stage of Oct4-null embryos are not pluripotent, instead these cells represent those of extraembryonic trophectoderm (Nichols et al., 1998). Over-expression of Oct4 induces primitive endoderm and mesoderm differentiation while suppressed expression induces trophectoderm production (Niwa et al., 2000). Thus, up-regulated expression of Oct4 alone can not relieve the requirement for LIF and therefore does not support self-renewal.

Nanog appears to be a master regulator of self-renewal. Unlike Oct4, over-expression of Nanog in mESCs, allows self-renewal to be maintained even in the absence of LIF (Mitsui et al., 2003, Chambers et al., 2003). Moreover, over-expression of Nanog can delay retinoic acid-induced differentiation. In the absence of Nanog, ESCs differentiate towards extraembryonic endodermal lineages (Mitsui et al., 2003). Nanog was identified in a screen for genes expressed exclusively in ESCs that acted independently of the LIF/Jak/Stat pathway. Each of the nine genes identified were expressed constitutively in ESCs but only Nanog could maintain self-renewal in the absence of LIF (Mitsui et al., 2003). Nanog is reported to form homodimers by self-association of a region of Nanog protein that displays a tryptophan repeat every fifth residue (Mullin et al., 2008). Furthermore, this region of Nanog protein was demonstrated to be functionally critical for Nanog transcriptional activity, required for the maintenance of mESC self-renewal (Mullin et al., 2008). In an independent study, Nanog was reported to both positively and negatively regulate expression of target genes by the formation of a homodimer (Wang et al., 2008). Indeed forced expression of dimerised Nanog, connected via C-terminal domains, maintained self-renewal in the absence of endogenous Nanog and in the absence of LIF (Wang et al., 2008).

Nanog and Oct4 have been reported to form a weak or transient complex to allow co-binding to numerous sites within the mESC genome for both activation and suppression of transcription (Zhang et al., 2007). The products of some genes bound by Nanog and Oct4 are down-regulated upon induced differentiation, consistent with a decrease in expression of Nanog and Oct4 themselves (Loh et al., 2006). Inducible Sox2-null ESCs exhibit a loss of self-renewal coupled with the expression of trophectoderm markers. It was noted that forced expression of Oct4, rescued Sox2-null ESC pluripotency. Furthermore, Oct4 and Sox2 were shown to cooperatively activate enhancers of ESC-specific genes, including Nanog, Oct4 and Sox2 (Masui et al., 2007). Consistent with this, Oct4 and Sox2 were found to co-bind the Nanog promoter in both mouse and human ESCs, to enhance Nanog mRNA expression (Rodda et al., 2005).

Kruppel-like factor 4 (Klf4) was shown to cooperate with both Oct4 and Sox2 to activate the core promoter of Lefty1, a target gene of Oct4 (Nakatake et al., 2006). Interestingly, although Klf4 was reported to be dispensable for maintaining the undifferentiated state of mESCs (Nakatake et al., 2006), the importance of Klf4 in inducing pluripotency in somatic cells is increasingly apparent (Lin et al., 2005, Takahashi and Yamanaka, 2006, Takahashi et al., 2007, Silva et al., 2008). Consistent with this, Klf2, Klf4 and Klf5 were demonstrated to be functionally redundant but simultaneous knockdown of all three of these Klf family members resulted in the differentiation of ESCs (Jiang et al., 2008). Furthermore, Klf4 was reported to not only share common targets with Nanog but also to bind genes encoding transcription factors important for maintaining self-renewal, including *Pou5f1*, *Nanog* and *Sox2* (Jiang et al., 2008). Interestingly, Nanog and Sox2 appear to regulate Rex1 transcription by binding to the Rex1 promoter. Indeed, Nanog was shown to promote Rex1 expression in differentiated cells as well as ESCs by co-transfection of Rex1 and Nanog genes (Shi et al., 2006a). Additionally, Oct4 is also reported to promote Rex1 expression in ESCs (Ben-Shushan et al., 1998).

Alongside promoting the expression of pluripotency genes, Oct4 also acts to repress differentiation. FoxD3, a member of the forkhead box (Fox) family of transcription factors is implicated in early linage commitment, particularly endodermal differentiation (Hromas and Costa, 1995). Oct4 was shown to interact with the DNA-binding domain of FoxD3 thereby preventing FoxD3 from binding lineage-specific gene promoters (Guo et al., 2002). Additionally, Nanog is known to repress differentiation and early lineage commitment genes GATA1 and GATA6 (Chambers et al., 2003, Mitsui et al., 2003).

Further genes that contribute to maintaining self-renewal have also been identified. These genes, *Esrrb*, *Tbx3* and *Tcl1*, were down-regulated following induced differentiation (Ivanova et al., 2006). Interference of these three genes with short-hairpin RNA (shRNA) was used to ascertain their roles in maintaining self-renewal and knock-down of Esrrb was found to prevent differentiation of embryoid bodies towards neuroectodermal and mesodermal lineages. In contrast, forced expression of Sox2 and Tbx3 prevented mesodermal differentiation, while Tcl1 was shown to prevent the expression of certain neural crest genes. However, over-expression of Nanog could overcome the effects of reduced *Esrrb*, *Tbx3* and *Tcl1* expression, further confirming Nanog as one of the master regulators of self-renewal (Ivanova et al., 2006).

Much evidence exists to support the fact that the major pluripotency-maintaining transcription factors act as a gene regulatory network to control pluripotency at a transcriptional level (reviewed by Niwa, 2007). Furthermore, extrinsic factors present in culture medium and secreted by ESCs activate a network of signalling pathways that converge in the nucleus at a seemingly minimal number of transcription factors. The activity of the main transcription factors, Nanog, Sox2 and Oct4, appear to regulate both their own expression and expression of genes for self-renewal and lineage directed differentiation. This observation was incorporated into a model by Niwa et al., which encompasses self-renewal and proliferation governed by epigenetic processes, which in turn are under the control of a transcription factor network found in pluripotent cells (Niwa, 2007). In light of this, Yamanaka et al., sought to identify the fewest number of genes that could be used to revert differentiated somatic cells into cells with stem cell-like properties. Forced expression using viral-mediated transduction of *Oct4*, *Sox2*, *Klf4* and *c-Myc* reprogrammed a subpopulation of mouse and human fibroblasts to ESC-like cells known as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006, Takahashi et al., 2007) Of interest, Nanog appears to be dispensable in this reprogramming cocktail of genes. Although, it was suggested that Nanog expression in iPSCs might arise by the suppression of p53 by Klf4, since Nanog expression is repressed by p53 following DNA damage (Lin et al., 2005). Interestingly, c-Myc activation is reported to result in the differentiation of hESCs (Sumi et al., 2007) and reprogramming without c-Myc proved to be possible but less efficient (Nakagawa et al., 2008). In a separate study, *Oct4*, *Sox2*, *Nanog* and *Lin28* were demonstrated to be sufficient to reprogram human fetal and postnatal fibroblasts, and human ESC-derived somatic cells towards pluripotent cells with ESC morphology, hESC marker expression and normal karyotypes (Yu et al., 2007). *Lin28* was included by this group since it encodes a DNA modifying protein which is expressed in undifferentiated hESCs but upon differentiation Lin28 expression is decreased (Richards et al., 2004).

However, it was observed that *Lin28* was absent in some of the iPSC clones generated by retroviral transduction, indicating that *Lin28* is not essential for reprogramming human somatic cells (Yu et al., 2007).

Selection of iPSCs from the total population by detection of endogenous Oct4 or Nanog is reported to be the most efficient way of selecting iPSCs. Following selection, iPSCs are demonstrated to display properties that are thus far indistinguishable from ESCs (Okita et al., 2007). Pluripotency markers such as alkaline phosphatase, endogenous Oct4 and Nanog are sequentially activated in mouse somatic cells following induction towards iPSCs. The time course for these pluripotency markers to be detected, 10-30 days after transfection, indicates that reprogramming is a slow and complex process for cells to undergo and one that requires forced conditions (Brambrink et al., 2008). Nevertheless, iPSCs demonstrate the ultimate control over cell fate that core pluripotency regulating transcription factors have in ESCs.

### *1.3.5 Murine embryonic stem cell proliferation*

ESCs have the capacity to undergo continuous division with great proliferation potential. Indeed the transplanting of self-renewing ESCs into adult mice produces the formation of tumours, containing all three germ layers called teratomas (Martin and Evans, 1975, Martin, 1980). Unsurprisingly, this perpetual proliferation has been likened to the aggressive growth of cancerous tumours resulting from uncontrolled proliferation of tumour cells. However, ECCs, isolated from teratomas, contain chromosome abnormalities but ESCs, derived from the ICM of a blastocyst, have no genetic abnormalities.

Mammalian cells require cues from growth factors to pass the $G_1$ phase of the cell cycle. In the absence of growth factors, mammalian cells exit $G_1$ phase and enter $G_0$ quiescence (Pardee, 1989). However, ESCs are unlike other mammalian cell types in that they have a short $G_1$ phase and do not enter quiescence. ESCs rapidly proliferate, with a cell cycle time of 10 hours, and an observed 70% of ESCs are in S-phase of the cell cycle at any given time (Sun et al., 1999, Savatier et al., 1994). In differentiated cell types, retinoblastoma protein (RP) is reported to be important for $G_1$/S transition, in that hypophosphorylated RP, found in $G_1$ phase cells, sequesters E2F transcription factors and, as a result, inhibits the expression of genes required for entry into S phase (Harbour et al., 1999). Phosphorylation of RP by complexes of cyclins and cyclin-

dependent kinases (CDKs) allows the release of some E2F transcription factors and subsequent transcription of *cyclin E* and *cdc25A* genes. Inhibitory phosphatases are removed from CDK2 by the cdc25A phosphatase allowing CDK2 to form a complex with Cyclin E, which further phosphorylates RP. The remaining sequestered E2F is released prompting transcription of target genes and entry of cells into S phase (reviewed by Bartek and Lukas, 2001, Harbour and Dean, 2000). However, in ESCs hypophosphorylated RP was reported to be undetectable with RP seemingly constitutively phosphorylated, leading authors to suggest that either RP was rephosphorylated immediately after mitosis in ESCs or that ESC cell cycle regulation was RP-independent (Savatier et al., 1994, Burdon et al., 2002). It is likely that ESCs do not depend on RP regulation for cell cycle control since disruption of *RP* or two *RP*-related genes did not significantly affect mESC proliferation (Dannenberg et al., 2000, Sage et al., 2000). Additionally, in contrast to differentiated cell types, ESCs display low expression of cyclin D1, which may explain the observed constitutive phosphorylation of RP in ESCs. Furthermore, cyclin D1 protein levels are reported to be independent of regulation by Ras/Erk signalling but dependent on PI3K activity, and to be uncoupled from specific mitogen activation (Jirmanova et al., 2002).

Uniquely, serum withdrawal does not substantially alter ESC proliferation (Schratt et al., 2001) but does down-regulate Erk activity (Lianguzova et al., 2007). However, activation of the classical MAPK pathway is not required for $G_1$ to S phase progression in ESCs. In fact, inhibition of Mek does not alter the proliferation rate of ESCs (Jirmanova et al., 2002), while inhibition of Mek or p38 was not sufficient to change the proportion of cells in $G_1$ or S phases (Lianguzova et al., 2007, Fluckiger et al., 2006). Consistent with this, expression of inactive Grb2 in ESCs does not affect proliferation (Cheng et al., 1998). Mitogenic stimuli are not required for S-phase entry, explaining the shortened $G_1$ phase characteristic of ESCs. Uncoupling of cell cycle control from the MAPK pathway, a usual activator of proliferation in other cell types, explains the ability of mESCs to proliferate in the absence of serum or growth factors (Lianguzova et al., 2007, Burdon et al., 1999). Furthermore, mouse embryos deficient for serum response factor (SRF), which regulates gene activation depending on the stage of the cell cycle, grow and develop normally until E6, and ESCs derived from these embryos exhibit normal proliferation rates and unaltered cell cycle progression (Schratt et al., 2001).

Having ascertained that typical mitogen-activated Ras/Erk signalling did not regulate mESC proliferation, research focus turned to mTOR, which activates S6K and S6, a pathway known to be important for protein synthesis and cell growth (Abraham, 1996).

It was demonstrated that the expression of mTOR was greatest in ESCs, testis and kidneys (Murakami et al., 2004). Induced inactivation of mTOR in ESCs or inhibition with rapamycin resulted in severely limited proliferation (Murakami et al., 2004). Consistent with the findings in ESCs, studies on mTOR deficient mouse embryos revealed embryonic lethality due to impaired cell proliferation and a failure of cells of the ICM to proliferate (Murakami et al., 2004, Gangloff et al., 2004).

### *1.3.6 The role of PI3K in mESC fate*

During the pre-implantation stage of embryo development, the cells comprising the embryo depend on both autocrine and paracrine factors for growth and development. p85 and p110 PI3K isoforms are expressed and localised to the plasma membrane of embryos from the one-cell stage to the blastocyst stage of development (Riley et al., 2005). More importantly, using GFP-tagged PH domains to monitor $PI(3,4,5)P_3$ synthesis, PI3Ks were demonstrated to be functionally active in one-cell embryos through to early blastocyst stage embryos (Halet et al., 2008). To date, two roles for PI3K in mESCs have been described: the maintenance of self-renewal and the regulation of proliferation.

#### *1.3.6.1 The contribution of PI3K to self-renewal*

Accruing evidence defining the role of PI3Ks in self-renewal comes from the use of a number of techniques (summarised in Figure 1.17). Treatment of mESCs with 5μM LY294002 led to a reduction in alkaline phosphatase staining and a change in ESC morphology to that resembling differentiated cells (Paling et al., 2004). Consistent with these findings, embryoid body formation can be inhibited by the presence of LY294002 (Chen et al., 2000). Treatment with LY294002 was also shown to decrease Nanog RNA levels in mESCs after 48 hours and protein levels within 8 hours (Storm et al., 2007). LIF was shown to activate signalling downstream of PI3Ks, namely PKB, Gsk-3 and S6. Activation of these signalling molecules down stream of PI3Ks by LIF was shown to be inhibited by pre-treatment with LY294002 (Paling et al., 2004). Consistent with the involvement of PI3Ks in self-renewal, a gain-of-function screen for genes that when over-expressed could maintain self-renewal in the absence of LIF, highlighted PKB as one such gene (Pritsker et al., 2006). Adding weight to this, expression of a myristolated, active form of PKB was reported to promote self-renewal in the absence of LIF (Watanabe et al., 2006).

Gsk-3 is known to participate in signalling networks other than the canonical Wnt pathway. For example, Gsk-3 is negatively regulated by PI3K-induced PKB activity following insulin stimulation (Cross et al., 1995). Negative regulation of Gsk-3 requires phosphorylation at serine 21 (Ser21) for Gsk-3α and serine 9 (Ser9) for Gsk-3β. In PDK1-null ESCs, Gsk-3 is not phosphorylated and thus remains active (Williams et al., 2000). An inhibitor of Gsk-3, 6-bromoindirubin 3'-oxime (BIO), which is derived from mollusc Tyrian Purple (Meijer et al., 2003), mimics the actions of PKB on Gsk-3. Treatment with BIO results in the up-regulation of Rex1 expression and activation of Wnt signalling and has been reported to enhance self-renewal of mESCs in the absence of LIF (Sato et al., 2004). Additionally, BIO treatment reverses the effects of LY294002 on Nanog protein expression levels (Storm et al., 2007) (Figure 1.17). A series of bisindolylmaleimides, which inhibit Gsk-3, were analysed alongside BIO and were also shown to enhance self-renewal of mESCs (Bone et al., 2009). Expression of an activated form of Gsk-3β in mESCs reduced Nanog expression, consistent with the non-phosphorylated form being in an active state. Expression of a dominant negative form of Gsk-3β, which imitates the effects of Gsk-3 inhibitors, enhanced Nanog expression (Storm et al., 2007). The link between Nanog and PI3K was strengthened by the findings that LY294002 reduced the expression of Nanog target genes. Conversely, induced expression of Nanog rescued the effect of LY294002 on self-renewal (Storm et al., 2007).

The tumour suppressor p53 responds to cell stress and induces pro-apoptotic signalling. Gsk-3 is reported to phosphorylate and interact with p53 in the nucleus, leading to cytoplasmic localisation and the prevention of p53-induced apoptosis (Qu et al., 2004). However, in mESCs, DNA damage results in enhanced p53 protein levels and decreased Nanog mRNA expression after just 4 hours, a feature not observed in p53 null ESCs. Furthermore, p53 was found to bind the Nanog promoter and inhibit expression of Nanog following induced DNA damage or retinoic acid-induced differentiation (Lin et al., 2005). Taken together, these reports indicate that PI3Ks maintain self-renewal via PKB and possibly via Gsk-3, although Gsk-3 is also regulated by other signalling pathways.

Inhibition of PI3Ks with LY294002 was also found to lead to an enhancement in Erk phosphorylation in mESCs (Paling et al., 2004). Activation of this pathway is reported to induce differentiation (Burdon et al., 1999). Treatment with both LY294002 and U0126, an inhibitor of Mek, reversed the effect of reduced alkaline phosphatase staining observed with LY294002 alone (Paling et al., 2004). However, U0126 was not sufficient to reverse the effect of LY294002 on Nanog expression (Storm et al., 2007).

Nevertheless, these results suggest that PI3Ks may promote self-renewal, at least in part, by regulation of the MAPK pathway.

The class $I_A$ PI3Ks were implicated in regulating mESC self-renewal by the expression of an inducible dominant negative form of p85, which lacks the p110 binding site (Hara et al., 1994). This dominant negative mutant, Δp85, acts as a competitive inhibitor of p110 isoforms. Induced expression of Δp85 in mESCs reduces PKB, Gsk-3 and S6 phosphorylation and results in a reduction in the percentage of alkaline phosphatase positive colonies, consistent with a loss of self-renewal (Paling et al., 2004).

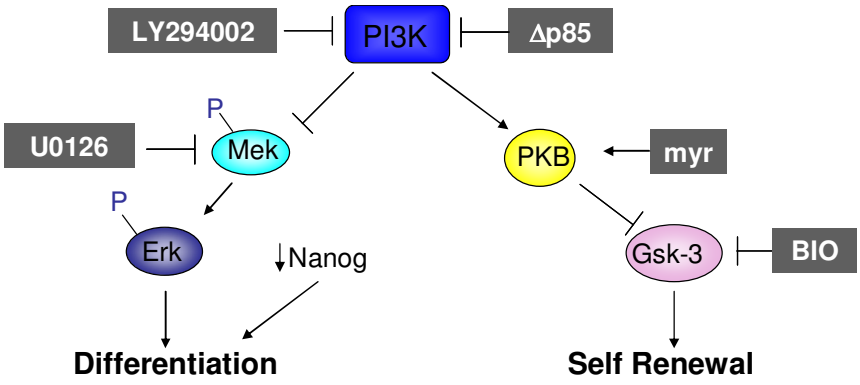



**Figure 1.17 Evidence of a role for PI3Ks in maintaining mESC self-renewal**
PI3Ks have been shown to participate in maintaining the undifferentiated state of mESCs. The approaches taken to reveal this role for PI3K are shown in grey boxes and referred to in the text. PI3K appears to maintain self-renewal via PKB and Gsk-3 and may prevent differentiation by exerting an inhibitory effect on MAPK signalling.

### *1.3.6.2 The role of PI3K in mESC proliferation*

The tumour-like growth properties observed in ESCs might be partly attributed to the activity of PI3Ks (summarised in Figure 1.18). Unconvincing evidence of a role for PI3Ks in mESC proliferation came from the use of 20-40μM LY294002 which yielded only a small increase in the proportion of cells in $G_1$ phase and a reduction in cell growth following 48 hours treatment (Lianguzova et al., 2007). Additionally, an increase in mESCs in $G_1$ phase following 24-hour treatment and a dramatic reduction in cell growth after 4 days treatment was reported using 25μM LY294002 (Jirmanova et al., 2002). Such concentrations of LY294002 are likely to have strong off-target effects

which could be toxic to cells, explaining the results obtained. Notably, inhibition of mTOR is evident at 1-30μM LY294002 (Brunn et al., 1996) and mTOR has been demonstrated to be essential for the proliferation of mESCs (Murakami et al., 2004).

Paling et al., demonstrated that the metabolic activity of mESCs was unchanged using modest doses of LY294002 (less than 10μM) and that growth rates were unaffected by 5μM LY294002 (Paling et al., 2004). Additionally, 5μM is close to the published $IC_{50}$ value for PI3Ks (Knight et al., 2006, Vlahos et al., 1994) and effects on PI3K effector phosphorylation are visible at this concentration in mESCs (Paling et al., 2004, Storm et al., 2007). Furthermore, LIF is important to maintain self-renewal and is reported to activate PI3Ks in ESCs. However, LIF concentration had little or no effect on the proliferation rate of a number of mESC lines (Raz et al., 1999). Interestingly, 10μM LY294002 treatment of embryos from the one-cell stage resulted in only 40% of embryos surviving to the 8-cell stage and only 10% surviving to the blastocyst stage of development. Importantly, this developmental restriction imposed by PI3K inhibition with LY294002 was not attributable to apoptosis (Halet et al., 2008).

More convincing evidence of a role for PI3Ks in mESC proliferation can be drawn from the characteristics of the tumour suppressor PTEN, a regulator of cellular $PI(3,4,5)P_3$ levels and thus a negative regulator of PI3K activity (Figure 1.5). Mutations or deletions of PTEN are often associated with human cancers (Li et al., 1997, Cantley and Neel, 1999). PTEN-deficient ESCs exhibit a faster growth rate and maintained growth even in the absence of serum. The mechanism of enhanced growth rate is attributed to reduced levels of $p27^{KIP1}$, a cyclin-dependent kinase inhibitor which inhibits $G_1$ cell cycle kinases that promote S-phase entry (Sun et al., 1999). Reduced expression of $p27^{KIP1}$ can arise due to phosphorylation and inhibition of forkhead transcription factors of the FOXO family by PKB (Stahl et al., 2002). In particular, FoxO3 has been implicated in interleukin-2 induced phosphorylation and subsequent regulation of $p27^{KIP1}$ expression (Stahl et al., 2002). Although, activated PKB is reported induce phosphorylation of $p27^{KIP1}$ leading to cytosolic sequestration, prevention of nuclear localisation and attenuating the cell-cycle inhibitory effects of $p27^{KIP1}$ (Liang et al., 2002, Shin et al., 2002, Viglietto et al., 2002). Furthermore, PKB activity is implicated in maintaining cell survival by negative regulation of the Bcl-2 family of pro-apoptotic proteins. For example, PKB-mediated phosphorylation of the Bcl-2 family member, BAD, induces binding of 14-3-3 proteins to BAD, releasing BAD from its target proteins (Datta et al., 2000). Constitutive expression of Bcl-2 in a transgenic mESC line reportedly supported the proliferation of mESCs in the absence of feeder cells or serum, however LIF was still required to maintain self-renewal and pluripotency (Yamane et al., 2005).

The activity of PI3Ks and subsequent downstream effectors such as PKB are enhanced in PTEN-null ESCs (Williams et al., 2000, Sun et al., 1999). In mESCs deficient in both PTEN and PKB, phosphorylation of Gsk-3β and S6K was reduced and cell growth was slower than either wild-type or PTEN-null cells. Unlike PTEN knockout ESCs, that form rapidly proliferating teratomas when injected into adult mice, PTEN/PKB double knockout ESCs formed teratomas with slower growth rates. However, these teratomas contained more differentiated cell types compared to teratomas formed from wild-type ESCs (Stiles et al., 2002).

If PTEN is important for regulating PI3K-induced proliferation in ESCs but serum or growth factors are not essential for ESC proliferation, how is PI3K activated? In a screen for genes expressed exclusively in ESCs, but not in differentiated cells, a Ras-like gene with 40-50% homology to Ras isoforms (HRas, KRas and NRas) was identified. This Ras-like gene, termed ERas (Takahashi et al., 2003), contains amino acid residues akin to those in activated, oncogenic HRas mutants, commonly found in human tumours (Fasano et al., 1984). ERas was confirmed to be predominantly GTP-bound and therefore active in both human and mouse ESCs. Over-expression of ERas in ESCs further enhances growth rates and transfection with antisense ERas cDNA significantly suppresses cell growth (Takahashi, 2003). ERas localises to the plasma membrane where it interacts with PI3Ks to promote PKB phosphorylation (Takahashi et al., 2005). In ERas-null ESCs, the level of PKB phosphorylation was reduced compared to wild-type ESCs. Both the reduced levels of PKB phosphorylation and the proliferative defects observed in ERas-null ESCs could be rescued by the forced expression of a myristolated, active form of p110 (Takahashi et al., 2003).

ESCs were found to express very low levels of p85α and targeted deletion of p85α in ESCs resulted in no change in PI3K activation (Hallmann et al., 2003). It was suggested that this effect was the result of compensation by utilisation of p85β instead. Despite this compensation, in order to maintain PI3K activation, PKB phosphorylation was lowered and cell growth rates were reduced (Hallmann et al., 2003).

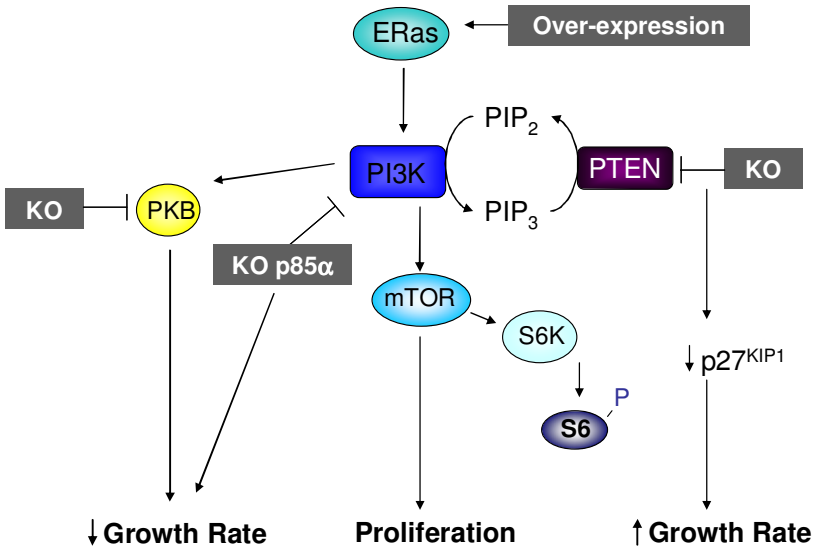



**Figure 1.18 Evidence of a role for PI3Ks in mESC proliferation**

Much research has been done to investigate the unique proliferation, growth and cell cycle characteristics of mESCs. The approaches used to implicate PI3Ks in mESC proliferation are shown in grey boxes.

### *1.3.6.3 Knockout studies in mice and ESCs*

Knocking out a gene provides valuable information regarding the normal physiological function of the protein products encoded by that gene. Since ESCs provide an *in vitro* model of cells of the early embryo, much can be learned by investigating knockout mice as well as knockout studies using ESCs. In the present study, the roles of the PI3K class I catalytic isoforms p110α, p110β, p110γ and p110δ in regulating mESC fate were of particular interest. Some clues could be drawn from previous work using knock-out mice (summarised in Table 1.2).

A profound proliferative defect was observed in embryos with a homozygous deletion of *Pik3ca,* the gene encoding p110α. *Pik3ca*$^{del/del}$ embryos were not viable and did not survive beyond E9.5. Furthermore, cells isolated from *Pik3ca*$^{del/del}$ embryos could not be expanded in culture, even with the addition of serum or growth factors (Bi et al., 1999). Thus, functional p110α is required for growth and development of the embryo. However, two drawbacks with this knockout exist. Firstly, the technique used to produce this knockout involved replacing the p110α start codon and p85-binding domain with a neomycin selection cassette, giving rise to a hybrid p110α mRNA, which contains a downstream start codon and an open reading frame. It is unknown if this truncated p110α mRNA is translated in these cells but, theoretically, expressed truncated hybrid p110α protein could display Ras-binding and catalytic activity. Secondly, the expression of p85 was enhanced in *Pik3ca*$^{del/del}$ embryos. This excess of free p85 may act as a negative regulator of PI3K activity by competing for phosphorylated tyrosine residues with p85 bound to other isoforms of p110. To avoid these complications, transgenic knock-in mice were generated expressing p110α with a single mutated amino acid residue rendering this isoform catalytically inactive (Foukas et al., 2006). Mice homozygous for this mutation exhibit embryonic lethality whereas heterozygous mice, with reduced p110α activity demonstrate impaired insulin signalling, implying a major role for p110α in insulin signal transduction (Foukas et al., 2006).

Mice in which *Pik3cb*, the gene encoding p110β, had been knocked out were reported to be embryonic lethal with *Pik3cb*$^{del/del}$ embryos not surviving past E3.5 (Bi et al., 2001). Interestingly, E3.5 pre-implantation embryos, at the blastocyst stage, are used to derive self-renewing, pluripotent mESC lines for culture (Evans and Kaufman, 1981). In comparison, mice generated to express a catalytically inactive form of p110β were demonstrated to exhibit lethality but with incomplete penetrance (Ciraolo et al., 2008,

Guillermet-Guibert et al., 2008). Authors explained the disparate results observed with knock-in mice compared to knock-out mice (Bi et al., 2001) on the non-catalytic functions mediated by p110β. A non-catalytic, structural or association role for p110β has been described for epidermal growth factor receptor endocytosis, which is reported to be impaired when the expression of p110β is reduced (Ciraolo et al., 2008). Moreover, the small GTPase Rab5, which is mainly located on endosomes, associates with p110β (Christoforidis et al., 1999).

Both knock-in and knock-out approaches were adopted to investigate p110δ, encoded by *Pik3cd*. Two *Pik3cd* knockout lines and a catalytically inactive p110δ knock-in line all displayed similar phenotypes. KO mice were reported to have defective lymphocyte, mast cell and neutrophil function (Clayton et al., 2002, Jou et al., 2002). Interestingly knocking out p110δ resulted in a reduction in the expression of p85α, p55α and p50α regulatory subunits in B cells. Using an alternative approach, a catalytically inactive p110δ knock-in was created producing mice that were viable but developed inflammatory bowel disease and had impairment of antigen receptor signalling in B and T cells. In cells expressing inactive p110δ, the expression and activity of other PI3K subunits was similar to that of wild-type cells (Okkenhaug et al., 2002). This role for p110δ is consistent with expression of this isoform being greatest in haematopoietic cells.

**Table 1.2 PI3K catalytic and regulatory subunit gene-targeting studies in mice**

| PI3K Isoform | Embryonic Lethality | Other Effects |
|---|---|---|
| p110α KO | E9.5 | Proliferative defects.<br>Elevated expression of p85 in embryo.<br>(Bi et al., 1999) |
| p110α KI | | Homozygotes – embryonic lethal.<br>Heterozygotes - impaired insulin signalling.<br>(Foukas et al., 2006) |
| p110β KO | E3.5 (Blastocyst stage) | Homozygotes – embryonic lethal.<br>Heterozygotes – live born.<br>(Bi et al., 2001) |
| p110β KI | Embryonic lethal with incomplete penetrance | Growth retardation of a proportion of surviving mice with low expression of p110β.<br>Indication that kinase-independent functions are sufficient to allow embryonic development leading to viable adult mice.<br>(Ciraolo et al., 2008) |
| p110δ KO | Viable | Impaired neutrophil function.<br>Decreased T-cell dependent antibody production.<br>Reduced expression of p85α, p55α and p50α in B cells.<br>(Clayton et al., 2002, Jou et al., 2002) |
| p110δ KI | Viable | Impaired antigen signalling in B and T cells.<br>Develop intestinal inflammation.<br>No alteration in p110 isoforms.<br>(Okkenhaug et al., 2002) |
| p110γ KO | Viable | Impaired neutrophil and macrophage inflammatory reactions.<br>Increased cardiac contractility.<br>No alteration in p110 isoforms.<br>(Hirsch et al., 2000) |
| p110γ KI | Viable | Impaired neutrophil and macrophage inflammatory reactions.<br>No alteration in p110β expression in macrophages.<br>(Patrucco et al., 2004) |
| p85α only | Viable | Increased insulin sensitivity.<br>Elevated expression of p55α and p50α, p50α.<br>(Terauchi et al., 1999) |
| pan-p85α | Perinatal lethality | Reduced insulin sensitivity.<br>Elevated expression of p85β.<br>Reduced expression of p110α and p110β.<br>(Fruman et al., 1999) |

The knock-out and kinase-dead knock-in approach was also used to investigate p110γ. Both techniques exhibited impaired neutrophil and macrophage inflammatory reactions (Hirsch et al., 2000, Patrucco et al., 2004). Only p110γ knockout mice displayed increased cardiac contractility and enhanced cAMP levels in cardiac cells. This was attributed to the kinase activity-independent coupling of p110γ to phosphodiesterase 3B in order to degrade cAMP in cardiac cells and reduce contraction. Therefore, in p110γ kinase-dead knock-in mice, p110γ expression allows coupling to

phosphodiesterase 3B and is unaffected by the lack of kinase activity (Hirsch et al., 2000, Crackower et al., 2002).

Knock-outs of the p85 regulatory subunit tend to have increased insulin sensitivity coupled to increased cellular $PI(3,4,5)P_3$ levels and enhanced PKB phosphorylation at Ser473 (Vanhaesebroeck et al., 2005). Mice lacking p85α only (expression of splice variants is intact) are viable but display hypoglycaemia and increased insulin sensitivity. This enhanced insulin sensitivity is explained by an increase in the expression of p55α and p50α. Additionally, p50α is capable of coupling to p110α to mediate insulin signal transduction in adipocytes and skeletal muscle (Terauchi et al., 1999). Furthermore, p50α-associated PI3K activity was shown to be greater than that for p55α or p85α (Ueki et al., 2000). Consistent with this, pan-p85α knock-out mice (no expression of splice variants) are perinatal lethal and have reduced sensitivity to insulin. Moreover, expression of p85β was elevated, while p110α and p110β expression were reduced in pan-p85α knock-out mice (Fruman et al., 1999).

Further information is provided from knockouts of effectors of PI3K signalling and phosphatases which regulate the cellular levels of PI3K inositol lipid products. PDK1 knock-out mice die at E9.5 resulting from underdevelopment of somite, forebrain and neural crest tissues. $PDK1^{-/-}$ mice are also smaller than wild-types but this is reported to be the result of a developmental block rather than a direct effect on cell proliferation. Therefore, PDK1 is important for mouse embryonic development (Lawlor et al., 2002). In contrast, embryonic lethality occurs at E6.5 in mice lacking mTOR as a direct result of reduced growth and proliferation (Murakami et al., 2004). PTEN deficient mice die at E9.5, while heterozygotes exhibit reduced survival rates and enhanced occurrence of tumours (Suzuki et al., 1998, Stambolic et al., 1998), suggesting a role for PTEN in growth and development.

Knock-out studies of individual p110 and p85 isoforms provide useful clues as to the necessity of PI3K isoforms for embryonic development and in ESC fate. Nonetheless, these studies have been hampered by compensatory expression of other catalytic and regulatory PI3K subunit isoforms. Additionally embryonic lethality clearly highlights an essential role for p110α and p110β in embryonic development. However, due to this lethality further investigation and clarification of p110α and p110β functions in embryo development, ESC self-renewal, proliferation and differentiation is restricted. Moreover, insights gained from knock-outs of PI3K effectors and regulatory phosphatases lack the details of PI3K isoform coupling to a specific functional role.

### *1.3.7 Human ESCs: similarities and differences to mESCs*

While human and mouse ESCs share common properties of perpetual self-renewal, pluripotency and the ability to differentiate into more specialised cell types, they differ somewhat in their cell-surface markers of self-renewal, in the extrinsic factors required to maintain pluripotent self-renewal and in the response to these factors.

Human ESCs are reported to express the self-renewal markers alkaline phosphatase and Rex1 (Draper and Fox, 2003, Eiges et al., 2001). Furthermore, human and murine ESCs share similar transcription factors that regulate self-renewal such as Nanog, Oct4 and Sox2 (Richards et al., 2004, Boyer et al., 2005). Indeed, forced expression of Oct4, Sox2, Klf4 and c-Myc, the same set of four genes used for mouse cells, induces reprogramming of human fibroblasts to iPSCs (Takahashi et al., 2007) although more efficient reprogramming was achieved when Klf4 and c-Myc were replaced with Nanog and the hESC-specific gene Lin28 (Yu et al., 2007). Further evidence of the conserved function of Oct4 in mouse and human ESCs comes from a report indicating that RNAi-mediated knockdown of Oct4 induced the expression of endoderm markers in ESCs from both species (Hay et al., 2004).

One notable difference in cell surface marker expression of human and mouse ESCs is that of the stage-specific embryonic antigens (SSEAs). Three forms of SSEA exist and each have different expression patterns throughout development. Self-renewing, pluripotent hESCs express SSEA-3 and SSEA-4. This expression is lost upon differentiation when hESCs begin to express SSEA-1 (Thomson and Marshall, 1998, Thomson et al., 1998). Conversely, undifferentiated mESCs and the ICM of mouse pre-implantation embryos express SSEA-1 (Solter and Knowles, 1978).

Unlike mESCs, hESC self-renewal is not maintained by LIF (Thomson et al., 1998, Dahéron et al., 2004, Humphrey et al., 2004). In fact, hESCs exhibit low expression of LIFR, Jak and Stat3 and high expression of suppressor of cytokine signalling (SOCS) which negatively regulates LIF signalling (Wei et al., 2005). Basic fibroblast growth factor (bFGF) is required to maintain hESC self-renewal, this, as with LIF for mESCs, can be provided by a layer of feeder cells on which ESCs can be cultured, or by addition to ESC optimised culture media (Thomson et al., 1998, Amit et al., 2004). BMP4 treatment of hESCs is reported to stimulate mesoderm, trophoblast and extraembryonic endoderm differentiation (Schuldiner et al., 2000, Xu et al., 2002, Pera et al., 2004). This is consistent with BMP-induced differentiation towards non-neural cell types in mESCs (Nakayama et al., 2000, Ying et al., 2003b, Wiles and Johansson,

1999). BMP-induced signalling activity and loss of self-renewal in hESCs can be counteracted by higher doses of bFGF or by the expression of Noggin, a BMP antagonist (Xu et al., 2005).

As in mESCs, a role for PI3Ks in regulating hESC fate is emerging. Pharmacological inhibition of PI3Ks in hESCs is reported to result in a reduction in the percentage of alkaline phosphatase positive colonies (Pyle et al., 2006). A large scale transcriptional comparison of hESC lines revealed that a high proportion of transcripts belonged to signalling pathways, notably the Ras/MAPK, PI3K/PKB and NFκβ pathways (Armstrong et al., 2006). Furthermore, treatment of hESCs with LY294002 led to differentiated colony morphologies and reduced expression of the self-renewal markers Nanog, Sox2 and Oct4 (Armstrong et al., 2006). For the feeder-free culture of hESCs, proliferation and pluripotency maintenance were shown to be dependent upon extracellular matrix molecules (Xu et al., 2001). Expression of tropomyosin-related kinase (TRK) receptors was detected in hESCs. What is more, three neurotrophin ligands of TRK receptors were shown to enhance clonal survival, maintain pluripotency and suppress apoptosis of hESCs, mediated via TRK, PI3K and PKB activation (Pyle et al., 2006).

In mESCs, c-Yes, a Src family non-receptor tyrosine kinase, was found to be activated by LIF and to partially contribute to mESC self-renewal independently from the Jak/Stat pathway (Annerén et al., 2004). Although hESC self-renewal is independent from LIF, higher levels of c-Yes were detected in hESCs compared to differentiated somatic cell types (Annerén et al., 2004), however, a role for non-receptor tyrosine kinases in hESCs is yet to be established. S1P, a GPCR agonist, has been shown to maintain the undifferentiated state of hESCs (Pébay et al., 2005). In contrast, lysophospholipids have been shown to promote the differentiation of mESCs. Indeed, S1P is reported to promote cardiac differentiation in embryoid bodies formed from mESCs (Sachinidis et al., 2003) and sphingosylphosphorylcholine was shown to induce cardiac and neural differentiation of self-renewing mESCs (Kleger et al., 2007).

Although mESCs and hESCs share mechanisms that maintain self-renewal and pluripotency, not all mechanisms are present in both cells. Using mESCs to investigate self-renewal, pluripotency and survival has led to many discoveries regarding extrinsic stimuli, signalling pathways and transcriptional control. Investigation into whether these same mechanisms are conserved in hESCs is a rapidly advancing area of research. Thus, gaining knowledge from mESCs is leading to the faster advancement in hESC knowledge and making the ideal of ESCs as a regenerative medicine more feasible.

## 1.4 Aims

Expansion of undifferentiated ESCs to sufficient numbers that would be of use for regenerative medicine is required if the therapeutic potential of ESCs is to be realised. Such expansion requires the maintenance of self-renewal and pluripotency prior to directed differentiation. PI3K function in the survival and self-renewal of hESCs is beginning to be unravelled. Separate roles for PI3Ks in proliferation and maintenance of self-renewal of mESCs have already been described. However, the contribution to proliferation and the maintenance of self-renewal by PI3Ks are largely addressed as individual issues. Thus, the present study endeavours to further characterise the role of PI3Ks in mESC fate. This was tackled by addressing the following aims:

- *To determine if inhibition of PI3Ks influence differentiation towards a particular lineage.* PI3Ks have been shown to induce differentiation in mESCs, observed by a reduction in alkaline phosphatase staining, a decrease in Nanog expression and a change in the morphology of mESC colonies formed in the presence of the broad spectrum PI3K inhibitor LY294002 (Paling et al., 2004, Storm et al., 2007). The contribution of PI3Ks to early lineage commitment of 2D cultured mESCs has not been reported.

- *To elucidate which factors, present in culture media or secreted by mESCs, contribute to the activation of PI3Ks*. LIF is known to activate signalling downstream of PI3Ks in mESCs. ESCs are routinely cultured in media containing a serum-replacement product of undefined components in order to maintain self-renewal (Burdon et al., 1999) and ESCs are known to secrete factors into culture media that aids their survival and dictates their fate (Guo et al., 2006, Singla et al., 2008). Consequently factors present in culture media, other than LIF, or those secreted by mESCs, may contribute to the activation of PI3Ks.

- *To investigate upstream regulators and downstream effectors of PI3Ks in mESCs.* The signalling pathways downstream of PI3Ks in other cell types are varied and exhibit a high degree of cross-talk (Vanhaesebroeck and Waterfield, 1999). Signalling pathways involving PI3Ks will be investigated using identified activators of PI3Ks in mESCs.

- *To analyse the roles of PI3K subunit isoforms in mESC fate*. PI3Ks have been investigated as a whole with regards to self-renewal and survival using the broad spectrum PI3K inhibitor LY294002. However Paling et al., identified the class $I_A$ PI3Ks as regulators of self-renewal in mESCs. Additionally, gene knockout studies in mice indicate that the class I isoforms may have distinct, redundant or overlapping roles (Vanhaesebroeck et al., 2005). The individual contribution of class I PI3K isoforms to mESC fate will be addressed in the present study. Of particular interest is p110β given that the embryonic lethality of catalytically inactive p110β knockout mice at E3.5 (Bi et al., 2001) coincides with the isolation of mESCs from blastocysts at the same stage of development (Evans and Kaufman, 1981).

# 2. MATERIALS AND METHODS

## 2.1 Cell lines, culture and storage

All cells were incubated in humidified incubators at 37°C with 5% (v/v) $CO_2$.

### *2.1.1 E14tg2A murine embryonic stem cell (mESC) line*

E14tg2A murine embryonic stem cell (mESC) line (Clone R63) was a kind gift of Dr Owen Witte, UCLA, California (Era and Witte, 2000). E14tg2A cells are stably transfected with a tetracycline-regulated transactivator construct pCAG20-1.

### *2.1.2 Mek* mESC clones*

Cloned E14tg2A mESCs expressing an inducible version of constitutively activated, histidine-tagged Mek1 (mitogen-activated extracellular signal-regulated kinase) were generated by Professor Welham (unpublished data). Constitutively active Mek1 was constructed by substitution mutations of serines 218 and 222, within the catalytic domain, to glutamic acid residues, mimicking activating phosphorylation (Treinies et al., 1999). This activated form of Mek (Mek*) was inserted into a gene expression vector under the control of a tetracycline-off expression system. Tetracycline binds the tetracycline-sensitive transactivator (tTA) to suppress expression. In the absence of tetracycline, tTA is expressed from the regulator plasmid which acts via the tTA-dependent promoter to drive expression of exogenous Mek*.

Cloned cells were routinely cultured in KO complete media supplemented with LIF and 500ng/ml tetracycline to suppress Mek* expression.

### *2.1.3 Murine embryonic stem cell culture*

E14tg2A and Mek* mESCs were routinely cultured on 92 x 17mm NUNC dishes coated with 0.1% (w/v) porcine gelatine. Cells were grown in Knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% (v/v) knockout serum replacement, 2mM Glutamine, 50μM 2-mercaptoethanol, 1% non-essential amino acids. Knockout DMEM plus supplements is referred to as KO complete media from here on in. For culture, KO complete media was supplemented with 1000U/ml Leukaemia inhibitory factor (LIF), or 4μl/ml recombinant LIF conditioned media to maintain self-renewal.

To passage, cells were washed with phosphate buffered saline (PBS) and 0.5ml of trypsin/Ethylenediaminetetraacetic acid (EDTA) applied. Dishes were incubated for 5 minutes at 37°C with 5% (v/v) $CO_2$. Cells were resuspended in KO complete media and pelleted at 1000rpm. The supernatant was removed and cells resuspended in fresh KO complete media. Cells were plated at $0.5x10^6$ cells per dish and routinely cultured every two days to maintain a sub-confluent density ( $<1.5x10^6$ cells per dish) and to limit differentiation. Cultures were passaged for no longer than 3 weeks (Paling et al., 2004).

#### *2.1.3.1 LIF-conditioned media*

Conditioned media was collected from the HEK293LIFV5 cell line (Park et al., 2003b) generated by stable expression of a V5 epitope-tagged LIF gene in HEK293 cells. (a kind gift from Dr. Konstantinos Anastassiadis, University of Technology, Dresden). Media was sterilised by filtration and batch tested in-house for the capacity to maintain self-renewal and stimulate LIF-mediated signalling.

#### *2.1.3.2 N2B27 chemically defined serum-free media*

E14tg2A cells were cultured in N2B27-defined media containing 1:1 Neurobasal media to DMEM F12 media supplemented with N2 and B27 supplements (Bottenstein and Sato, 1979, Brewer and Cotman, 1989, Brewer et al., 1993)) (Table 2.1), 2mM glutamine, 50µM Bovine Serum Albumin (BSA) fraction V, 0.0125% (v/v) Monothioglycerol (MTG), 1000U/ml LIF and 10ng/ml recombinant BMP4. To maintain a high cell density, cells were plated at no fewer than $0.5x10^6$ cells/92cm NUNC-tissue culture dish coated with 0.1% (w/v) porcine gelatine. Stimulation experiments were conducted using cells cultured for at least 2 days in N2B27-defined media.

**Table 2.1 Composition of N2 and B27 supplements**

| N2 SUPPLEMENT | B27 SUPPLEMENT |
|---|---|
| 25µg/ml Insulin | Biotin |
| 100µ/ml Apo-transferrin | L-camitine |
| 30ng/ml Sodium Selenite | Corticosterone |
| 16µg/ml Putrescine | Ethanolamine |
| 6ng/ml Progesterone | D(+)-galactose |
| | Glutathione (reduced) |
| | Linoleic acid |
| | Linolenic acid |
| | Progesterone |
| | Putrescine |
| | Retinyl acetate |
| | Selenium |
| | T3 (triodo-1-thyronine) |
| | DL-α-tocopherol (Vitamin E) |
| | DL-α-tocopherol acetate |
| | Albumin (bovine) |
| | Catalase |
| | Insulin |
| | Superoxide dismutase |
| | Transferrin |

#### *2.1.3.3 Murine ESC freezing and thawing*

Freezing media consisted of Glasgow Modified Eagle Medium (GMEM) supplemented with 2mM glutamine, 50µM 2-mercaptoethanol, 1% (v/v) non-essential amino acids, 1mM sodium pyruvate. E14tg2A cells were suspended at $2x10^6$ cells/ml in freezing media plus 10% ES screened Fetal Bovine Serum (FBS). Cold freezing media plus 10% (v/v) DMSO was added drop wise to give a final suspension of $1x10^6$ cells/ml. Aliquots of 1ml were added to NUNC cryovials and stored at -80$^oC$ for 24 hours. Cryovials were transferred to liquid nitrogen for long-term storage.

Cells were thawed rapidly and resuspended drop wise in 10ml KO complete media to wash. Pelleted cells were resuspended and plated onto gelatine coated, 92 x 17mm NUNC dishes. After 24 hours media was replaced and cells routinely cultured as previously described.

### *2.1.4 BaF/3 cell line*

BaF/3 cells are an immortalised murine bone marrow derived pro-B cell line with interleukin-3 (IL-3)-dependent proliferation (Palacios and Steinmetz, 1985).

BaF/3 cells were cultured in non-tissue culture treated plastic in RPMI 1640 medium supplemented with 10% (v/v) Fetal Calf Serum (FCS), 20µM 2-mercaptoethanol, 100U penicillin/streptomycin, 2mM glutamine and 5-10% JWW3 conditioned media, as a source of IL-3. Cells were passaged regularly by serial dilution.

#### *2.1.4.1 JWW3 conditioned media*

JWW3 cells expressing murine IL-3 (mIL-3) were cultured in 175 $cm^2$ tissue culture flasks for 1 week. Media was collected and filtered to remove cells and cell debris then sterilised using 0.2µm bottle top filter (Nalgene). JWW3 conditioned media was tested using an XTT dye reduction assay (section 2.4.1) with BaF/3 cells to determine the optimum concentration of conditioned media for maximum BaF/3 cell growth (typically 5-10% JWW3). JWW3 conditioned media was stored at -20$^oC$.

#### *2.1.4.2 BaF/3 freezing and thawing*

BaF/3 cells were suspended at $2x10^6$/ml in 90% FCS and 10% DMSO. Aliquots of 1ml were added to NUNC cryovials and stored at -80$^oC$ for 24 hours. Cryovials were transferred to liquid nitrogen for long-term storage.

BaF/3 cells were thawed rapidly, resuspended in supplemented RPMI 1640 medium and pelleted. The pelleted cells were resuspended in supplemented RPMI 1640 culture media for expansion.

**Table 2.2 Tissue culture consumables**

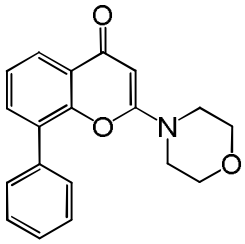
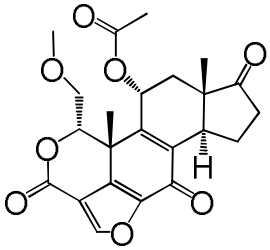
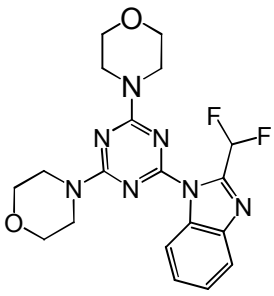
| PRODUCT | SUPPLIER | CATALOG # |
|---|---|---|
| **GROWTH MEDIUM** | | |
| Glasgow's Modified Eagle Medium (GMEM) | Invitrogen, Paisley, UK | 21710-025 |
| Knockout Dulbecco's Modified Eagle Medium (DMEM) | Invitrogen | 10829-018 |
| RPMI 1640 | Invitrogen | 21875-059 |
| **SERUM** | | |
| Knockout Serum Replacement | Invitrogen | 10828-028 |
| Bovine Serum Albumin (BSA) Fraction V (for N2B27-defined medium) | | |
| Fetal Calf Serum (for BaF/3 cell line) | Sigma, Dorset, UK | |
| ES Screened Fetal Bovine Serum (FBS) (Hyclone) | Perbio, Hyclone, UK | SH30070.03E |
| ES Screened Fetal Bovine Serum (FBS) (Biosera, South America origin) | Biosera, Ringmer, East Sussex, UK | S181S |
| **MEDIUM SUPPLEMENTS** | | |
| ESGRO LIF | Chemicon, Hampshire, UK | ESG1106 |
| N2 Supplement (100X) | Fisher Scientific, Leicestershire, UK | 07152 |
| B27 Supplement (50x) | Fisher Scientific | 07153 |
| Glutamine | Invitrogen | 25030-024 |
| 2-mercaptoethanol | Bio-Rad, Hemel Hempstead, Hertfordshire | 161-0710 |
| 100x Non-essential amino acids (NEAA) | Invitrogen | 11140-050 |
| Monothioglycerol | Sigma | M6145 |
| Recombinant Human BMP4 | R&D Systems, Abingdon, UK | 314-BP |
| Sodium Pyruvate | Fisher Scientific | 11360 |
| Tetracycline Hydrochloride | Sigma | T7660 |
| Penicillin/streptomycin | Invitrogen | 15140-122 |
| **OTHER** | | |
| Porcine Gelatine | Sigma | G1890-110G |

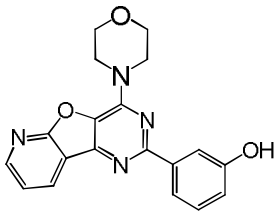
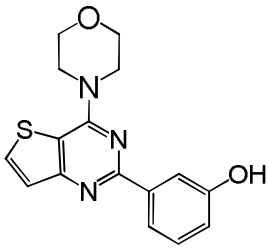
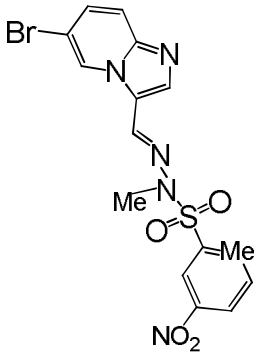
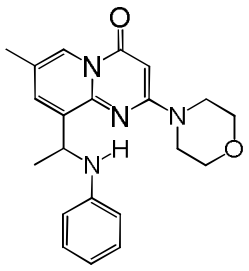
| | | |
|---|---|---|
| Trypsin-Ethylenediaminetetraacetic acid (EDTA) | Fisher Scientific | 25300-062 |
| DMSO | Sigma | D2650 |
| Phosphate Buffered Saline (PBS) | Invitrogen | 14200-067 |
| 10x Hank's Balanced Salt Solution (HBSS) | Invitrogen | 14060-040 |
| **TISSUE CULTURE PLASTIC WARE** | | |
| 15ml Centrifuge Tubes | Greiner Bio-one (GBO) Gloucestershire, UK | 188271 |
| 50ml Centrifuge Tubes | GBO | 227261 |
| NUNC Cryovials | Fisher Scientific | CRY-960-070B |
| NUNC Tissue Culture dish 60x15mm | Fisher Scientific | TKT-110-010S |
| NUNC Tissue Culture dish 92x17mm | Fisher Scientific | TKT-110-070A |
| NUNC T75 Tissue Culture Flasks | Fisher Scientific | TKT-130-130R |
| 3ml Pasteur Pipettes | GBO | 612398 |
| Petri (non-tissue culture treated) dishes 60x15mm | GBO | 628160 |
| 10ml Single-wrapped Sterile Pipettes | GBO | 607180 |
| 25ml Single-Wrapped Sterile Pipettes | GBO | 760180 |
| 150mm unplugged glass pipettes | Fisher Scientific | FB50251 |

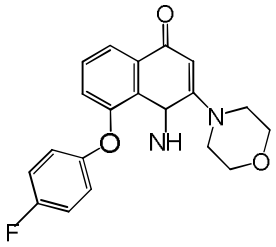
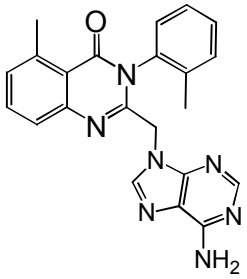
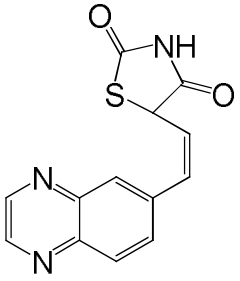
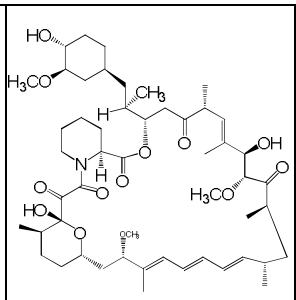
## 2.2 Inhibitors

A variety of inhibitors were used in this study to assess the role of PI3Ks in mESC fate (Table 2.3).

**Table 2.3 Pharmacological Inhibitors**

| INHIBITOR | STRUCTURE | INFORMATION | SUPPLIER |
|---|---|---|---|
| **BROAD SPECTRUM PI3K INHIBITORS** | | | |
| LY294002 |  | $IC_{50}$<br>PI3K 1.4 µM<br>p110α 9.3 µM<br>p110β 2.9 µM<br>p110δ 6.0 µM<br>p110γ 38 µM<br>mTOR 8.9 µM<br><br>Inhibition of $PIP_2$ production in platelets $IC_{50}$ 2.5-5 µM<br><br>(Vlahos et al., 1994, Walker et al., 2000, Jackson et al., 2005) | Calbiochem |
| Wortmannin |  | $IC_{50}$<br>PI3K 2-4 nM<br>Class $I_A$ 4.2nM<br><br>Inhibition of agonist-mediated respiratory-burst activation $IC_{50}$ 12nM<br><br>(Arcaro and Wymann 1993, Knight et al., 2004, Powis et al., 1994, Walker et al., 2000, Wymann et al., 1996) | Calbiochem |
| ZSTK474 |  | $IC_{50}$<br>PI3K 37 nM<br>p110β 17 nM<br>p110δ 6 µM<br>p110γ 53 µM<br><br>Inhibition of growth of HUVECs 0.146 µM<br><br>(Dan et al., 2008, Kong and Yamori, 2007, Yaguchi et al., 2006, Kong et al., 2009) | Zenyaku Kogyo Co, Tokyo, Japan.<br>Generous gift from Tom Crabbe, UCB, Slough UK |

| | | | |
|---|---|---|---|
| **DUAL PI3K/mTOR INHIBITOR** | | | |
| PI-103 |  | $IC_{50}$<br>p110α 0.008 μM<br>p110β 0.088 μM<br>p110δ 0.048 μM<br>p110γ 0.15 μM<br>mTORC1 0.02 μM<br>mTORC2 0.083 μM<br><br>(Fan et al., 2006, Hayakawa et al, 2001b, Knight et al., 2004, Knight et al., 2006) | Generous gift from Tom Crabbe, UCB, Slough, UK<br><br>Patent WO01083456 |
| **PI3K p110 CATALYTIC SUBUNIT ISOFORM INHIBITORS** | | | |
| **p110α Selective** | | | |
| Compound 15e |  | Inhibition of A375 cell proliferation $IC_{50}$ 580nM<br><br>(Hayakawa et al., 2006) | Alexis Biochemicals, Nottingham, UK |
| PIK-75 |  | $IC_{50}$<br>p110α 0.0058 μM<br>p110β 1.3 μM<br>p110δ 0.51 μM<br>p110γ 0.076 μM<br>mTORC1 1 μM<br>mTORC2 10 μM<br><br>(Hayakawa et al., 2001a, Knight et al., 2001) | Generous gift from Peter Shepherd, University of Auckland, New Zealand<br><br>Patent WO2001083481 |
| **p110β Selective** | | | |
| TGX-221 |  | $IC_{50}$<br>p110α 5 μM<br>p110β 5-50 nM<br>(for ATP 50 μM-1 mM)<br>p110γ 10 μM<br><br>Inhibition of $PIP_2$ production in platelets 50nM<br><br>(Condliffe et al., 2005, Hennessy et al., 2005, Jackson et al., 2004, Jackson et al., 2005) | Generous gift from Peter Shepherd<br>Patent WO2004016607 |

| | | | |
|---|---|---|---|
| TGX-121 |  | $IC_{50}$<br>PI3K 0.05 μM<br>p110β 0.05 μM<br>p110γ 5 μM<br><br>(Robertson et al., 2001, Kuang et al., 2006) | Generous gift from Tom Crabbe<br><br>Patent WO0153266 |
| **p110δ Selective** | | | |
| IC87114 |  | $IC_{50}$<br>p110α >200 μM<br>p110β 16 μM<br>p110δ 0.13 μM<br>p110γ 61 μM<br><br>(Knight et al., 2006, Sadhu et al., 2001, Sadhu et al., 2003) | Generous gift from Tom Crabbe<br><br>Patent WO0181346 |
| **p110γ Selective** | | | |
| AS605240 |  | $IC_{50}$<br>p110γ 0.008 μM<br><br>Inhibition of C5a mediated PKB phosphorylation in RAW264 mouse macrophages $IC_{50}$ 0.09 μM<br><br>(Barber et al., 2005, Camps et al., 2005) | Generous gift from Tom Crabbe<br><br>Patent WO2004007491 |
| **mTOR INHIBITOR** | | | |
| Rapamycin |  | Inhibition of p70 S6K activity in 3T3 cells $IC_{50}$ 50 ρM<br><br>(Kuo et al., 1992) | Calbiochem |

| G PROTEIN INHIBITOR | | | |
|---|---|---|---|
| Pertussis toxin (PTX) | $AB_5$-type exotoxin produced by the bacterium *Bordetella pertussis* | PTX inhibition of norepinephrine-induced inositol phosphate production in primary cultures of neuronal and glial cells, $IC_{50}$ 7 ng/ml<br><br>(Burns, 1988, Wilson and Minneman 1990) | Calbiochem |

$IC_{50}$ values represent the concentration of an inhibitor that is required for 50% inhibition. However, it is difficult to compare $IC_{50}$ values since they are derived in different ways. Assays can be cell based or cell-free but can also differ in the conditions used to derive this value. For example, variations in the concentration of ATP between assays can alter the $IC_{50}$ value obtained for an ATP-competitive inhibitor in a kinase assay. Furthermore, cell type, relative expression of target proteins and off-target proteins, cell permeability and stability of inhibitors under tissue culture conditions and the measurement of a biological process can all cause additional variability.

## 2.3 Biochemical analysis techniques

### *2.3.1 Cell stimulations*

#### *2.3.1.1 mESC stimulations*

A number of different media were used to assess intracellular signalling in mESCs relating to PI3K activation, inhibition of specific PI3K isoforms and siRNA-mediated knockdown of PI3K catalytic isoforms. Figure 2.1 details the different media used for mESC stimulation experiments.

E14tg2A mESCs or Mek* transfected mESCs were plated at $3x10^5$ cells per 60mm gelatin coated NUNC-tissue culture dish and incubated for 24-48 hours. Prior to stimulation, cells were washed three times with PBS and serum starved by incubating for 4 hours in GMEM supplemented with 2mM glutamine, 50µM 2-mercaptoethanol, 1% (v/v) non-essential amino acids and 1mM sodium pyruvate. No serum starvation period was required for cells cultured in N2B27-defined media since it is serum-free. Instead, cells were incubated for 4 hours in 1:1 Neurobasal media to DMEM F-12 media for 4 hours. Where stated, cells were pre-treated with inhibitors for 30 minutes. The stated dose of cytokine was diluted in GMEM and 1:1 Neurobasal media to DMEM F-12 media and applied to cells. During the stimulation period, dishes were incubated at 37°C and 5% (v/v) $CO_2$.

For stimulation of GPCRs, 10% (v/v) KO SR in GMEM was boiled for 5 minutes and allowed to cool to room temperature prior to stimulations. Murine ESCs were stimulated for 5 minutes with boiled 10% (v/v) KO SR in GMEM and washed thoroughly with ice cold PBS to ensure all serum was removed from cells prior to lysing. Where pertussis toxin was used, mESCs were incubated with 10ng/ml pertussis toxin over-night. A 4 hour serum starvation period was carried out prior to stimulation.

For long-term stimulations (1-5 days) and analysis of basal signalling, cells were plated at $1x10^5$ cells per 60mm gelatin coated NUNC-tissue culture dish in KO complete media supplemented with 1000U/ml LIF or N2B27-defined media supplemented with 1000U/ml LIF and 10ng/ml BMP4. Inhibitors were added after 4 hours, which allowed cells to adhere to the tissue culture dish.

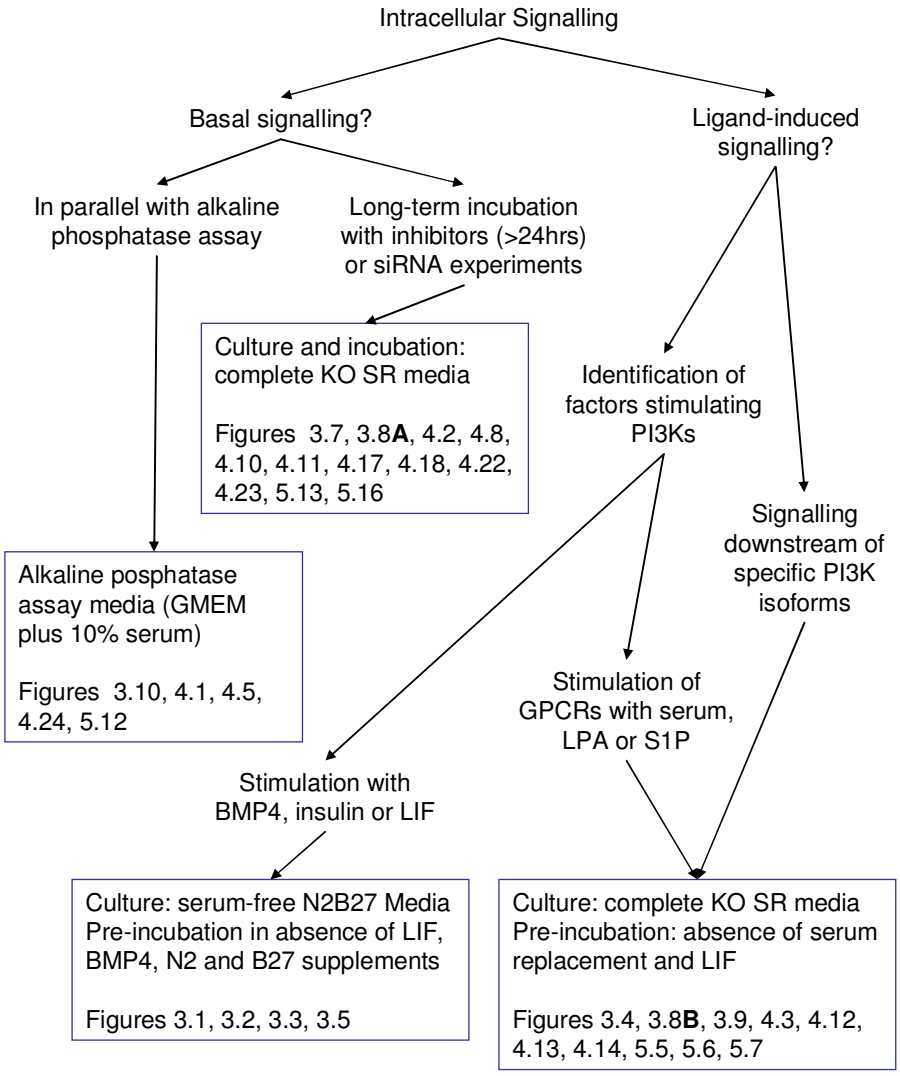



**Figure 2.1 Experimental design and medium used for mESC stimulations**
Different media were used for the culture and stimulation of mESCs depending on the aim of a particular experiment. The culture media and pre-incubation media, where appropriate, are indicated in boxes.

Following stimulations, dishes were washed twice with ice-cold PBS and cells solubilised in 30µl/$10^6$ cells of ice-cold lysis buffer (50mM Tris-HCl pH 7.5, 150mM sodium chloride, 1% (v/v) Nonidet P40, 10% (v/v) glycerol, 5mM EDTA, 1mM sodium vanadate, 1mM sodium molybdate, 10mM sodium fluoride, 0.7µg/ml Pepstatin A, 40µg/ml phenylmethylsulphonyfluoride (PMSF), 10µg/ml Aprotinin and 10µg/ml soyabean trypsin inhibitor). Dishes were scraped using a cell scraper and cell extracts transferred to 1.5ml tubes. Samples were spun at 14000rpm at 4 °C for 1 minute in a microfuge to remove cell debris. Supernatants were transferred to clean tubes for storage at -20 °C.

#### *2.3.1.2 BaF/3 cell stimulations*

BaF/3 cells were washed three times with Hanks buffered saline containing 20mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and resuspended at $1x10^7$ cells/ml in RPMI 1640 and 20mM HEPES lacking serum and IL-3 (JWW3 conditioned media). Cells were serum starved for 45 minutes in a 37°C water bath. Cells were pre-treated with inhibitors for 30 minutes prior to stimulation. Following stimulation with 20ng/ml IL-3 for 10 minutes, cells were spun briefly. The supernatant was aspirated and the cell pellet lysed with ice-cold lysis buffer at $2x10^7$ cells/ml. Samples were spun at 14000 rpm at 4°C for 1 minute to remove cell debris. Supernatants were transferred to clean tubes for storage at -20°C.

#### *2.3.1.3 Bradford assay*

A Bradford assay (Bradford, 1976) was performed to determine lysate protein concentrations. This colorimetric assay relies on a directly proportional relationship between the absorbance of Coomassie brilliant blue dye at 595nm and the amount of protein present. Absorbance of the dye increases according to the amount of dye bound to arginine and hydrophobic residues of proteins.

Bradford reagent was diluted 1:10 in milli-Q® $H_2O$. To derive a standard curve, 0, 2, 4, 6, 8, 10 or 12µg/ml Bovine Serum Albumin (BSA) were added to 1ml aliquots of 1:10 diluted Bradford reagent. In separate tubes, 3-5µl of cell extract samples were added to 1ml aliquots of 1:10 diluted Bradford reagent. Standard and sample tubes were vortexed briefly and 200µl per well plated in triplicate on flat-bottomed 96 well plates. The absorbance at 595nm of each well was measured using a Versamax plate reader (Molecular Devices). Sample protein concentrations were extrapolated from the standard curve established from known BSA dilutions.

The same amount of protein (10-20µg) was taken from each sample and diluted with lysis buffer to a maximum volume of 20µl. SDS-sample buffer (5X sample buffer: 10% SDS (w/v), 50% glycerol (v/v), 200mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol and 2% bromophenol blue) was added to each sample and tubes were boiled for 5 minutes at 95°C to denature proteins.

### *2.3.2 Protein resolution and immunoblotting*

#### *2.3.2.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

Sample proteins were separated according to molecular size by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Boiling of protein samples in reducing conditions (containing 2-mercaptoethanol) denatures protein structure and breaks disulphide bonds. SDS binds linear protein at approximately 1.4g per 1g protein to give all denatured polypeptide chains an equal mass to charge ratio. Subsequent separation by SDS-PAGE is therefore independent of intrinsic electrical charge and dependent on polypeptide relative molecular mass alone.

Samples were loaded into individual lanes of a stacking gel of large pore size and pH 6.8. At this pH, glycine, a component of the running buffer, is weakly ionised with a low mobility but NaCl is highly ionised and has a greater mobility through the gel. When a constant voltage is applied, current passes through the gel and protein polypeptides are trapped between the glycine and NaCl phases within the stacking gel. This arrangement allows sample polypeptides to be collected between the NaCl/glycine moving boundaries and focused into a thin band of migration. Thus, all polypeptides within the samples enter the resolving gel at approximately the same time. The resolving gel has smaller pore sizes to allow polypeptides to separate according to relative molecular mass. The resolving gel of pH 8.8 causes glycine to become highly ionised and allows it to migrate much faster through the gel without restricting the migration of polypeptides (Laemmli, 1970).

A broad range molecular weight marker, diluted in 1x SDS sample buffer and boiled, was loaded into one lane of each gel and run in parallel to the samples. This was used to identify proteins of interest, and known molecular weight, following immunoblotting.

SDS-PAGE gels were prepared using Mini Protean III Gel Electrophoresis Apparatus (Bio-Rad). The percentage of acrylamide used in the resolving gel was determined according to the size of the proteins to be resolved (Table 2.4).

**Table 2.4 Proteins resolved according to acrylamide Content**

| RESOLVING GEL ACRYLAMIDE % | RESOLVED PROTEIN SIZE (kDa) |
|---|---|
| 7.5 | 40-200 |
| 10 | 30-200 |
| 12 | 15-100 |

The resolving gel solution (Table 2.5) was made according to the required acrylamide percentage (Table 2.4). TEMED (Tetramethylethylenediamine) was added last and just prior to pouring to initiate the cross-linking reaction. The resolving gel solution was poured into the gel casting apparatus (4.5ml per gel) and overlaid with milliQ® $H_2O$.

**Table 2.5 Resolving gel solution**

| ACRYLAMIDE %: | 7.5 | 10 | 12 |
|---|---|---|---|
| Acrylamide/bisacrylamide 30% (w/v)/ 0.8% (w/v) (ml) | 3.75 | 5.0 | 6.0 |
| milliQ $H_2O$ (ml) | 5.6 | 4.35 | 3.35 |
| 1M Tris-HCl pH 8.8 (ml) | 5.6 | 5.6 | 5.6 |
| 10% (w/v) SDS (ml) | 0.25 | 0.25 | 0.25 |
| 10% (w/v) Ammonium persulphate (APS) (µl) | 50 | 50 | 50 |
| Tetramethylethylenediamine (TEMED) (µl) | 20 | 20 | 20 |

The upper stacking gel solution consisted of 1.67ml acrylamide/bisacrylamide, 6ml milliQ® $H_2O$, 1.25 1M Tris-HCl, 150µl 10% (w/v) SDS, 50µl 10% (w/v) APS and 20µl TEMED. When the resolving gel had set, the overlaying milliQ® $H_2O$ was aspirated and the stacking gel solution poured onto the resolving gel. Combs were inserted to form wells until the stacking gel had set. The wells were washed briefly with milliQ® $H_2O$ and aspirated. The gel running apparatus was assembled and the reservoir filled with 1x SDS-PAGE running buffer (10x 3% (w/v) Tris, 0.1% (w/v) SDS, 14.4% (w/v) glycine).

Samples were loaded into individual wells and a molecular weight marker into a separate well. A constant voltage of 80mV was applied during stacking and 180mV to resolve.

#### *2.3.2.2 Immunoblotting*

The process of immunoblotting transfers resolved proteins from SDS-PAGE gels onto nitrocellulose paper to allow antibody detection of proteins. Proteins from SDS-PAGE gels were transferred onto nitrocellulose paper by semi-dry transfer. Graphite electrodes of Pharmacia LKB Novoblot electroblotting apparatus were dampened with semi-dry transfer buffer (39mM glycine, 48mM Tris base, 0.0375% (w/v) SDS, 20% (v/v) methanol). Nitrocellulose blotting membrane and 3MM Whatman chromatography paper were cut to the same size as the resolving gel (8.5x5.5cm) and soaked in semi-dry transfer buffer. Four dampened sheets of Whatman paper, a sheet of nitrocellulose blotting membrane, an SDS-PAGE gel and four sheets of dampened Whatman paper were sandwiched between the upper and lower graphite electrode transfer apparatus. Immunoblotting was performed with a current of 0.8mA per $cm^2$ nitrocellulose paper for 1 hour.

After transfer, nitrocellulose membranes were rinsed briefly in $dH_2O$. Ponceau S solution was used to stain nitrocellulose-bound protein to assess transfer, equal loading of samples and to visualise the broad range molecular markers. Ponceau stain was washed off with Tris-buffered Saline (TBS) (150mM NaCl, 20mM Tris-HCl pH 7.5). Nitrocellulose membrane blots were blocked for 1 hour in either 5% BSA block (5% (w/v) BSA, 1% (w/v) ovalbumin, 0.05% (w/v) sodium azide in TBS) or 2% ECL (Enhanced chemiluminescence) Advance blocking solution (200mg ECL advance blocking agent/10ml TBS) at room temperature. Nitrocellulose blots were incubated with primary antibodies (Table 2.6) at 4°C for 16 hours with gentle agitation.

Blots were washed once in TBS, three times with TBST (TBS with 0.05% (v/v) Tween) for 15 minutes then once again with TBS wash. Blots were incubated with an appropriate horse radish peroxidase (HRP) conjugated secondary antibody diluted in TBST with gentle agitation. After 1-2 hours, blots were washed as before with an additional final TBS wash. ECL Western blotting detection reagent or ECL Advanced was applied for 1 minute. Blots were wrapped in Clingfilm and placed in an autoradiography cassette. In a dark room, blots were exposed to autoradiography film for 1-10 minutes, and developed with an RGII Fuji X-Ray film developer.

**Table 2.6 Primary antibodies for immunoblotting**

| ANTIBODY | SOURCE | BLOCK | DILUTION | DILUENT | SIZE kDa | SUPPLIER |
|---|---|---|---|---|---|---|
| **Primary Antibodies** | | | | | | |
| α-p110α | Rabbit | 5% BSA | 1:3000 | 1% BSA | 110 | SCB 7174 |
| α-p110β | Rabbit | 5% BSA | 1:1000 | 1% BSA | 110 | SCB 602 |
| α-p85 | Rabbit | 5% BSA | 1:5000 | 1% BSA | 85 | UBI 06-195 |
| α-Nanog | Rabbit | ECL Adv. | 1:5000 | ECL Adv. | 34/39 | AC 21603 |
| α-Oct4 | Rabbit | ECL Adv. | 1:1000 | ECL Adv. | 43 | SCB 9081 |
| α-phospho-p44/42 MAPK (Thr202/Tyr204) (referred to as pErk-1/2) | Rabbit | 5% BSA | 1:1000 | 1% BSA | 42/44 | SCB 93 |
| α-phospho-Mek 1 (Ser217/221) | Rabbit | 5% BSA | 1:1000 | 1% BSA | 45 | CST 9121 |
| α-His Tag (27E8) | Mouse | 5% BSA | 1:1000 | 1% BSA | | CST 2366 |
| α-pStat3 (Tyr 705) | Rabbit | 5% BSA | 1:1000 | 1% BSA | 79 | SCB 9131 |
| α-pStat3 (Ser 668) | Mouse | 5% Milk | 1:1000 | PBS | 79 | BL |
| α-pStat3 (Ser 727) | Rabbit | 5% BSA | 1:2500 | 1% BSA | 79 | BL |
| α-phospho-ribosomal S6 protein (Ser235/236) (referred to as pS6) | Rabbit | 5% BSA | 1:2000 | 1% BSA | 32 | CST 2211 |
| α-pPKB (Ser 473) | Rabbit | 5% BSA | 1:1000 | 1% BSA | 60 | CST 4058 |
| α-pPKB (Thr 308) | Rabbit | ECL Adv. | 1:2000 | ECL Adv. | 60 | CST 4056 |
| α-pGSK-3 α/β (Ser21/9) | Rabbit | ECL Adv. | 1:2000 | ECL Adv. | 49/51 | CST 9331 |
| α-pSmad1 (Ser 463/465) | Rabbit | 5% BSA | 1:1000 | 1% BSA | 60-65 | UBI 06-702 |
| **Primary Antibodies used for Reprobing** | | | | | | |
| α-p44/42 MAPK (referred to as Erk-1/2) | Rabbit | 5% BSA | 1:1000 | 1% BSA | 42/44 | CST 9102 |
| α-Stat3 | Rabbit | 5% BSA | 1:2000 | 1% BSA | 79 | SCB 482 |
| α-PKB | Rabbit | 5% BSA | 1:1000 | 1% BSA | 60 | CST 9272 |
| α-GSK-3 β | Rabbit | 5% BSA | 1:1000 | 1% BSA | 46 | CST 9315 |
| α-SH2 domain tyrosine phosphatase (referred to as Shp-2) | Rabbit | 5% BSA | 1:2000 | 1% BSA | 70 | SCB 280 |
| α-Mek 1 (N-terminus) | Mouse | 5% BSA | 1:1000 | 1% BSA | 45 | |
| α-Mek 1 | Rabbit | 5% BSA | 1:1000 | 1% BSA | 45 | CST 9122 |
| **Suppliers** | | | | | | |
| AC | AbCam, Cambridge, UK | | | | | |
| BL | H Boeuf Laboratory, CNRS, Bordeaux, France | | | | | |
| CST | Cell Signaling Technologies, New England Biolabs (NEB), Hitchin, UK | | | | | |
| SCB | Santa Cruz Biotechnology, Autogen Bioclear, Calne UK | | | | | |
| UBI | Upstate Biotechnology Incorporated (Millipore), UK | | | | | |

**Table 2.7 Secondary antibodies for immunoblotting**

| ANTIBODY | SOURCE | DILUTION | DILUENT | SUPPLIER |
|---|---|---|---|---|
| Mouse-HRP | Goat | 1:10000 | TBST | Dako Cytomation #P0447 |
| Rabbit-HRP | Goat | 1:20000 | TBST | Dako Cytomation #P0778 |

#### *2.3.2.3 Stripping and reprobing nitrocellulose membranes*

Blots were stripped of initial probing reagents in stripping buffer (6.25% (v/v) 1M Tris-HCl pH 7.5, 2% (w/v) SDS, 0.77% (v/v) 2-mercaptoethanol) at 55°C for 45 minutes. Blots were washed thoroughly in TBST (2-mercaptoethanol waste was disposed of appropriately) and incubated in 5% blocking buffer for 1 hour. Appropriate primary and secondary antibodies were used to re-probe bound proteins, as before.

### *2.3.3 Immunochemistry*

#### *2.3.3.1 Cytospin preparation of slides*

Mek* transfected mESCs were washed twice with PBS, dissociated and resuspended at $1x10^5$ cells/ml in GMEM plus 5% ESC screened FBS. After securing a glass slide, filter paper and cuvette in a metal holder, 100µl of resuspended cells were loaded per slide. This was spun at 500rpm for 10 minutes under slow acceleration in a Cytospin 3 (Shandon). The glass slide was carefully retrieved and the area around the cytocentrifuged cells marked with a PAP hydrophobic barrier pen. Cells were fixed in 90% ethanol for 10 minutes and rinsed in TBS.

#### *2.3.3.2 Immunochemistry staining*

Fixed cells were blocked for 1 hour in 1% block (1% (w/v) BSA in TBS) then incubated overnight with diluted primary His-Tag Antibody 1:800 in 1% block at 4°C. Slides were washed in TBS for 15 minutes. Two drops of peroxidise block were applied per slide for 15 minutes followed by a brief rinse in TBS. The secondary antibody, goat anti-mouse HRP diluted 1:200 in 1% block was applied for 1 hour and rinsed twice with TBS. Slides were developed with DAB (3,3'-diaminobenzidine) (1ml DAB buffer with two drops of DAB reagent) for 30 minutes then briefly rinsed with water. Cells were counterstained for 20 seconds with Mayer's Hematoxylin then rinsed with water, air-dried and mounted with Pertex mounting media.

**Table 2.8 Biochemical consumables**

| PRODUCT | SUPPLIER | CATALOG # |
|---|---|---|
| **Cell Stimulations** | | |
| Aprotinin | Roche Biochemicals, Burgess Hill, UK | 236624 |
| Bromophenol blue | BD electran | 44305 |
| Bradford Reagent | Bio-Rad | 500-006 |
| Glasgow's Modified Eagle medium | Invitrogen | 21710-025 |
| 200mM Glutamine | Invitrogen | 25030-024 |
| Glycerol | Sigma | G5150 |
| 1M HEPES | Invitrogen | 15630-056 |
| Leupeptin | Sigma | L8511 |
| Nonidet P40 | VWR, Leicestershire, UK | 560092-L |
| Pertussis toxin | Calbiochem | |
| Pepstatin | Sigma | P5318 |
| Phosphate buffered saline (PBS) | Invitrogen | 18912-014 |
| PMSF | Sigma | H0891 |
| Sodium Chloride | Sigma | S7653 |
| SDS | VWR | 442444-H |
| Sodium Fluoride | Sigma | S6521 |
| Sodium Molybdate | VWR | 102542-Q |
| Sodium Vanadate | Sigma | S6508 |
| Soybean Trypsin Inhibitor | Sigma | T9003 |
| Trizma (Tris) base | Sigma | T8404 |
| **Protein Resolution and Immunoblotting** | | |
| 30% Acrylamide/bisacrylamide | Bio-Rad | 161-0158 |
| Ammonium Persulphate | Sigma | A7460 |
| Bovine Serum Albumin (BSA) | Roche Biochemicals | 735-108 |
| ECL | Amersham/GE Healthcare, Buckinghamshire, UK | RPN-2106 |
| ECL Advance | Amersham/GE Healthcare | RPN-2135 |
| Glycine | Sigma | G8790 |
| Hybond ECL Nitrocellulose membrane | Amersham/GE Healthcare | RPN203D |

| | | |
|---|---|---|
| Ovalbumen | Sigma | A5378 |
| Ponceau S | Sigma | P7170 |
| Sodium azide | Fisher Scientific | S2380-48 |
| TEMED | Sigma | T9281 |
| 3MM Whatman paper | VWR | 3030917 |
| **Immunochemistry** | | |
| His-Tag Antibody | Cell Signalling Technologies | 2366 |
| Peroxidase Block | Dako Cytomation | 002428 |
| DAB | Dako Cytomation | K3465 |
| Mayer's Hematoxylin | BDH | |
| Pertex Mounting Media | Cell Path | SEA-0104-00A |

## 2.4 Functional assay techniques

### *2.4.1 XTT dye reduction metabolism assay*

This technique was adapted from that used to assay for activated T cell and murine cytokine-dependent cell line viability and proliferation (Roehm et al., 1991, Scudiero et al., 1988). The assay relies on the cleavage of yellow tetrazolium salt XTT (sodium 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) by mitochondrial dehydrogenases present in metabolically active cells, to produce an orange, water-soluble, formazan dye product. Optical density is measured at 450nm and the bioreduction of XTT is potentiated by the addition of phenazine methosulfate (PMS), an electron coupling agent.

#### *2.4.1.1 XTT assays for BaF/3 cells*

BaF/3 cells were plated at $1x10^3$ cells/well in NUNC flat bottomed 96 well plates in serum-free AIM-V media supplemented with recombinant mIL-3 to a final volume of 100µl. Cells were incubated with inhibitors for 48-72 hours at 37°C.

PMS-XTT solution was made by adding 5µl of 1.53mg/ml PMS in PBS to 1ml of 1mg/ml XTT in warmed medium. 25µl of PMS-XTT was added per well and incubated at 37°C for 4 hours. The soluble formazan product was measured at 450nm on a Versamax plate reader.

#### *2.4.1.2 XTT assays for mESCs*

Murine ESCs were plated on a NUNC flat bottomed 96 well plate at $2.5x10^3$ cells per well in GMEM plus 10% Hyclone serum supplemented with 1000U/ml LIF. Inhibitors were added after cells had adhered to give a final volume of 200µl. Plates were incubated for 24-48 hours at 37°C. 50µl of PMS-XTT solution was added per well. After 4 hours incubation, the absorbance at 450nm was read on a Versamax microplate reader.

#### *2.4.1.3 Analysis of XTT assay*

The mean absorbance of wells containing GMEM, Hyclone and PMS-XTT solution but no cells was subtracted from the absorbance of each well containing cells. Data were analysed by a one-way ANOVA, with Dunnet's post-hoc test where appropriate, to detect any effect of treatment on cell metabolism.

### *2.4.2 Alkaline Phosphatase self-renewal assay*

Self-Renewal of mESCs was assessed by assaying for mESC surface alkaline phosphatase activity. Self-renewing mESCs express alkaline phosphatase, but cell-surface expression is lost upon differentiation (Scutt and Bertram, 1999).

E14tg2A or Mek* transfected mESCs were plated at $1.5x10^3$ cells per well of 6 well NUNC-tissue culture plates coated with 0.1% (w/v) porcine gelatin in GMEM, or complete KO SR medium where stated, supplemented with 10% Hyclone serum (or 10% Biosera serum) and 1000U/ml LIF (Chemicon). For some experiments, limited LIF concentrations were used, these are indicated accordingly. Where indicated, 500ng/ml tetracycline was added. Inhibitors were added after 4 hours when cells had adhered. Plates were incubated for 4-5 days at 37°C with 5% (v/v) $CO_2$.

Cells were washed twice with PBS and fixed with 100% methanol for 10 minutes. Plates were allowed to air-dry at room temperature. Staining was conducted by applying a solution of 0.1M Tris-HCl pH 9.2, 200µg/ml Napthol AS-MX and 1mg/ml Fast Red TR Salt, for 20 minutes. Wells were washed twice with milliQ® $H_2O$ and air-dried.

All colonies were counted in each well and scored according to the extent of staining and appearance. Pure self-renewing colonies stain bright red and are compact with a smooth outline. Self-renewing colonies stain strongly in the centre with a paler outer area and often have a rough perimeter to the colony. Differentiated cells stain weakly or do not stain at all and have a flattened, rough morphology. The number of pure self-renewing colonies alone or total self-renewing colonies (the sum of pure self-renewing and self-renewing colonies) were expressed as a percentage of the total number of colonies in each well. The means plus S.E.M of a number of independent experiments are shown. Where a treatment was found to alter the total number of colonies, alkaline phosphatase assay data are represented graphically by the number of self-renewing colonies and the number of non-self-renewing colonies observed on the same graph, thus giving the total number of colonies for that treatment. Statistical significance of the results was determined using ANOVA and Dunnet's post hoc test or a Student's t-test where appropriate, where * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

### *2.4.3 Cell Viability Counts*

E14tg2A mESCs were plated at 2 x $10^4$ cells per 60mm tissue culture dish and incubated with $10^3$ U/ml LIF in the presence of vehicle (DMSO), LY294002, Compound 15e or PIK-75, in triplicate, for 1-4 days. At 24 hour time points, adherent cells were harvested by dissociation using trypsin/EDTA. Live cells were determined using the vital dye trypan blue. Cells were counted using a haemocytometer in triplicate and data represented graphically with standard deviations.

### *2.4.4 Analysis of apoptosis using $DiOC_6$ staining*

Mitochondrial membrane integrity was assessed by $DiOC_6$ (3,3' – dihexyloxacarbocyanide iodide) staining. Used at low concentrations $DiOC_6$ is a cell-permeable, green fluorescent dye that preferentially stains the mitochondria of live cells. Inner mitochondrial membrane impermeability aids the maintenance of a proton gradient, essential for oxidative phosphorylation. Mitochondrial membrane permeabilisation is both a marker of and an irreversible point in programmed cell death (Petit et al., 1995, Rottenberg and Wu, 1998). Mitochondrial membrane permeabilisation causes dissipation of the proton gradient, dissipation of mitochondrial membrane potential and functional failure of the mitochondria. $DiOC_6$ dye is lipophilic and accumulates in the mitochondrial matrix of live cells with intact inner mitochondrial

membranes. A decrease in $DiOC_6$ staining and a corresponding decrease in FL1 fluorescence measured by flow cytometry are indicative of reduced mitochondrial membrane potential and apoptosis.

Cells were treated with vehicle (DMSO), LY294002, Compound 15e or PIK-75 for 16 hours. Media was collected from each treatment dish to ensure that cells that may have detached from a colony or the tissue culture dish surface were collected. Cells were dissociated using trypsin/EDTA and resuspended in the corresponding media. Cells were washed twice in PBS and resuspended at $1x10^6$ cells in 1ml PBS. As a positive control, a sample of vehicle treated cells were pre-treated with 100µM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for 20 minutes. CCCP is a mitochondrial-uncoupling agent that induces mitochondrial membrane permeability and apoptosis.

CCCP treated and inhibitor treated cells were treated with 1nM $DiOC_6$ (in DMSO). A sample of unstained control cells was also retained. All cell samples were incubated in the dark at room temperature for 30 minutes. Cells were washed three times with PBS and resuspended in 1ml PBS. Fluorescence was measured using a FACS Canto on parameter FL1 (Galluzzi et al., 2007, Metivier et al., 1998). $DiOC_6$ staining data were analysed using Cell Quest flow cytometry software (BD).

**Table 2.9 Functional analysis technique consumables**

| PRODUCT | SUPPLIER | CATALOG # |
|---|---|---|
| **XTT Dye Reduction Assay** | | |
| XTT Salt | Sigma | X4626 |
| Phenazine methosulfate (PMS) | Sigma | P5812 |
| AIM-V Media | Gibco (Fisher Scientific) | 31035 |
| **Self-Renewal Assay** | | |
| Napthol-AS MX | Sigma | N4875 |
| Fast Red TR Salt | Sigma | F2768 |
| **$DiOC_6$ Staining** | | |
| $DiOC_6$ | Calbiochem | 305110 |
| CCCP | Calbiochem | 215911 |

## 2.5 Molecular techniques

### *2.5.1 Virtual northern expression data*

Expression information for *Pik3ca*, *Pik3cb*, *Pik3cd*, *Pik3cg* and LPA/S1P receptors was gained from virtual Northern data available from the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/unigene). Searches were made for genes of interest and the data available scrutinised for expression during mouse embryo development. Details of this information are referenced in the text and the date the database was accessed is also given.

### *2.5.2 Expression analysis*

Cells were incubated with appropriate inhibitors for 3-7 days as indicated. Expression of self-renewal and early lineage marker mRNA was assessed to determine the fate of mESCs. Expression of PI3K catalytic subunit isoform mRNA was used to confirm a decrease in expression following siRNA-mediated knockdown.

#### *2.5.2.1 RNA isolation*

Cells were washed twice with PBS, resuspended at 1ml Trizol per $1x10^6$ cells and transferred to sterile RNase-free 1.5ml Eppendorf tubes. After 5 minutes, 200µl chloroform was added and tubes were shaken vigorously. The two phases were allowed to separate for 2-3 minutes at room temperature followed by centrifugation at 10000 rpm for 15 minutes at 4°C. The aqueous upper phase was transferred to sterile 1.5ml tubes and 500µl isopropanol added to precipitate RNA. After 10 minutes, tubes were centrifuged as before to pellet the RNA. The supernatant was discarded and the pellet washed with ice-cold 70% ethanol. Tubes were centrifuged at 10000 rpm for 5 minutes at 4°C. The ethanol was aspirated and pellets allowed to air dry briefly. The RNA pellet was resuspended in 10-30µl RNase-free water. To denature secondary structures and aid solubilisation, tubes were incubated at 55°C for 5 minutes. RNA was stored at -80°C.

#### *2.5.2.2 RNA quantification*

RNA was quantified by measuring the absorption of light at 260nm. Protein absorbs light at 280nm, therefore measuring the absorption at both 260nm and 280nm allows quantification of RNA and assessment of protein contamination.

RNA was diluted 1:100 in milliQ® $H_2O$ and absorbance measured using a GeneQuant II spectrophotometer.

RNA concentration was calculated using Beer's law:

$$A_{260} = \varepsilon C l$$

Where $A_{260}$ is the absorbance at 260nm, ε is the RNA extinction coefficient (25µg/µl/cm), C is the concentration of the diluted sample and l is the path length (1cm for the cuvette used).

Ideally the ratio of absorption $A_{260}/A_{280}$ should be close to 2. However, this ratio is pH sensitive so values of 1.6-2.0 were accepted since milliQ® $H_2O$ was used to dilute samples prior to quantification. If purity was questionable, ($A_{260}/A_{280}$ <1.6) a sample of RNA was separated by agarose gel electrophoresis. Electrophoresis equipment was made RNAse free by washing in 3% $H_2O_2$. Non-degraded RNA produces two bands corresponding to 28s and 16s rRNA with the latter being approximately half as intense as the former.

#### *2.5.2.3 Reverse transcription polymerase chain reaction*

Reverse transcription was used to make cDNA for expressional analysis from mRNA. Contaminating genomic DNA was removed by DNase treating RNA. 1µg RNA was incubated with 0.5µl of 10x DNase buffer, 1µl of DNase and an appropriate volume of RNase-free water to give a final volume of 5µl at 37°C, for 30 minutes. 1µl of DNase Stop Solution (0.5M EDTA, pH 8.0) was added and tubes were incubated at 65°C for 10 minutes to denature DNase enzymes. 0.5µl RNasin Plus and 0.5µl Oligo dT was added and incubated for a further 5 minutes at 65°C. A reverse transcription master mix was made with 6µl RNase-free water, 4µl First Strand sample buffer, 5mM DTT, and 50µM dNTPs (dATP, dTTP dGTP and dCTP) per sample. Samples were incubated on ice for 2-3 minutes then 13µl of reverse transcription master mix was added to each sample tube. Finally 1µl Superscript Reverse Transcriptase III was added to each sample.

Duplicate negative control samples were set up in the absence of reverse transcriptase enzyme. The reverse transcription reaction was performed by incubating tubes for 50 minutes at 50°C followed by 15 minutes at 70°C in a Techne Touchgene gradient PCR machine. The resulting cDNA products were stored at -20°C.

#### *2.5.2.4 Primer design*

Primers were designed to detect expression of PI3K catalytic isoform subunits p110α, p110β and p110δ. Where possible, sense (S) and antisense (A) primers (Table 2.10) were designed according to the following guidelines (Innis et la., 1990). Primers should be 17-28 base pairs in length with 3' terminal bases G, C, GC or CG. GC content should be 50-60% to produce a melting temperature of 55-80°C but runs of 3 or more G's or C's are undesirable. 3' complementarity or internal self complementarity should also be avoided.

Melting temperature was calculated according to the base content of primers (Rychlik and Rhoads, 1989):

$$T_m = 4(G+C) + 2(A+T)$$

Possible primer sequences were checked for the potential formation of dimers and hairpins using Net Primer, an online tool by Premier Biosoft (http://www.premierbiosoft.com/netprimer/)

**Table 2.10 Primers**

| MARKER GENE | PRIMER SEQUENCES | ANNEALING TEMP °C | CYCLES | SOURCE |
|---|---|---|---|---|
| **Self-Renewal Markers** | | | | |
| Nanog | S: 5'-CTCTTCAAGGCAGCCCTGAT-3'<br>A: 5'-CCATTGCTAGTCTTCAACCAC-3' | 60 | 25-30 | Paling, N (designed in house) |
| Rex1 | S: 5'-CGTGTAACATACACCATCCG-3'<br>A: 5'-GAAATCCTCTTCCAGAATGG-3' | 55 | 25-30 | (Faloon et al., 2000, Lacaud et al., 2004) |
| Oct4 | S: 5'-GCGTTCTCTTTGGAAAGGTGTTC-3'<br>A: 5'-CTCGAACCACATCCTTCTCT-3' | 58 | 25-30 | Paling, N. (Designed in house) |
| **Early Lineage Markers** | | | | |
| GATA1 | S: 5'-ACTCGTCATACCACTAAG-3'<br>A: 5'-AGTGTCTGTAGGCCTCAG-3' | 56 | 30 | (Ogawa et al., 1999) |
| GATA6 | S: 5'-GCAATGCATGCGGTCTCTAC-3'<br>A: 5'-CTCTTGGTAGCACCAGCTCA-3' | 56 | 30 | (Fujikura et al., 2002) |
| Pax6 | S: 5'-CGGAGAAGACTCGGATGAAG-3'<br>A: 5'-GGCCCTTCGATTAGAAAACC-3' | 56 | 30 | (Yoshida-Koide et al., 2004) |
| Nodal | S: 5'-ACGTTCACCGTCATTCCTTC-3'<br>A: 5'-TCAGCTTCCCAAAGCAAAGT-3' | 62 | 30 | (Yoshida-Koide et al., 2004) |
| FGF 5 | S: 5'-AAAGTCAATGGCTCCCACGAA-3'<br>A: 5'-CTTCAGTCTGTACTTCACTGG-3 | 62 | 30 | (Cartwright et al., 2005, Oka et al., 2002) |
| **PI3K Catalytic Isoforms** | | | | |
| p110α | S: 5'-AAATGGCGACGACTTACG-3'<br>A: 5'-TTGTTCTTGTCCTTGAGC-3' | 54 | 30 | Kingham, E. Designed according to guidelines (Section 2.5.2.4) |
| p110β | S: 5'-TAATGTGTCAAGTCGTGG-3'<br>A: 5'-CAGCCTACAGCGTATTCC-3' | 58 | 30 | |
| p110δ | S: 5'-CTGGACCTGAGGATGACG-3'<br>A: 5-GGCTCAAGTCCAAGAACC-3' | 58 | 30 | |
| **Loading Markers/House-Keeping** | | | | |
| β-actin | S: 5'-TAGGCACCAGGGTGTGATGG-3'<br>A: 5'-CATGGCTGGGGTGTTGAAGG-3' | 62 | 25 | Storm, M. (designed in house) |

### *2.5.2.5 Quantitative polymerase chain reaction*

PCR exponentially amplifies DNA enzymatically using GoTaq, a modified form of Taq DNA polymerase lacking 5' 3' exonuclease activity. PCR was conducted by combining 10µl 5X Green GoTaq Flexi buffer or colourless GoTaq Flexi buffer, 2mM $MgCl_2$, 250µl dNTPs, 5pmol sense primers, 5pmol antisense primers and 0.5U GoTaq DNA polymerase. 2µl of DNA from the reverse transcription reaction was added to give a final volume of 20µl. Reactions were performed using a Techne Touchgene gradient PCR machine.

**Table 2.11 PCR program for expression analysis**

| STAGE | TEMPERATURE | DURATION | CYCLES |
|---|---|---|---|
| Initial Elongation | 94°C | 5 min | |
| Denaturation | 94°C | 30 s | |
| Annealing | Primer Dependent | 30 s | 25-30 Primer Dependent |
| Elongation | 72°C | 60 s | |
| Final Elongation | 72 °C | 5 min | |
| Hold | 4 °C | | |

### *2.5.2.6 Agarose gel electrophoresis*

Agarose gel electrophoresis was used to separate DNA in order to visualise PCR products. Typically, 2% Agarose gels were used to resolve linear DNA of 0.05-1.5kb. Agarose was dissolved in Tris-acetate EDTA (TAE) buffer (50x TAE buffer: 2M Tris, 50mM $Na_2$ EDTA (pH8.0) adding glacial acetic acid (to pH 7.6)) by boiling. Once cooled, the agarose solution was poured into a pre-assembled agarose gel casting support with a well forming comb inserted. Once set, the gel was placed into an electrophoresis tank containing TAE. When Green GoTaq Flexi buffer was used in PCR reactions, no DNA loading buffer with dye was required, when colourless GoTaq Flexi buffer was used, DNA loading buffer (6x loading buffer: 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue) was added to each PCR product tube. PCR products were loaded into individual wells and agarose gels run at 70-90V until sufficient separation was achieved. Agarose gels were submersed in 0.5µg/ml ethidium bromide in TAE for 15 minutes. Ethidium bromide intercalates DNA and can be visualised under UV light. Agarose gels were photographed in a Syngene UV transluminator using Genesnap software.

#### *2.5.2.7 Real-time quantitative PCR*

Real time quantitative PCR (QPCR) was conducted using a 1X solution of reaction buffer containing 2.5mM $MgCl_2$, 5pmol sense primers, 5pmol antisense primers and 1µl SYBR Green enzyme to a final volume of 8µl with PCR sterile $H_2O$. 8µl of this reaction mix was added to glass LightCycler capillaries (Roche) and 2µl of 1:5 diluted sample cDNA from RT-PCR reactions as described in section 2.5.2.3. Capillaries were sealed with plastic caps and centrifuged at 4000 rpm for 20 seconds at 4°C.

Capillaries were loaded into the carousel and placed into a Roche Molecular Biochemical LightCycler. LightCycler amplification using SYBR Green was performed for each sample in duplicate, together with a no-template negative control. The LightCycler program for acquiring real-time quantitative PCR data is detailed in Table 2.12. Briefly, samples were incubated at 95 °C for 10 minutes at the start of each run to activate Taq DNA polymerase. Real-time quantitative PCR utilises the double-stranded DNA intercalator SYBR Green. Fluorescence emitted by SYBR green intercalation during amplification of DNA was measured at 530nm during the elongation phase by the LightCycler. Increasing fluorescence, when amplification increases the amount of DNA present, was monitored over 40 PCR cycles. Annealing temperatures for primers are given in Table 2.13.

**Table 2.12 LightCycler program**

| STAGE | TEMPERATURE | DURATION | CYCLES |
|---|---|---|---|
| **Hot Start** | 95 °C | 10 minutes | |
| **Initial Denaturation** | 95 °C | 30 seconds | |
| **Denaturation** | 95 °C | 10 seconds | 40 cycles |
| **Annealing** | Primer Dependent (Table 2.13) | 5 seconds | |
| **Elongation** | 72°C | 16 seconds | |
| | | | |
| **Melting Curve** | 65-95 °C | 0.1 °C/sec | |

**Table 2.13 QPCR annealing temperatures**

| PRIMERS | ANNEALING TEMPERATURE |
|---|---|
| β-actin | 60 °C |
| P110α | 54 °C |
| P110β | 60 °C |
| P110δ | 58 °C |
| Nanog | 60 °C |

Following a run, melt curve analysis was performed to confirm the presence of only the desired PCR product. Melt curves were constructed by programming the LightCycler to slowly heat the PCR product from 65-95°C. This induces melting of the double-stranded DNA and a decrease in SYBR Green fluorescence, which was continuously monitored. Melt curve analysis produces melting peaks (examples of which are shown in Figure 2.2) that are specific to particular DNA products. Where necessary, PCR products were subsequently analysed by agarose gel electrophoresis.

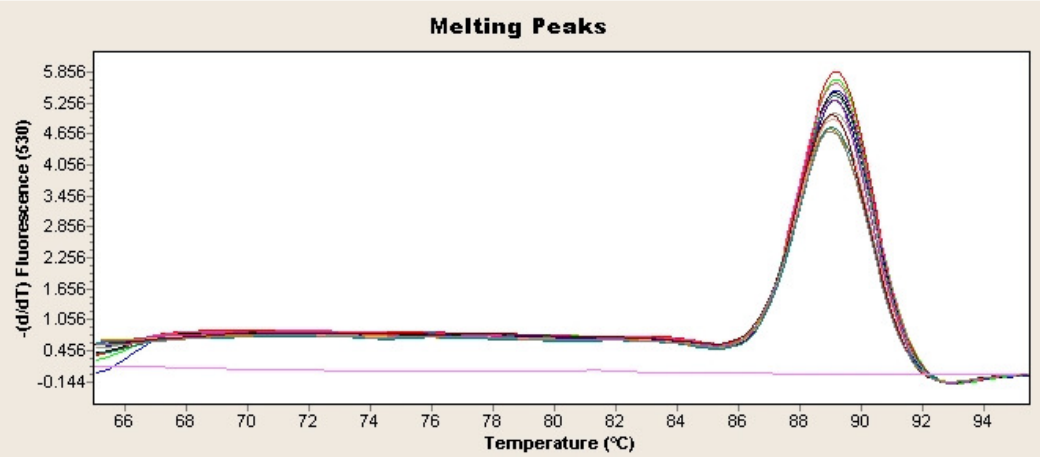



**Figure 2.2 Melting peaks are specific to particular DNA products**

The melting peaks shown here are specific to a particular DNA product. The presence of abnormal or additional peaks for samples may indicate contamination of the capillary samples or poor PCR primer design leading to the amplification of a non-specific region of the template DNA. Furthermore, melt curve analysis confirms the lack of PCR product in the no template negative control capillaries, represented here by the pink trace.

Variations in cDNA synthesis efficiency of samples, differences in the amount of starting cDNA, RNA degradation, pipetting errors and loading errors were corrected for by comparing the data accumulated for target genes (for example p110 isoform genes) with that of a reference gene (β-actin). Crossing point values were determined by the LightCycler Version 4.0 software (Roche, Idaho Technology Inc.) using data obtained by monitoring fluorescence intensity per PCR cycle. A crossing point value for a reaction is the cycle at which the fluorescent signal becomes detectable as greater than the threshold background signal (Figure 2.3). Thus, crossing point values are different for each sample depending on the amount of target gene in the starting reaction mix. Crossing point values appear within the logarithmic-linear phase where a logarithmic increase in fluorescence is detected (Figure 2.3). Furthermore, the cycle at which a crossing point occurs is presumed to be proportional to the amount of starting material of the target gene. Efficient PCR reactions are considered to have one replication of every PCR product every cycle. Within the logarithmic region, amplification efficiencies of each reaction are constant and can therefore be compared. Crossing points are assumed to be reliably proportional to the initial starting amount of target gene. Therefore, a target to reference ratio was calculated from the crossing point values derived. Measurements taken at the plateau phase do not provide information regarding the amount of starting material since reactions with low or high amounts of target gene or different efficiencies can reach the same plateau.

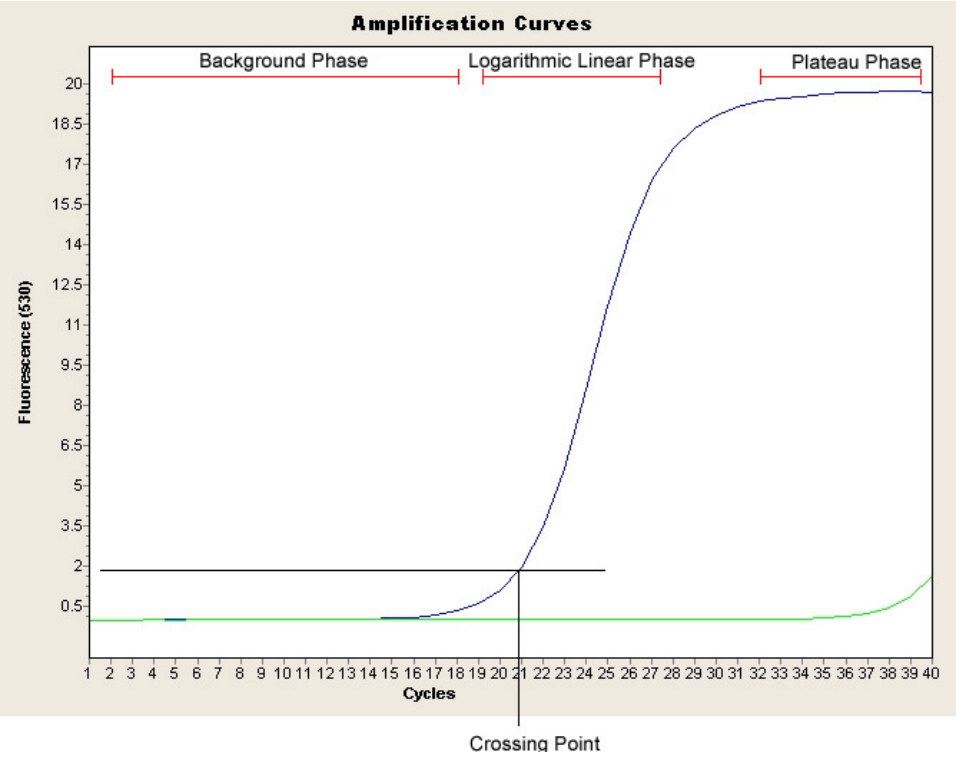



**Figure 2.3 A typical amplification curve**

The fluorescence intensity versus the number of cycles of PCR are plotted to obtain an amplification curve. Here the blue trace represents a positive control where the DNA product is amplified from the template DNA and the green trace represents a no template negative control sample. The number of cycles where the fluorescent intensity of the target gene is distinguishable from the background fluorescent signal is described as the crossing point (indicated on the amplification curve) and occurs within the logarithmic linear phase.

Duplicate calibrator cDNA samples were included for all real time QPCR runs. Calibrator cDNA from a single batch of RT-PCR cDNA product was diluted 1:5 and used for all LightCycler runs in this study. This allows the data for each run to be compensated to account for batch variations in SYBR Green and differing experimental sample RT-PCR efficiencies.

Standard curves for target and reference genes were determined to allow relative quantification of the unknown samples. Serial dilutions (typically 1:5 1:50 1:500 etc.) of a standard template cDNA were used in triplicate to determine a standard curve for each gene used in the present study. Serial dilutions were set up in separate capillaries but within the same LightCycler run. The LightCycler software was used to generate

standard curves for each gene where the cycle number at the crossing point for each dilution of standard template was plotted versus the log concentration of starting cDNA material (Figure 2.4). Therefore, the crossing point value for any given sample, determined after a LightCycler run, allows the relative concentration of starting material to be determined. Furthermore, PCR efficiencies (E) are generated from the slope of the standard curve for each gene investigated using the formula:

$$E = 10^{-1/slope}$$

Under optimal conditions, PCR efficiency E=2. However, most PCR reactions do not occur at maximum efficiency and, as a result, the error gets greater with increasing PCR cycles. In the example shown in Figure 2.4, E= 1.824 and can be corrected for by an efficiency algorithm, part of the LightCycler Version 4.0 software.

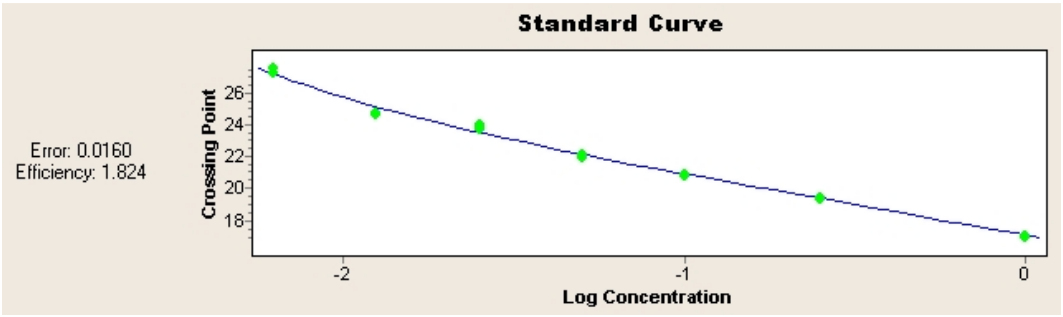



**Figure 2.4 Standard curves were plotted for all target and reference genes**

For each target gene and the reference gene, a standard curve was determined by plotting the crossing point versus the log concentration of serially diluted cDNA. From the slope of the standard curve, PCR efficiency values are derived and are used to correct data collected from unknown samples. Error values are also derived from the standard curve (mean squared error, $\Sigma (\Delta x)^2/n$ where $\Delta x$ is the vertical distance between data points and the regression line and n the number of data points), indicating replicate variations such as pipetting errors between LightCycler capillary reactions. An error value ≤0.1 is acceptable.

#### *2.5.2.7.1 QPCR relative quantification by calibrator normalisation and efficiency correction*

In the present study, QPCR data were analysed by relative quantification, where the target concentration is expressed as a ratio of target (p110) to reference gene (β-actin). Using the PCR efficiency and crossing point values allow quantification of both a target and a reference gene. To derive the QPCR values, expressed as histogram graphs in the present study, LightCycler software divides the ratio of target to reference gene relative concentrations by the calibrator relative concentration in order to normalise the data from each run:

$$\text{Normalised ratio} = \frac{\text{concentration target sample}}{\text{concentration reference sample}} \Big/ \frac{\text{concentration target calibrator}}{\text{concentration reference calibrator}}$$

This corrects variations in replicate samples within the same run and for different runs. Additionally, PCR efficiency correction is conducted to account for sub-maximal PCR efficiencies. Finally the mean and standard deviation of corrected and normalised ratio values (in duplicate per run and two runs conducted per experiment) are used to plot histogram graphs representing the relative normalised amount of the target gene of interest.

### *2.5.3 Transient transfection*

#### *2.5.3.1 Transfection of mESCs with siRNA*

Transient transfections were conducted using short interfering RNA (siRNA) to knockdown the expression of PI3K catalytic p110 subunit isoforms and Nanog. The method of conducting RNA interference (RNAi) using siRNAs was first identified by investigations into post-transcriptional gene silencing in plants (Hamilton and Baulcombe, 1999) and later adapted for use in mammalian cells (Elbashir et al., 2001).

Briefly, double stranded RNAs enter a cell aided by a transfection reagent. This RNA is cleaved into fragments of 21-25 nucleotides of siRNA duplexes by Dicer (in drosophila), an RNAse III-like enzyme (Figure 2.5) (Bernstein et al., 2001, Elbashir et al., 2001). Following this, siRNAs are incorporated into the RNA induced silencing complex (RISC) in an ATP-dependent manner (Nykänen et al., 2001). When siRNAs become single stranded, the antisense strand remains bound to RISC and activates the complex. The activated complex then binds to the complementary endogenous mRNA sequence. Once bound, mRNA is cleaved by Slicer (in drosophila), a component of RISC and targeted for degradation by nucleases therefore, preventing translation into functional protein (Martinez et al., 2002).

For the transfection of mESCs with siRNA, 5nmol siRNA (Dharmacon or Ambion) was resuspended in RNase-free siRNA buffer (20mM KCl, 6mM HEPES pH7.5 and 0.2mM $MgCl_2$) to a working concentration of 20µM. Aliquots were stored at -80$^{o}$C.

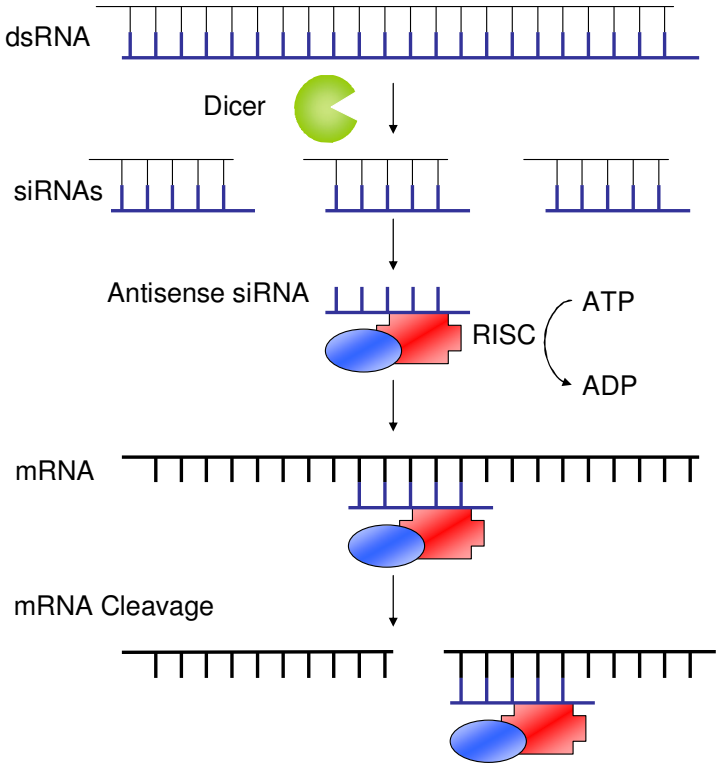



**Figure 2.5 Gene silencing with siRNA**

Double stranded RNA is cleaved by Dicer to form siRNAs. These subsequently bind to RISC and unwind. Antisense siRNA bound to RISC binds to complementary mRNA. Slicer, a member of RISC, cleaves mRNA rendering it unsuitable for translation.

Lipofectamine transfection reagent was incubated with Knockout DMEM (lacking Knockout Serum Replacement) (3.1µl lipofectamine per 50µl Knockout DMEM) for 5 minutes at room temperature. In individual tubes, 2.5, 5.0 or 7.5µl siRNA was diluted with Knockout DMEM to a total volume of 50µl. Next 50µl of lipofectamine/Knockout DMEM solution was added to tubes containing siRNA and incubated at room temperature for 20 minutes. Meanwhile, mESCs were washed and resuspended at $8.9 \times 10^4$ cells/ml in KO complete media. Following incubation, 900µl of cell suspension ($8 \times 10^4$ cells) was added to 100µl lipofectamine/siRNA solution and plated onto 6 well NUNC plates coated with 0.1% gelatine. 1ml of KO complete media was added to each well to give a final volume of 2ml and a final dilution of lipofectamine of 1:650. Plates were incubated at 37°C with 5% (v/v) $CO_2$.

For transfection with Dharmacon smartpool siRNA targeting *Pik3ca*, *Pik3cb*, *Pik3cd* and *Nanog*, and non-targeting siRNA, final concentrations of 25, 50 and 75nM siRNA were used. Cells were initially transfected for 48 hours followed by re-transfection. For re-transfection, lipofectamine and siRNA were incubated in Knockout DMEM as before. Cell media was replaced with fresh KO complete media plus 100µl of lipofectamine/siRNA solution. Plates were incubated at 37°C with 5% (v/v) $CO_2$ for 24 hours after re-transfection then harvested for use. Harvested cells were either used to set up self-renewal assays, which were incubated for a further 4 days before fixing and staining colonies for alkaline phosphatase, lysed immediately with solubilisation buffer or resuspended immediately in TRIzol for RNA isolation. Knockdown of targeted genes was confirmed by quantitative and semi-quantitative PCR.

Ambion siRNA (Applied Biosystems) was also used to target *Pik3cb* at two independent regions. Additionally Ambion siRNAs were selected to targeted regions different from those targeted by Dharmacon siRNA (Figure 2.6) For transfection with Ambion siRNA, *Pik3cb* targeting and negative control siRNA, a final siRNA concentration of 10nM was used. Cells were plated at $1x10^5$ cells/well with lipofectamine/siRNA solution prepared as described above. For Ambion siRNA experiments, a 48h transfection period was sufficient to induce knockdown of target genes. Following this, cells were harvested then lysed immediately for protein analysis, resuspended immediately in TRIzol for RNA isolation or used for self-renewal assays, which were incubated for a further 4 days before fixing and staining colonies for alkaline phosphatase.

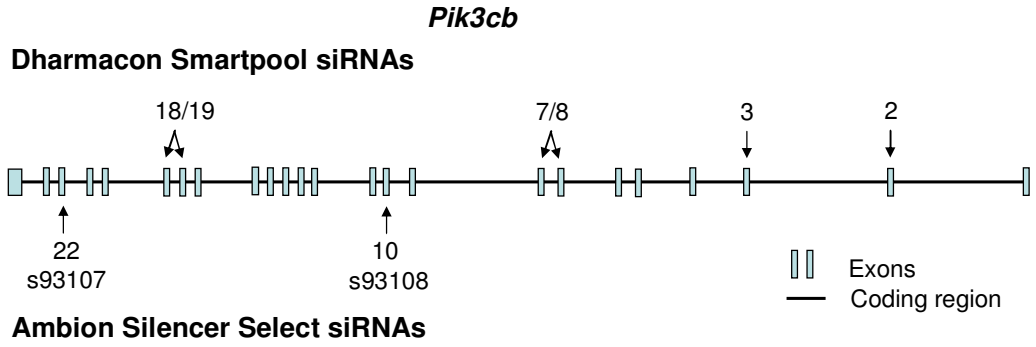



**Figure 2.6 Pik3cb regions targeted by Dharmacon and Ambion siRNAs**
Dharmacon Smartpool siRNAs contain four individual siRNAs which target four different regions of Pik3cb at different exons (2 and 3) or different exon boundaries (7/8 and 18/19). Two Ambion Silencer Select siRNAs were also used in this study; s93107 and s93108 which target exons 22 and 10 respectively.

**Table 2.14 Molecular techniques consumables**

| PRODUCT | SUPPLIER | CATALOG # |
|---|---|---|
| **Expression Analysis** | | |
| Agarose | Invitrogen | 15510-027 |
| DNase | Promega | M6101 |
| dNTPs | Invitrogen | 10297-018 |
| Ethidium Bromide | Bio-Rad | 1610433 |
| Oligo $(dT)_{15}$ Primer | Promega | C1101 |
| Primers | Invitrogen | Made to order |
| RNase Free Eppendorfs | Eppendorf | 3810X |
| RNasin Plus | Promega | N2611 |
| Superscript III reverse transcriptase (including RT buffer and DTT) | Invitrogen | 18080-044 |
| Taq Polymerase | Promega | M1661 |
| TRIzol | Invitrogen | 15596-018 |
| **siRNA-Mediated Knockdown** | | |
| Lipofectamine 2000 | Invitrogen | 11668-019 |
| 5X siRNA Buffer | Dharmacon | B-002000-UB |
| **siGENOME SMARTPool siRNA:** | | |
| Mouse Nanog siRNA | Dharmacon | NM-057004-00 |
| Mouse *Pik3ca* siRNA | Dharmacon | NM-008839-00 |
| Mouse *Pik3cb* siRNA | Dharmacon | NM-008840-00 |
| Mouse *Pik3cd* siRNA | Dharmacon | NM-029094-00 |
| Non-Targeting Control siRNA | Dharmacon | D-001206-13-20 |
| **Silencer Select siRNA Pre-designed siRNA:** | | |
| Mouse *Pik3cb* Targeting siRNA s93107 | Ambion (Applied Biosystems) Warrington UK | 4390771 s93107 |
| Mouse *Pik3cb* Targeting siRNA s93108 | Ambion | 4390771 s93108 |
| Negative Control siRNA | Ambion | 4390843 |

# 3 RESULTS: PI3K activation, regulation of the MAPK pathway and lineage commitment of mESCs

## 3.1 Introduction and aims

PI3Ks have been implicated in both the maintenance of self-renewal and in the promotion of proliferation of mESCs (Sections 1.3.6.1 and 1.3.6.2). Inhibition of PI3Ks leads to a decrease in alkaline phosphatase positive colonies and a reduction in Nanog expression demonstrating a loss of self-renewal (Paling et al. 2004, Storm et al., 2007). Faster growth rates are observed in mESCs lacking PTEN, the endogenous antagonist of PI3K activity (Sun et al., 1999), and enhanced growth rates were also observed following over-expression of ERas, a Ras isoform that activates PI3Ks in mESCs (Takahashi et al., 2003).

A variety of external stimuli, particularly growth factors and cytokines, are known to activate pathways that facilitate the maintenance of self-renewal and other cellular functions. For example, LIF is known to promote the maintenance of self-renewal and pluripotency via activation of Signal Transducer and Activator of Transcription 3 (Stat3) (Niwa et al., 1998, Boeuf et al., 1997) and by the activation of PI3Ks (Paling et al., 2004). However, in the absence of serum, in a chemically defined media, both LIF and BMP4 are required to maintain self-renewal (Ying et al., 2003a, Ying et al., 2003b). BMP4 maintains self-renewal by activation of Smad proteins and subsequent regulation of Id (Inhibitor of differentiation) gene expression (Hollnagel et al., 1999, Ying et al., 2003b) and also prevents differentiation by the inhibition of Erk and p83 of the Mitogen Activated Protein Kinase (MAPK) pathway (Qi et al., 2004). Previously, only LIF has been shown to activate PI3Ks in mESCs thus the present investigation sought to identify other such factors. Of interest were factors that might be present in routine culture media or factors that are known to be secreted by mESCs, which could be involved in autocrine signalling.

It has previously been reported that inhibition of PI3Ks with LY294002 in mESCs enhances basal Erk phosphorylation (Paling et al., 2004) and that inhibition of the MAPK pathway, by the inhibition of Mek (directly upstream of Erk) with PD098059, can promote self-renewal (Burdon et al., 1999). Conversely, the effect of activating the MAPK pathway, as observed following PI3K inhibition, was investigated in the present study.

Self-renewal appears to be a balance of inhibition of differentiation and promotion of self-renewal. In some cases, particular signalling pathways direct differentiation towards a particular lineage. For example, BMP4, in the absence of LIF and serum, promotes the differentiation of mESCs towards non-neural cell types (Wiles and Johansson, 1999).

Although the activity of PI3Ks support self-renewal and inhibition of PI3Ks with the broad selectivity inhibitor LY294002 leads to a loss of self-renewal (Paling et al., 2004, Storm et al., 2007), the effect of inhibiting PI3Ks in terms of directed differentiation has not been assessed. Therefore, a role for PI3Ks in lineage commitment was also addressed.

## 3.2 Stimulation of PI3Ks by factors present in culture medium

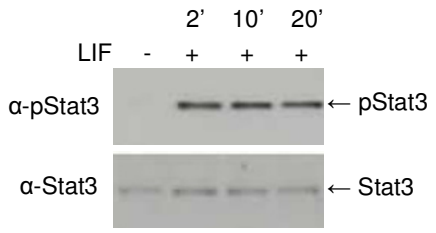
### *3.2.1 Serum-free chemically defined media*

The use of complex, serum containing or serum-replacement media impedes the identification of factors that may activate signalling pathways of interest. The presence of a variety of factors in routine culture media can mask the effects of stimulating cells with individual factors. In order to identify factors that activate PI3Ks in mESCs a serum-free, chemically defined media with known components was used. This media was initially described for the culture of a rat neuroblastoma cell line (Bottenstein and Sato, 1979, Brewer and Cotman, 1989, Brewer, 1993) but refined for the culture of mESCs (Ying et al., 2003a, Ying et al., 2003b). Supplemented with N2 and B27 (the components of which are listed in Table 2.1), this media can be used for the long-term culture of mESCs in a self-renewing, pluripotent state (Ying et al., 2003a).

#### *3.2.1.1 LIF stimulation of mESCs in chemically defined media*

The signalling response of mESCs to stimulation with LIF following culture in chemically defined media was assessed. Activation of the Stat3 pathway by LIF is important for the maintenance of self-renewal (Boeuf et al., 1997, Niwa et al., 1998) and pluripotency (Raz et al., 1999). Stat3 is phosphorylated following the binding of LIF to a heterodimer of the LIF receptor and the cytokine receptor gp130, and regulates the expression of the transcription factor c-myc (Cartwright et al., 2005). Prior to stimulation, mESCs were cultured in chemically defined medium containing supplements (detailed in Section 2.1.3.2) for at least 2 days then incubated for 4 hours in media lacking supplements. Unstimulated mESCs exhibited no detectable basal Stat3 phosphorylation on the activating tyrosine (Y705). LIF stimulation for 2, 10 and 20 minutes induced the phosphorylation of Stat3 at Y705 (Figure 3.1**A**). These observations are consistent with LIF stimulation of mESCs cultured in serum containing or serum-replacement media (Paling et al., 2004).

**A**




**B**

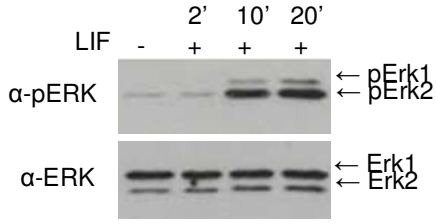



**Figure 3.1 LIF stimulates Stat3 and Erk phosphorylation in mESCs cultured in chemically defined media**

Prior to stimulation, mESCs were cultured in chemically defined culture media supplemented with N2, B27, LIF and BMP4. Cells were incubated for 4 hours in the absence of N2, B27, LIF and BMP4 to decrease basal signalling. 1000U/ml LIF was applied for 2, 10 or 20 minutes, as indicated. Following stimulation, cells were lysed and analysed by SDS-PAGE and immunoblotting with antibodies detecting pStat3 (Tyr705) (**A**) and pErk (Thr202/Tyr204) (**B**). Immunoblots were stripped and reprobed using antibodies detecting total Stat3 and Erk protein to ascertain equal loading. The data presented here are representative of three independent experiments.

In addition to activating the Stat3 pathway, LIF is reported to activate the MAPK pathway, inhibition of which enhances self-renewal (Burdon et al., 1999). Prior to stimulation, low basal levels of Erk2 phosphorylation were detectable but phosphorylated Erk1 protein was not detected. LIF stimulation for 2 minutes did not alter Erk1 or Erk2 phosphorylation at Thr202/Tyr204 from basal, unstimulated levels. Stimulation with LIF for 10 or 20 minutes induced phosphorylation of both Erk1 and Erk2 (Figure 3.1**B**).

The signalling response to LIF stimulation of mESCs cultured in chemically defined media was found to be similar to the reported signalling response to LIF in mESCs cultured in serum-containing media (Paling et al., 2004). Having ascertained that this

media was suitable for investigating signalling in mESCs, it was used to assess the activation of PI3K signalling following short-term cell stimulation experiments.

#### *3.2.1.2 BMP4 stimulation of mESCs cultured in chemically defined media stimulates signalling downstream of PI3K*

BMP4 contributes to the maintenance of mESC self-renewal by activation of Smad proteins and by inhibition of MAPK pathway activity (Qi et al., 2004, Ying et al., 2003a, Ying et al., 2003b, Suzuki et al., 2006). To ascertain if BMP4 could activate PI3Ks in mESCs, cells cultured in chemically defined medium were stimulated with BMP4 and signalling downstream of PI3Ks was assessed.

Murine ESCs were cultured in chemically defined media and incubated for 4 hours in chemically defined media lacking N2 and B27 supplements, and lacking LIF and BMP4. Unstimulated cells exhibited basal phosphorylation of PKB at Ser473, a site of phosphorylation important for the activation of PKB (Hanada et al., 2004). Additionally, basal phosphorylation of S6 (Ser235/236) and Erk2 (Thr202/Tyr204) were also detectable (Figure 3.2**A**). When stimulated with BMP4, no change in PKB phosphorylation was observed, where as, phosphorylation of Erk1, Erk2 and S6 were enhanced with 10ng/ml BMP4. Interestingly, stimulation of mESCs with BMP4 enhances S6 phosphorylation but not PKB (Figure 3.2**A**). Evidence exists to support both PKB-dependent and PKB-independent activation of S6K, upstream of S6, according to the membrane or cytosolic localisation of PKB respectively (Dufner et al., 1999). This may explain the observation of S6 phosphorylation in the absence of PKB phosphorylation, although further investigation would be required to confirm PKB localisation.

Treatment of mESCs with the broad selectivity PI3K inhibitor LY294002 did not alter basal PKB phosphorylation but did reduce basal S6 phosphorylation (Figure 3.2**A**). The phosphorylation of both Erk1 and Erk2 was enhanced by LY294002 treatment, consistent with previous reports in this mESC line (Paling et al., 2004). When mESCs were stimulated with BMP4 following pre-treatment with LY294002, no effect on PKB phosphorylation at Ser473 was detected (Figure 3.2**A**). LY294002 enhanced BMP4-stimulated Erk1 and Erk2 phosphorylation but inhibited BMP4-induced S6 phosphorylation, consistent with the activation of S6 via PI3K.

An alternative broad spectrum PI3K inhibitor was also used to investigate BMP4 induced signalling. Wortmannin is an irreversible inhibitor of PI3Ks that is less stable than LY294002 but still suitable for short-term investigations (Wymann et al., 1996). Unlike LY294002, treatment of mESCs cultured in chemically defined media with wortmannin produced a decrease in Erk2 phosphorylation (Figure 3.2**B**). This disparity might be the result of different inhibitor profiles or the way in which these inhibitors bind to PI3Ks (discussed in Section 1.2.3). Consistent with LY294002 treatment, wortmannin reduced basal phosphorylation of S6 (Figure 3.2**B**). BMP4-induced Erk phosphorylation was slightly reduced in cells pre-treated with wortmannin. Inhibition of PI3Ks with wortmannin resulted in reduced BMP4-induced S6 phosphorylation, in a similar manner to that observed using LY294002.

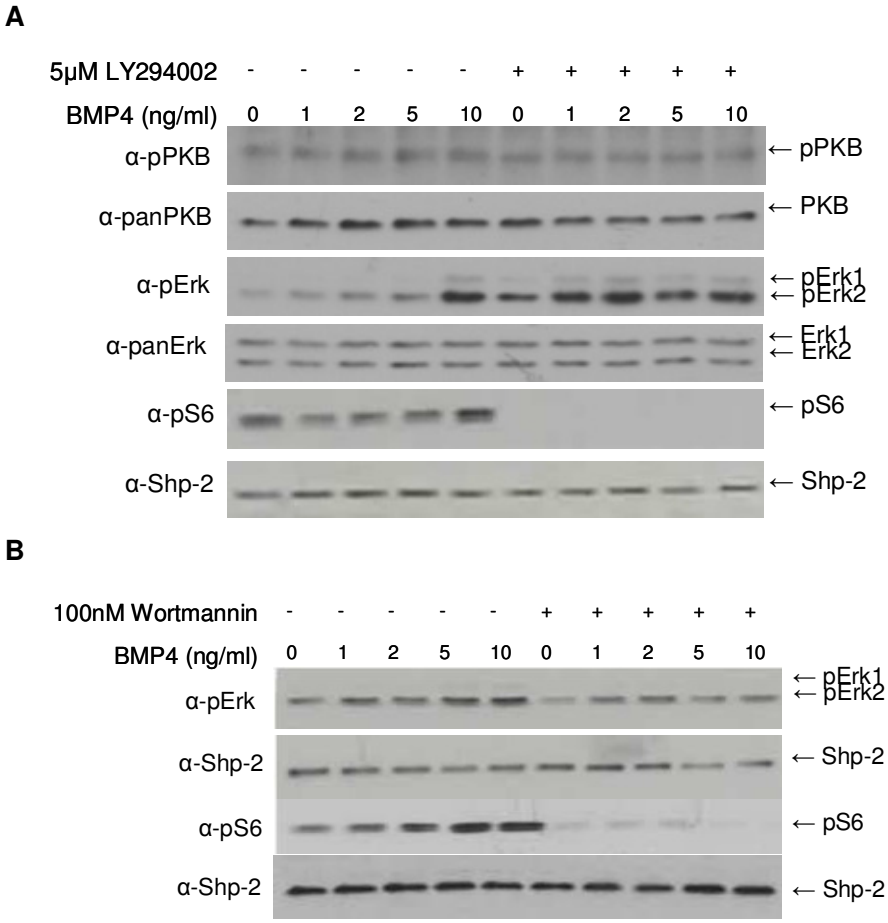



**Figure 3.2 Response of mESCs cultured in chemically defined media to BMP4 stimulation**

Murine ESCs grown in chemically defined media were incubated for 4 hours in the absence of LIF, BMP4, N2 and B27 supplements. Where indicated, cells were pre-incubated with 5μM LY294002 (**A**) or 100nM wortmannin (**B**) for 30 minutes prior to

stimulation. Cells were stimulated with 1, 2, 5 or 10ng/ml BMP4, where indicated, for 5 minutes. Signalling downstream of PI3Ks was assessed by SDS-PAGE and immunoblotting with antibodies to detect phosphorylated PKB (Ser473), Erk (Thr202/Tyr204) and S6 (Ser235/236). Equal loading was assessed by stripping and reprobing immunoblots with antibodies detecting PKB, Erk and Shp-2. The stimulation experiment shown is representative of four independent experiments.

#### *3.2.1.3 Insulin stimulation of mESCs cultured in chemically defined media stimulates signalling downstream of PI3Ks*

Activity of PI3Ks is reported to be essential for glucose metabolism and the survival of blastocyst stage embryos (Riley et al., 2006). Furthermore, the expansion of ICM cell numbers in preimplantation mouse embryos was reported to require PI3K activity (Harvey and Kaye, 1990). Typically, the class $I_A$ PI3Ks are activated upon stimulation of the insulin receptor (Vanhaesebroeck et al., 2005, Roche et al., 1998, Knight et al., 2006). It was this class of PI3Ks that were implicated in maintaining mESC self-renewal, since expression of Δp85, a dominant negative mutant of class $I_A$ PI3K regulatory subunit, leads to a reduction in alkaline phosphatase stained mESC colonies, indicating a decrease in self-renewal (Paling et al., 2004). Therefore, the activation of PI3K signalling by insulin was investigated in mESCs cultured in chemically defined media.

Following an incubation period in chemically defined media lacking LIF, BMP4, N2 and B27 supplements, stimulation of mESCs with insulin was conducted. Insulin stimulation induced phosphorylation of PKB at serine 473, which could be enhanced by increasing the concentration of insulin used to stimulate cells (Figure 3.3**A**). Insulin stimulation of mESCs also induced Erk1, Erk2 and S6 phosphorylation at Thr202/Tyr204 and Ser235/236 respectively. Enhancements in Erk1, Erk2 and S6 phosphorylation were prominent following stimulation with 10μg/ml insulin. Pre-treatment with LY294002, to inhibit PI3Ks, prevented insulin-induced PKB and S6 phosphorylation (Figure 3.3**A**). An enhancement in both basal and insulin stimulated Erk1 and Erk2 phosphorylation was observed following LY294002 pre-treatment. In contrast, pre-treatment with wortmannin did not alter insulin-induced Erk2 phosphorylation but did prevent insulin-induced S6 phosphorylation (Figure 3.3**B**).

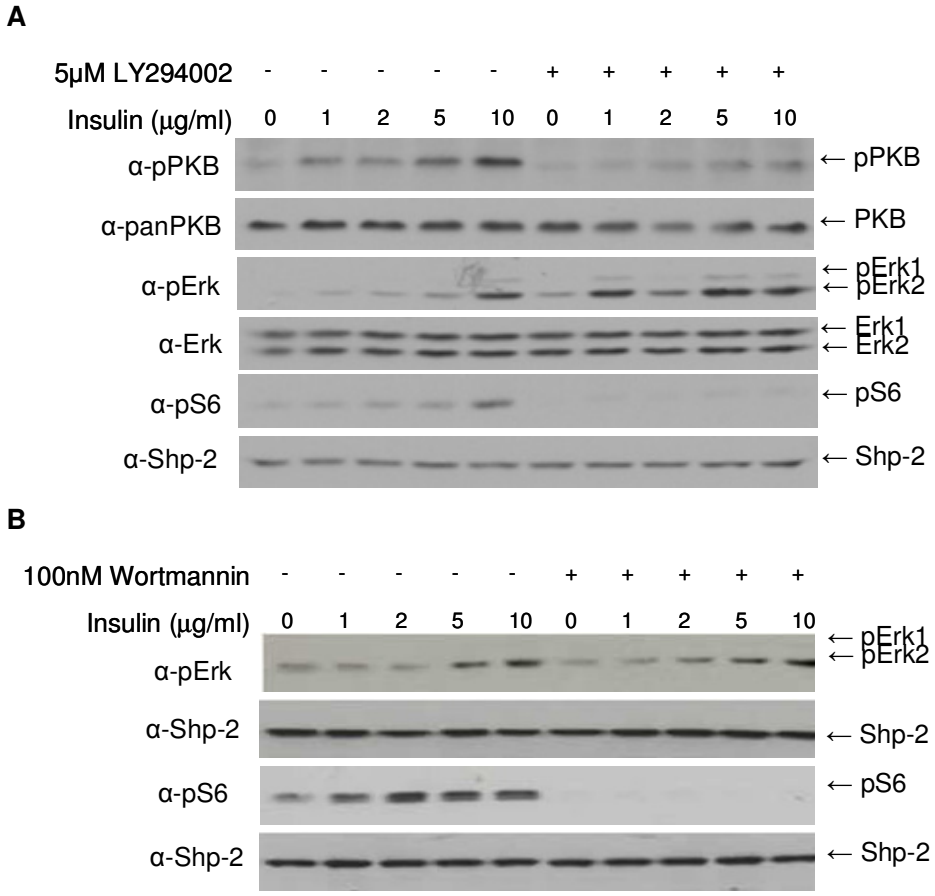



**Figure 3.3 Response of mESCs cultured in chemically defined media to insulin stimulation**

Murine ESCs grown in chemically defined media supplemented with LIF and BMP4 were incubated for 4 hours in the absence of LIF, BMP4, N2 and B27 supplements. Where indicated, cells were incubated with 5μM LY294002 (**A**) or 100nM wortmannin (**B**) for 30 minutes prior to stimulation. Stimulations were conducted using 1, 2, 5 or 10 μg/ml insulin for 5 minutes. Signalling downstream of PI3Ks was assessed by SDS-PAGE and immunoblotting with antibodies detecting phosphorylated PKB (Ser473), Erk (Thr202/Tyr204) and S6 (Ser235/236). Equal loading was assessed by stripping and reprobing immunoblots with antibodies detecting PKB, Erk and Shp-2. The stimulation experiment shown is representative of four independent experiments.

### *3.2.2 Stimulation of mESCs in knockout serum-replacement media*

Serum and serum-replacement media are used for the routine culture of mESCs and contain a cocktail of undefined components that may contribute to maintaining self-renewal. Having identified BMP4 and insulin as activators of PI3K signalling in mESCs cultured in chemically defined N2B27 media, the effect of BMP4 and insulin stimulation on mESCs grown in complete Knockout serum-replacement media was assessed.

Following a period of serum starvation, LIF stimulation of mESCs induced phosphorylation of Erk1 and Erk2 above basal levels and induced a small increase in S6 phosphorylation (Figure 3.4). Stimulation of mESCs with 2 or 5ng/ml BMP4 resulted in enhanced phosphorylation of Erk1 and Erk2 from unstimulated levels (Figure 3.4**A**). In contrast, higher concentrations of 10 or 20ng/ml BMP4 induced a small decrease in Erk1 and Erk2 phosphorylation when compared to unstimulated levels. This BMP4-induced decrease in Erk phosphorylation is consistent with reports indicating that BMP4 signalling can inhibit the MAPK pathway in order to maintain self-renewal (Qi et al., 2004, Yamaguchi et al., 1999). Similarly, the effects of BMP4 stimulation on S6 phosphorylation were only observed at higher concentrations, where an increase in S6 phosphorylation was observed (Figure 3.4**A**).

In mESCs cultured in complete Knockout serum replacement media, serum starved then stimulated with insulin, only the highest concentration of insulin used induced an enhancement in Erk phosphorylation (Figure 3.4**B**). High levels of basal S6 phosphorylation are often observed in cells cultured in serum-replacement media. Despite high basal S6 phosphorylation, an increase in phosphorylation was induced using 5µg/ml insulin (Figure 3.4**B**).

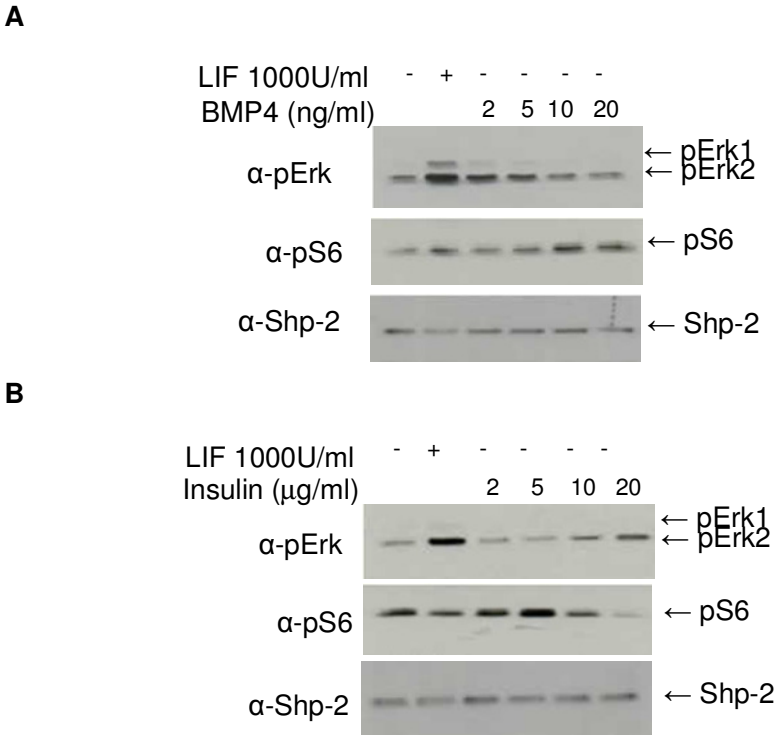



**Figure 3.4 BMP4 and insulin stimulation of mESCs cultured in serum-replacement media**

Cells cultured in complete Knockout serum-replacement media were serum and LIF starved for 4h prior to stimulation. Cells were stimulated for 5 minutes with LIF, BMP4 or Insulin as indicated. Immunoblotting was used to detect Erk protein phosphorylated at Thr202/Tyr204. Immunoblots were stripped and reprobed with appropriate antibodies detecting phosphorylated S6 (Ser235/236) and with antibodies detecting Shp-2 to confirm equal loading. The data shown are representative of two independent experiments.

### *3.2.3 BMP4 stimulated signalling in mESCs*

Smad1 is known to be directly phosphorylated by BMP type I receptors (Kretzschmar et al., 1997). In mESCs, reciprocal inhibition of BMP signalling and of signalling molecules of the MAPK pathway has been reported. For example, Erk is known to phosphorylate Smad1 and prevent nuclear accumulation (Fuentealba et al., 2007). Conversely, BMP4-activated XIAP is known to inhibit p38 MAPK signalling (Yamaguchi et al., 1999). Furthermore, inhibitors of p38 or Mek, of the MAPK family, mimic the effect of BMP4 on self-renewal (Qi e al., 2004). In section 3.2.1.2, BMP4 was shown to activate signalling

downstream of PI3Ks via S6 but not via PKB. Given that LY294002 treatment of mESCs elicits an enhancement in Erk phosphorylation, the effect of inhibiting PI3Ks on Smad1 activity was of interest.

In order to assess the effect of inhibition of PI3Ks on Smad1 activity, mESCs were incubated in the presence or absence of LY294002 or wortmannin and Smad1 phosphorylation detected by immunoblotting. High basal levels of Smad1 phosphorylation were observed even after 4 hours incubation in the absence of BMP4, LIF, N2 and B27 supplements (Figure 3.5**A**). Stimulation of mESCs with BMP4 did not alter levels of Smad1 phosphorylation. Additionally, no effect on Smad1 phosphorylation was seen following pharmacological inhibition of PI3Ks with either LY294002 or wortmannin.

These findings are inconsistent with Ying et al., (2003), who reported an induction in Smad1 phosphorylation in mESCs following addition of BMP4 for 15 minutes or 1 hour after an overnight incubation in the absence of BMP4. In mESCs incubated in the absence of BMP4, they detected no Smad1 phosphorylation (Ying et al., 2003b). In the present study, high basal levels of Smad1 phosphorylation were unchanged by BMP4 stimulation. An experiment was set up to determine whether this high level of basal Smad1 phosphorylation could be reduced by BMP4 withdrawal over a longer period of time. No change in Smad1 phosphorylation was observed following BMP4 withdrawal (Figure 3.5**B**). Even after 24 hours incubation in the absence of BMP4, Smad1 phosphorylation remained high. However, withdrawal of BMP4 enhanced S6 phosphorylation, peaking at 1 hour after withdrawal and enhanced Erk phosphorylation maximally at 2 hours post-BMP4 withdrawal. Both of which are consistent with early triggers to induce differentiation and consistent with a report demonstrating BMP4-induced inhibition of MAPK signalling (Qi et al., 2004).

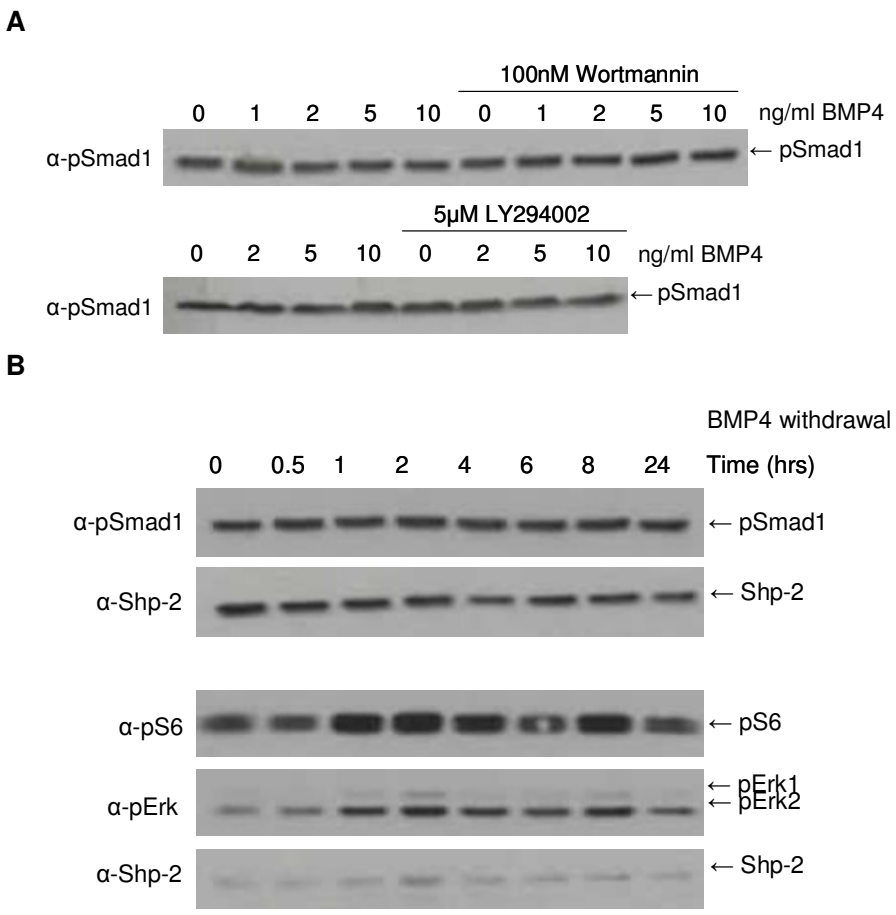



**Figure 3.5 Neither stimulation with BMP4 nor withdrawal of BMP4 alters Smad1 phosphorylation**

**A** Following a 4 hour incubation in the absence of LIF, BMP4, N2 and B27 supplements, mESCs were stimulated with BMP4 for 5 minutes. Lysates were resolved by SDS-PAGE and immunoblotting with antibodies detecting phosphorylated Smad1 (Ser463/465). **B** A BMP4-withdrawal time-course was conducted by washing mESCs with PBS and incubating with media lacking BMP4. Lysates were prepared at the times indicated following BMP4 withdrawal and resolved by SDS-PAGE. Immunoblotting was conducted using antibodies detecting phosphorylated Smad1 (Ser463/465) or Erk (Thr202/Tyr204). Immunoblots were stripped and reprobed to allow detection of phosphorylated S6 (Ser235/236) and Shp-2 to confirm equal loading.

## 3.3 Activation of the MAPK Pathway

The maintenance of self-renewal and pluripotency is governed by activation of pathways that maintain self-renewal and inhibition of pathways that promote differentiation. Whilst activating the Stat3 pathway to promote self-renewal, LIF also activates the MAPK pathway and inhibition of the MAPK pathway is known to enhance self-renewal (Burdon et al., 1999). Furthermore, inhibition of Mek, of the MAPK pathway, reverses the effects of LY294002 inhibition of PI3Ks on self-renewal (Paling et al., 2004). In the present study, BMP4 was identified as a factor that activated PI3Ks in mESCs (Welham et al., 2007) and reduced the activity of the MAPK pathway when applied to cells cultured in serum-replacement media, used for the routine culture of mESCs (Figure 3.4**A**). Thus, under normal culture conditions, given the right external stimuli such as BMP4 and LIF, PI3Ks may act to inhibit activity of the MAPK pathway.

To further address the role of Mek in self-renewal, mESC clones that can be induced to express a constitutively active form of Mek1 (Mek*) were used. Mek* clones were generated by Prof. M. J. Welham (unpublished data). Briefly, constitutively active Mek1 was created by substitution mutations within the catalytic domain. Serine 218 and serine 222 were substituted for glutamic acid residues to mimic activating phosphorylation (Treinies et al., 1999) (Figure 3.6**A**). This activated form of Mek, referred to as Mek*, was histidine-tagged and inserted into a gene expression vector under the control of a tetracycline-off expression system (Era and Witte, 2000). The CAG promoter drives expression of the tetracycline-sensitive transactivator (tTA) containing a tetracycline response element. Mek* expression is driven by a tTA-dependent promoter. Tetracycline binds to tTA and prevents it from binding to the tTA-dependent promoter, resulting in no transcription of Mek*. In the absence of tetracycline, tTA binds the tTA-dependent promoter to drive expression of exogenous Mek* (Figure 3.6**B**). Thus, under the tetracycline-off expression system, in the presence of tetracycline only endogenous Mek is expressed, while in the absence of tetracycline, both endogenous Mek and induced, exogenous Mek* are expressed.

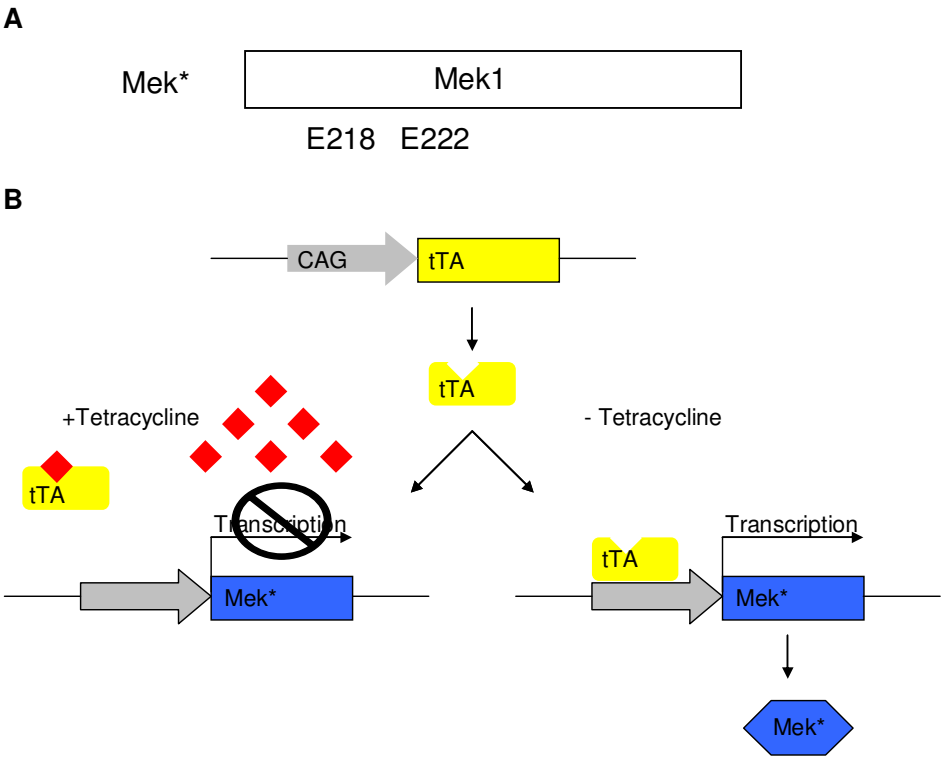



**Figure 3.6 Inducible expression of Mek* by the tetracycline-off expression system**
**A** Constitutively active Mek1 (Mek*) was generated by replacement of serine 218 and serine 222 with glutamic acid residues (E) within the catalytic domain of Mek1. **B** The tetracycline-sensitive transactivator (tTA) is expressed by a CAG promoter. In the absence of tetracycline, tTA binds the tTA-dependent promoter driving transcription of Mek*. However, in the presence of tetracycline, tTA is prevented from binding the tTA-dependent promoter and Mek* is not transcribed. Adapted from Treinies et al., 1999, Era and Witte, 2000.

The hypothesis that expression of constitutively active Mek would promote constitutive activation of the MAPK pathway leading to the loss of self-renewal and the promotion differentiation was tested using two independent Mek* clones, clone 28 and clone 34.

### *3.3.1 Assessment of Mek expression in cloned cells*

Expression of Mek was initially assessed in Mek* clones by immunoblotting (Figure 3.7**A**). Cultured in the presence of tetracycline, clone 28 and 34 expressed moderate levels of phosphorylated and total Mek. Tetracycline withdrawal for 24 hours induced enhanced levels of phosphorylated and total Mek protein expression. However, this method of assessing Mek expression does not distinguish between endogenous Mek expression and induced Mek* expression since the antibodies used are capable of detecting both types of Mek1.

Immunochemistry staining for histidine was used to assess expression of histidine-tagged Mek* (Figure 3.7**B**). In the presence of tetracycline, histidine-tagged Mek* was detected in neither clone 28 nor clone 34. When tetracycline was removed for 24 hours, histidine-tagged Mek* could be detected in both clone 28 and clone 34. Murine ESCs have large nuclei, which were evident in stained cells (Figure 3.7**B**). Despite this, staining for histidine-tagged Mek* was observed in the cytosol of mESCs cultured in the absence of tetracycline (Figure 3.7**B** right hand panel). Clone 28 exhibited a mosaic expression pattern where not all cells stained positive for histidine-tagged Mek* expression. Clone 34 did not exhibit such a phenomenon, all clone 34 cells stained positively for histidine-tagged Mek* in the absence of tetracycline.

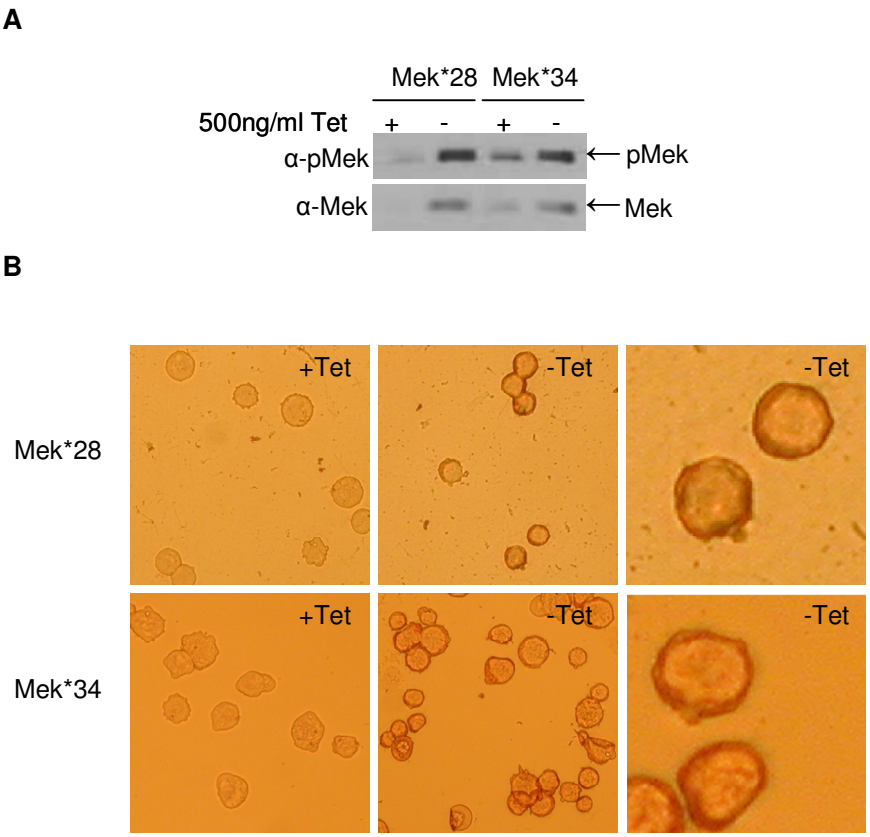



**Figure 3.7 Expression of Mek in Mek* cloned mESCs**

Mek* clones 28 and 34 (Mek*28 and Mek*34 respectively) were harvested after 24 hours incubation in the presence or absence of tetracycline. **A** Lysates were analysed by immunoblotting with antibodies that detect phosphorylated Mek (Ser217/221) (upper panel) and or total Mek protein (lower panel). The data shown are representative of two independent experiments. **B** Cytospin slides were prepared with fixed cells and incubated with an anti-histidine antibody. Transfected Mek* is histidine-tagged, thus positive cells stain brown. An enlarged image of stained cells demonstrates cytoplasmic staining, right hand panel.

Having ascertained that histidine-tagged Mek* expression was induced following withdrawal from tetracycline and that the majority of the cells within each clone expressed Mek* following induction, signalling downstream of Mek* was investigated. In the absence of tetracycline, when Mek* and endogenous Mek are expressed, Erk phosphorylation was greater than that in the presence of tetracycline, when cells express endogenous Mek alone (Figure 3.8**A**). This indicated that Mek* was functionally active since expression of Mek* enhanced Erk phosphorylation, consistent with Erk being a direct target of the kinase activity of Mek.

LIF promotes self-renewal and maintenance of pluripotency via induction of Stat3 phosphorylation but LIF also activates the MAPK pathway (Burdon et al., 1999, Niwa et al., 1998, Paling et al., 2004). Unstimulated Mek* clones cultured in the absence of tetracycline displayed elevated levels of phosphorylated Erk, consistent with expression of both endogenous Mek and induced Mek* (Figure 3.8**B**). Upon LIF stimulation, Erk phosphorylation was elevated above unstimulated levels. Erk phosphorylation was greater in cells expressing induced Mek* and endogenous Mek compared to cells expressing endogenous Mek alone. This indicates that Mek and Mek* are functionally active in mESC Mek* clones. Additionally, enhanced MAPK pathway activity is detectable and elevated in unstimulated and LIF-stimulated cells when Mek* is expressed.

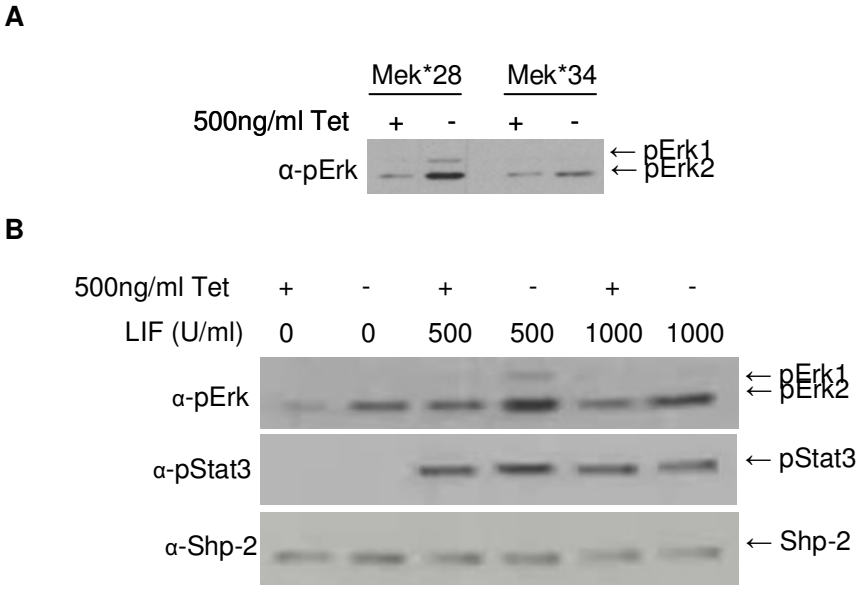



**Figure 3.8 Mek* is functionally active and enhances MAPK pathway activation**

Mek* clones 28 and 34 (Mek*28 and Mek*34 respectively) were harvested after 24 hours incubation in the presence or absence of tetracycline. **A** Lysates were analysed by immunoblotting with phosphorylated Erk antibodies. The data shown are representative of two independent experiments. **B** Mek* clones incubated for 24 hours in the presence or absence of tetracycline were serum starved for 4 hours prior to stimulation. Stimulations were conducted for 10mins with vehicle (DMSO), 500 and 1000U/ml LIF. Cell lysates were analysed by SDS-PAGE separation and immunoblotting with antibodies detecting phosphorylated Erk (Thr202/Tyr204). Immunoblots were stripped and reprobed using antibodies detecting phosphorylated Stat3 (Tyr705) and Shp-2. Mek* clone 34 data presented here are representative of two independent experiments for each Mek* clone.

LIF-induced Stat3 phosphorylation at Tyr705 is essential for dimerisation and nuclear translocation (Zhong et al., 1994). Stat3 phosphorylation at Tyr705 appeared unchanged by the presence of tetracycline (Figure 3.8**B**). However, on closer inspection a small change in the migration of the band corresponding to pStat3 was observed. Phospho-Stat3 protein bands detected by immunoblotting following SDS-PAGE separation of protein lysates appeared to correlate with protein of a greater size in the absence of tetracycline.

Migration of proteins through a polyacrylamide gel is dependent on the size of the protein. Retarded migration is often due to molecular weight variation as a result of additional phosphorylation. The molecular weight of Stat3 appeared to be altered following expression of Mek*, which might be explained by phosphorylation at another site, potentially mediated by activated Mek*. Therefore, the phosphorylation of Stat3 at alternative sites was investigated. Phosphorylation at Tyr705 is required for the dimerisation and nuclear translocation of Stat3 (Zhong et al., 1994) but phosphorylation at a second site, Ser727, is critical for optimal induction of transcriptional activity of Stat3 (Wen et al., 1995). Basal phosphorylation of Ser727 was similar in cells cultured with and without tetracycline (Figure 3.9). Therefore, the expression of Mek* does not appear to alter basal Ser727 phosphorylation of Stat3. Furthermore, LIF stimulation did not change Ser727 phosphorylation for Mek* clones 28 and 34 (Figure 3.9). Similarly, basal phosphorylation of Ser668, of unknown function, remained unchanged regardless of the presence of tetracycline or LIF stimulation for either clone.

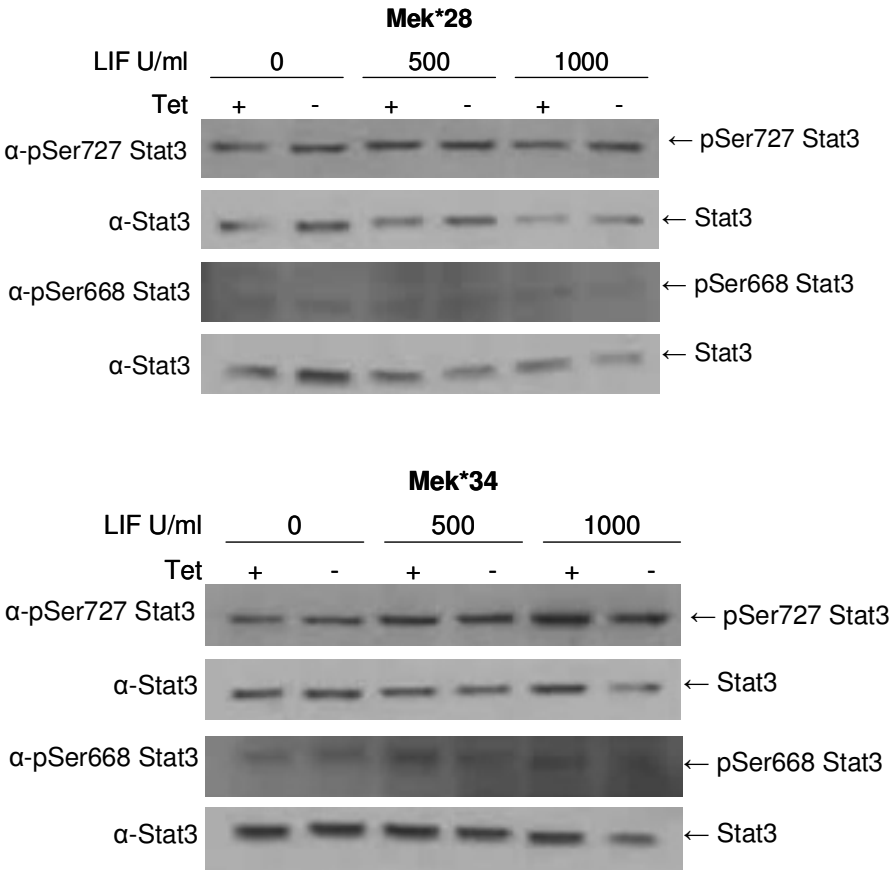



**Figure 3.9 LIF stimulation of Mek* clones does not elicit phosphorylation of Stat3 at alternative phosphorylation sites**

Mek* clones, incubated for 24 hours in the presence or absence of tetracycline, were serum starved for 4 hours prior to stimulation with LIF. Stimulations were conducted for 10mins with vehicle (DMSO), 500 or 1000U/ml LIF. Cell lysates were analysed by SDS-PAGE separation and immunoblotting with antibodies detecting phosphorylation sites at Ser727 and Ser668 of Stat3, loading was assessed by stripping and reprobing immunoblots with antibodies detecting all Stat3 proteins. Data are representative of two independent experiments for each Mek* clone.

### *3.3.2 Effect of constitutively active Mek expression on self-renewal*

Self-renewal of ESCs is important to maintain pluripotency, the ability of ESCs to differentiate into any adult cell type of the organism from which they were derived, and to maintain the undifferentiated state of ESCs in culture. However, self-renewal and differentiation involve a number of signalling pathways. Previously LIF was demonstrated to stimulate signalling of the Stat3, PI3K and MAPK pathways in mESCs (Niwa et al., 1998, Boeuf et al., 1997, Paling et al., 2004). Interestingly, inhibition of PI3Ks with LY294002 was shown to enhance MAPK pathway signalling (Paling et al., 2004) (Figure 3.2 and 3.3) demonstrating cross-talk between the pathways activated by LIF. However, inhibition of the MAPK pathway, via pharmacological inhibition of Mek, is reported to enhance mESC self-renewal (Burdon et al., 1999) and reverse the effects of LY294002 inhibition of PI3Ks on self-renewal (Paling et al., 2004). Therefore, the effect of enhanced MAPK pathway activation on mESC self-renewal was of interest. In order to address the effect of enhanced MAPK pathway activity on mESC fate, mESC Mek* clones were induced to express Mek* and the effect on self-renewal assessed by alkaline phosphatase assay. Alkaline phosphatase is a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions. It is expressed on the surface of pluripotent, self-renewing mESCs but surface expression is lost upon the initiation of differentiation (Scutt and Bertram, 1999, Pease et al., 1990). The alkaline phosphatase assay was used to assess self-renewal of mESC Mek* clones expressing endogenous Mek alone and both endogenous Mek and exogenous Mek*.

Mek* clones were cultured at clonal density for 4 days in the presence or absence of tetracycline then assayed for alkaline phosphatase activity (Figure 3.10**A**). In the presence of LIF and tetracycline, 80 to 90% of colonies were alkaline phosphatase positive. Most colonies stained bright red with a smooth rounded morphology (an example is depicted in Figure 3.10**Bi**), indicative of highly self-renewing, undifferentiated colonies. Other colonies stained strongly in the centre and were surrounded by pale or unstained cells (Figure 3.10**Bii**), classed as self-renewing or mixed colonies. In the absence of tetracycline, but in the presence of LIF, 70 to 85% of colonies were self-renewing and exhibited a similar morphology to colonies incubated with tetracycline. Statistically, there was no difference in the proportion of self-renewing colonies observed in the presence or absence of tetracycline when LIF was present, although a small but consistent decrease in self-renewal was noted in the absence of tetracycline. In the absence of LIF, the ability of cells to form self-renewing colonies was significantly reduced, consistent with the requirement of LIF to maintain self-renewal (Smith and

Hooper, 1987, Boeuf et al., 1997, Niwa et al., 1998, Raz et al., 1999, Cartwright et al., 2005). In the absence of LIF, colonies either stained pale pink (Figure 3.10**Biii**) or remained unstained (Figure 3.10**Biv**) and displayed an asymmetrical, flattened appearance with rough colony edges, indicative of differentiated colonies. In the absence of LIF, colonies expressing Mek* exhibited a moderate decrease in self-renewal compared to colonies expressing endogenous Mek alone (Figure 3.10**A**) but this was not statistically significant.

To confirm that induced cells were expressing Mek* at the time of harvesting the alkaline phosphatase assay, protein lysates were analysed for Mek protein expression after 4 days incubation in the presence or absence of tetracycline (Figure 3.10**C**). In the absence of tetracycline, higher Mek protein levels were detected in Mek* clones 28 and 34 on day 4, indicating expression of induced Mek* and endogenous Mek protein was maintained.

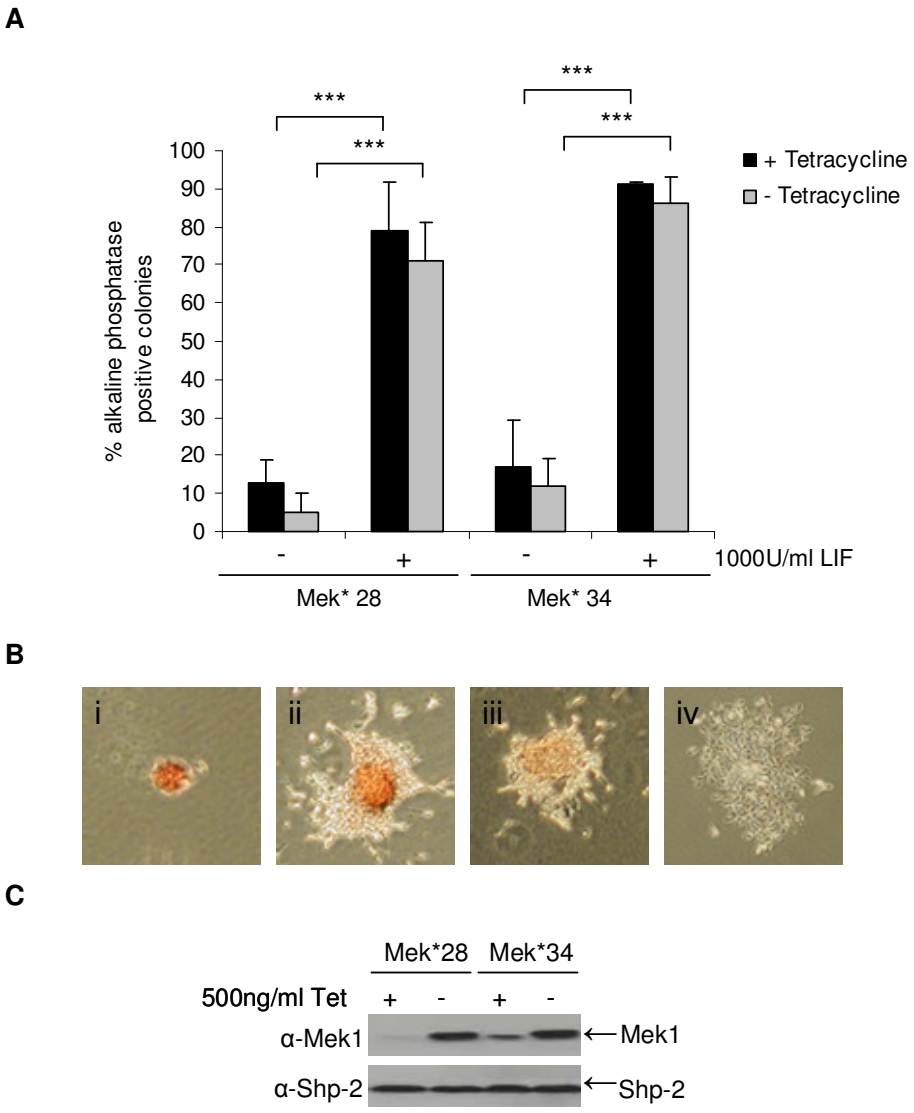



**Figure 3.10 Expression of Mek* does not significantly reduce alkaline phosphatase staining**

Mek* mESC clones were cultured in supplemented GMEM plus 10% (v/v) serum, in the presence or absence of 500ng/ml tetracycline for 4 days. **A** Self-renewal was quantified by staining for alkaline phosphatase activity, a cell-surface marker of undifferentiated self-renewing mESCs. Two independent Mek* mESC clones were used (Mek* 28 and Mek* 34). The mean data from three experimental repeats are shown with Standard Error of Mean (S.E.M.). Statistical significance was assessed using a Student's T-test where *** represents $p<0.001$. In the presence of LIF, no significant difference was found in the level of alkaline phosphatase staining of colonies cultured in the presence or absence of tetracycline (Mek* 28 $p=0.64$, Mek* 34 $p=0.61$). **B** Representative colonies are shown following alkaline phosphatase staining. **Bi** depicts a pure self-

renewing colony. Colonies with morphology and staining similar to **Bii** are also scored as self-renewing. **Biii** and **Biv** are representative of non-self-renewing, differentiated colony types. **C** After 4 days incubation in the presence or absence of tetracycline, cell lysates were prepared and resolved by SDS-PAGE. Mek1 was detected by immunoblotting with Mek1 antibody and Shp-2 used to confirm equal loading.

Expression of constitutively active Mek*, which activates the MAPK pathway downstream of Mek, produced a trend towards decreased alkaline phosphatase staining that was not found to be statistically significant. The assessment of self-renewal by alkaline phosphatase assay was conducted in the presence of 1000U/ml LIF. This concentration is used for the routine culture of mESCs but might be considered to be in excess of the concentration required to maintain self-renewal. In order to address this matter, the level of self-renewal of mESC Mek* clones was assessed in the presence and absence of tetracycline, and under reduced, limiting LIF concentrations. Additionally, if excess LIF was supporting self-renewal upon constitutive activation of MAPK signalling then limiting LIF concentrations might reveal a decrease in self-renewal upon activation of the MAPK pathway.

Mek* clones were plated at clonal density in the presence or absence of tetracycline, and in limiting LIF conditions then assayed for alkaline phosphatase assay after 4 days. Both Mek* clone 28 and clone 34 colonies exhibited reduced alkaline phosphatase staining when incubated with 10 and 20U/ml LIF compared to incubation with 1000U/ml (Figure 3.11). Moreover, in the absence of tetracycline, when Mek* is expressed, a further decrease in the proportion of alkaline phosphatase positive colonies was observed compared to colonies incubated in the presence of tetracycline, although again this was found not to be statistically significant. This trend towards decreased alkaline phosphatase activity when Mek* was expressed and mESCs cultured in low concentrations of LIF was particularly noticeable for clone 28.

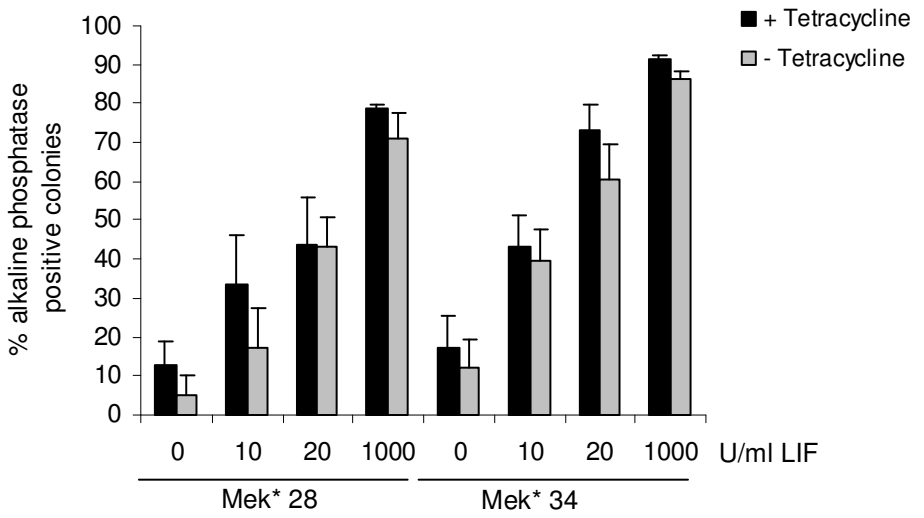



**Figure 3.11 A decrease in self-renewal is observed upon expression of Mek* in low LIF concentrations**

Mek* mESC clones were cultured in supplemented GMEM plus 10% (v/v) serum in the presence or absence of 500ng/ml tetracycline for 4 days and in the presence of 0, 10, 20 or 1000U/ml LIF. Self-renewal was quantified by staining for alkaline phosphatase activity. The mean data from three independent experiments are shown with S.E.M. The data were analysed statistically, using a Student's T-test to compare the presence or absence of tetracycline at each LIF concentration. No statistical significance was found despite a trend towards decreased alkaline phosphatase staining upon expression of Mek* in low LIF concentrations.

Self-renewal of Mek* mESC clones was also assessed using Knockout serum replacement (KO SR) media (used for the routine culture of mESCs) supplemented with 10% (v/v) serum to support the growth of differentiated cells (Figure 3.12). In this media and in the presence of LIF, alkaline phosphatase staining in the presence of tetracycline was indistinguishable from staining observed in the absence of tetracycline. In the absence of LIF, alkaline phosphatase staining was significantly reduced compared to the presence of LIF. In the absence of LIF, no consistent trend correlating with the presence or absence of tetracycline was observed (Figure 3.12).

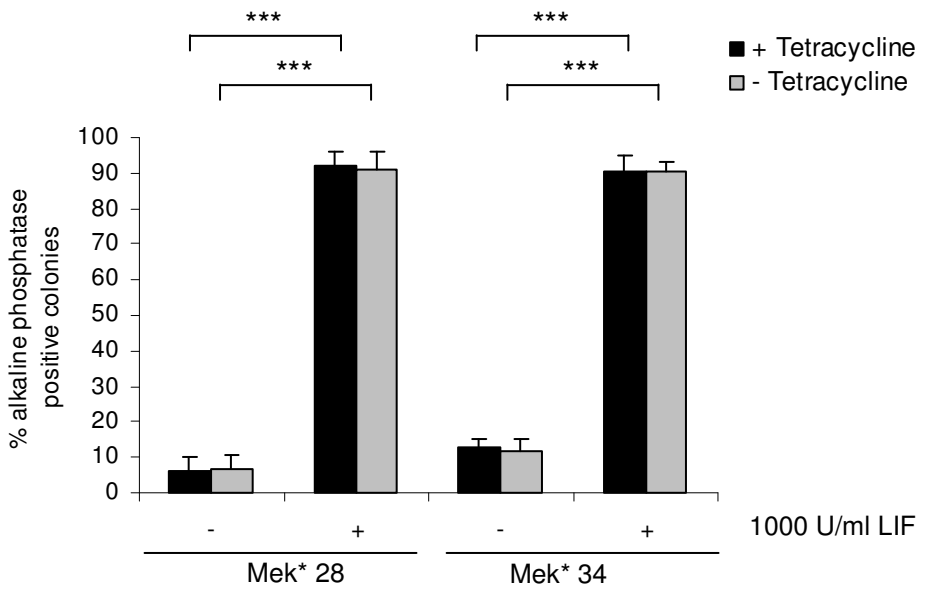



**Figure 3.12 The trend towards decreased alkaline phosphatase staining when the MAPK pathway is activated is not observed when clones are cultured in complete KO SR media**

Mek* mESC clones were cultured in complete KO SR media with 10% serum in the presence or absence of 500ng/ml tetracycline and in the presence or absence of 1000U/ml LIF for 4 days. Self-renewal was quantified by staining for alkaline phosphatase activity. The mean data from three experimental repeats are shown with S.E.M. Statistical significance was assessed using a Student's T-test where *** represents $p<0.001$. Alkaline phosphatase staining in the presence of LIF and tetracycline was not significantly different from staining in the presence of LIF and absence of tetracycline (Mek* 28 $p= 0.954$, Mek* 34 $p=0.953$).

## 3.4 Inhibition of PI3K and lineage commitment

LIF promotes self-renewal allowing mESCs to retain their pluripotency, therefore mESCs cultured with LIF remain largely undifferentiated. Conversely, culture in the absence of LIF causes spontaneous differentiation towards multiple lineages. While LIF maintains self-renewal and its withdrawal triggers differentiation, other factors direct commitment to particular cell lineages. For example, BMP4 promotes self-renewal by blocking neural differentiation (Ying et al., 2003b) and promoting non-neural differentiation (Ying et al., 2003b, Xu et al., 2002). PI3K inhibition, even in the presence of LIF, results in a loss of self-renewal with mESCs adopting a differentiated morphology, reduced alkaline phosphatase staining and a reduction in Nanog expression (Paling et al., 2004, Storm et al., 2007). However, whether inhibition of PI3Ks results in differentiation towards specific lineages is unknown.

To ascertain whether or not PI3K inhibition directs differentiation towards a particular lineage in adherent 2D monolayer culture, mESCs were incubated in the presence of the PI3K inhibitor LY294002. Usually, mESCs are replated every two days to maintain a sub-confluent cell density suitable for self-renewal and are cultured in a media suitable for the maintenance of undifferentiated cells. However, at higher densities, mESCs undergo spontaneous differentiation. For this experiment cells were plated on day 0 in media containing serum to support the growth of both undifferentiated and differentiated cells. RNA was isolated on days 3-7 without passaging cells in between. Additionally, cells were cultured in the presence or absence of LIF to demonstrate differentiation that is not restricted to a particular lineage. Primers detecting early lineage marker genes, GATA1, GATA6, Pax6, Nodal and FGF5, were used to determine the effects of these treatments on the differentiation potential of mESCs following inhibition of PI3K signalling (Figure 3.13). GATA1 belongs to the sub-family of haematopoietic GATA transcription factors, which are predominantly expressed in cells of the haematopoietic system (Ferreira et al., 2005). GATA1 is reported to be a suitable marker of mesodermal differentiation of ESCs (Robertson et al., 2000, Fujimoto et al., 2001) and is expressed by ES-derived cells undergoing mesodermal differentiation to haematopoietic and endothelial lineages (Robertson et al., 2000). In contrast, GATA6 belongs to the non-haematopoietic subfamily of GATA transcription factors and its expression correlates with its indispensability in the development of heart, lung and gastrointestinal tract tissues (reviewed by Maeda et al., 2005). With the exception of GATA6 expression in the heart, GATA6 is frequently used as a marker of endodermal lineage cells (Natira et al., 1997). Pax6 is a homeobox transcription factor that regulates the expression of

proteins important for the development of the central nervous system, eye, and endocrine glands. Thus, Pax6 expression can be used to detect the presence of neuroectodermal cell types (Yoshida-Koide et al., 2004). Nodal is a transforming growth factor β (TGFβ) related gene, which is detected at E5.5 in primitive streak ectoderm of developing mouse embryos, where its expression is required for mesoderm induction (Conlon et al., 1994). During implantation (E4.5) nodal expression is also detectable in the ICM and primitive endoderm (Mesnard et al., 2006). Therefore, nodal can be used as a marker for embryonic ectoderm and primitive endoderm (Leahy et al., 1999, Yoshida-Koide et al., 2004). Fibroblast growth factor 5 (FGF5) belongs to the FGF family of mitogens, which have a variety of roles in developmental processes (Goldfarb, 1990). FGF5 is important in embryogenesis and expressed in the epiblast and visceral endoderm at E5.25-7.5 during the development of the mouse embryo (Haub and Goldfarb, 1991). Thus FGF5 expression is frequently used as a marker of primitive ectoderm in the early stages of ESC differentiation.

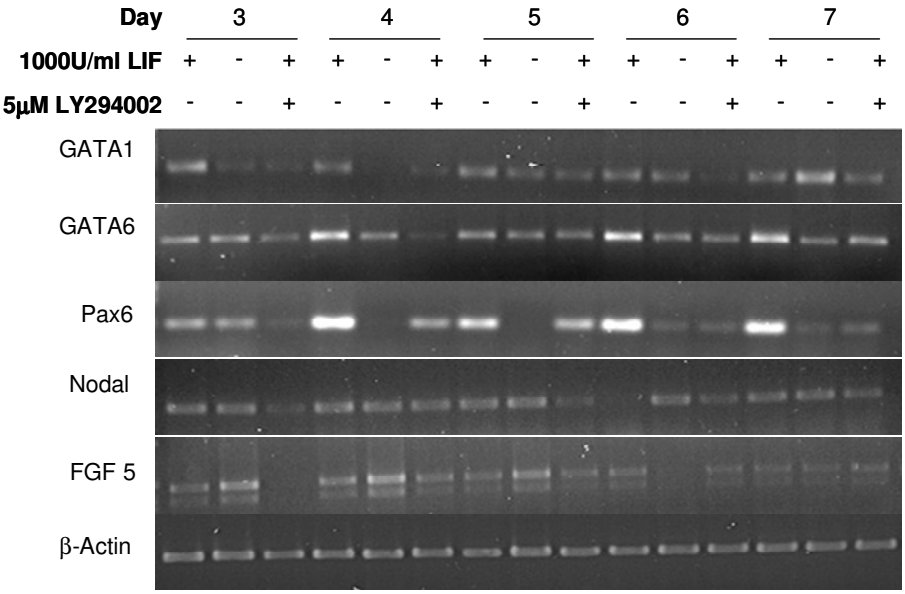



**Figure 3.13: PI3K inhibition results in differentiation towards all lineages**

Murine ESCs were cultured in the presence or absence of 1000U/ml LIF and in the presence or absence of 5µM LY294002. RNA was isolated daily from day 3 to day 7. Reverse-transcription was conducted to produce cDNA. Primers that mark early lineage differentiation were used in PCR reactions to determine cell fate. A typical expression profile, representative of three independent experiments, is depicted. The expression of β-Actin was used as a control and remained unchanged at different time points and regardless of treatment.

GATA1 expression, an indication of mesodermal differentiation, appeared to remain constant in the presence of LIF between days 4 and 7 (Figure 3.13). In the absence of LIF, GATA1 expression was low on days 3 and 4 but increased towards day 7. Treatment with LY294002 yielded low GATA1 expression with a notable peak on day 5. The GATA1 expression pattern for LY294002 treatment is similar to the pattern observed in the absence of LIF, indicating no change in spontaneous differentiation.

In all experimental repeats, GATA6, a marker of visceral and definitive endoderm (Narita et al., 1997), showed highest expression with LIF alone and a peak in expression at days 4 and 7 (Figure 3.13). Expression of GATA6 was detected for each treatment suggesting a degree of endodermal differentiation. RNA isolated from cells incubated without LIF exhibited constant GATA6 expression throughout the time course of the experiment. Treatment with both LIF and LY294002 showed lower GATA6 expression on days 3 and 4 but similar expression to cells differentiating in the absence of LIF on days 5-7. This pattern suggests that the time-course involved in differentiation towards endodermal lineages is altered but not prevented by PI3K inhibition.

Pax6 exhibited high expression in the presence of LIF particularly on day 4 (Figure 3.13). In contrast, Pax6 was detected but expression was lower in the absence of LIF or in the presence of LIF and LY294002 compared to presence of LIF alone. Interestingly, PI3K inhibition did not alter the observed increase in Pax6 expression on day 4 from levels detected on day 3. However, in the presence of LY294002, expression levels of Pax6 were consistently lower.

Nodal, a marker of embryonic ectoderm and primitive endoderm, showed an almost constant expression profile for each treatment, with one exception: Expression on day 3 in cells treated with LY294002 was consistently lower than that observed for the other two treatments (Figure 3.13). Small dips in nodal expression were observed in different treatments from day 5 onwards but the temporal nature of these varied across experimental repeats. These differences may represent slight variations in the differentiation of cells populating a particular culture dish.

In both humans and mice, two splice variants of FGF5 are known to be expressed (Ozawa et al., 1998), both mouse splice variants were detected by the primers used in the present study (Figure 3.13). FGF5 expression was similar to that of nodal, an alternative primitive ectoderm marker. FGF5 was undetectable or detectable at very low levels in cells treated with LY294002 at the day 3 time point (Figure 3.13). As with nodal expression, small fluctuations were observed on days 6 and 7 but these varied across

experimental repeats. Expression remained fairly constant between days 3 and 7 for cells incubated in the presence of LIF. A consistent elevation in FGF5 expression was observed in the absence of LIF on days 4 and 5.

Overall, markers representing mesoderm, endoderm, neuroectoderm and ectoderm were detected in each of the treatments indicating differentiation along these lineages (Figure 3.13). Differentiation in the presence of LIF can be explained by a number of factors including spontaneous differentiation exhibited by ESCs, the presence of differentiation-inducing serum and differentiation induced by the high density of mESCs following a number of days without passage. Differentiation in the presence of LIF appeared to be more towards neuroectodermal lineages but other lineages were represented. Differentiation in the absence of LIF resulted in the formation of cells representing all lineages. Importantly, inhibition of PI3Ks with LY294002 did not appear to favour differentiation towards any particular lineage since the expression of markers from all lineages were detectable. Furthermore, marker expression patterns in the presence of LY294002 were notably similar to those observed in the absence of LIF, suggesting that differentiation potential is not altered by inhibition of PI3Ks.

## 3.5 Summary and discussion: Identification of factors activating PI3K in mESCs

In the present study, a chemically defined media was used to determine factors that activate PI3K signalling in mESCs. The effects of BMP4 and insulin stimulation on mESCs are summarised in Table 3.1.

**Table 3.1 Responses to stimulations conducted in N2B27-defined media**

| | INHIBITOR | PHOSPHORYLATION RESPONSE | | |
|---|---|---|---|---|
| | | PKB | S6 | Erk |
| Control | Control | | High basal levels | Low basal levels |
| | LY294002 | No change | Decreased basal levels | Enhanced basal levels |
| | Wortmannin | | Decreased basal levels | Negligible decrease |
| Insulin | Control | Concentration-response | Enhanced | Enhanced from basal |
| | LY294002 | Inhibition | Inhibition | Further enhanced |
| | Wortmannin | | Inhibition | No change |
| BMP4 | Control | No change | Concentration-response | Concentration-response |
| | LY294002 | No change | Inhibition | Further enhanced |
| | Wortmannin | | Inhibition | No change |

BMP4 stimulation of mESCs resulted in enhanced S6 phosphorylation but no change in PKB phosphorylation. Furthermore, BMP4-induced S6 phosphorylation was inhibited with either LY294002 or wortmannin. Insulin stimulation of mESCs induced PKB and S6 phosphorylation, which was inhibited by either LY294002 or wortmannin. Insulin is present in both the N2 and B27 supplements, used in chemically defined media, and is likely to be present in undefined serum and serum-replacement. BMP4 is added to chemically defined media in order to maintain self-renewal alongside LIF, but may also be present in undefined serum-containing or serum-replacement media. Insulin and BMP4 were shown to stimulate PI3K signalling in mESCs.

Murine ESCs lacking endogenous BMP4 exhibit reduced Erk phosphorylation upon the addition of exogenous BMP4 (Qi et al., 2004). In contrast, LIF stimulation activates the MAPK pathway, yet both LIF and BMP4 are required in serum-free media to maintain self-renewal (Ying et al., 2003b). In the present study, and in a previous report (Paling et al., 2004), inhibition of PI3K with LY294002 enhanced basal Erk phosphorylation. Furthermore, in the present study, LY294002 enhanced BMP4-induced Erk phosphorylation. Taken together these results suggest that PI3K may exert a dampening effect on the MAPK pathway. It is unclear why wortmannin does not exert the same enhancement in Erk phosphorylation as observed with LY294002, but could be due to the different modes of action of these inhibitors. A handful of reports have noted the phenomenon of PI3K inhibition enhancing Erk phosphorylation in a number of cell types. In a pituitary gonadotroph cell line, treatment with either LY294002 or wortmannin enhanced Erk phosphorylation and enhanced the expression and promoter activity of the α subunit of gonadotrophins. Interestingly, inhibition of PKB did not enhance Erk phosphorylation and no change was observed in expression of promoter activity, suggesting that PI3K mediated this inhibitory action on the MAPK pathway (Kanasaki et al., 2008). LY294002 was also shown to enhance Erk phosphorylation in differentiating Caco-2/15 cells (an intestinal cell line) but not in undifferentiated Caco-2/15 cells (Laprise et al., 2004). The mechanism by which PI3K might exert the inhibition of MAPK signalling is still unclear. Laprise et al., demonstrated that PI3K inhibition can enhance Mek activity (Laprise et al., 2004). In a different study, using pituitary cells, inhibition of PI3Ks resulted in enhanced Raf-1 kinase and Rap-1 activity, both of which are upstream of Mek and Erk (Romano et al., 2006).

Insulin was also demonstrated to enhance Erk phosphorylation. Consistent with this observation, insulin has been shown to induce Erk phosphorylation via activation of type 1 insulin-like growth factor (IGF-1) receptors in mESCs (Nguyen et al., 2007). Stimulation of insulin receptors or IGF-1 receptors results in activation of insulin receptor substrate-1 (IRS-1), which contains PH and SH2 domains. IRS-1 expression and activation have been reported to be required for mESC self-renewal since targeting IRS-1 with siRNA led to a decrease in alkaline phosphatase staining, a decrease in Oct4 expression and a decrease in PKB phosphorylation indicating signalling via PI3K (Rubin et al., 2007). Interestingly, in cell types other than mESCs, the actions of IRS-1 are reported to be mediated through PI3K, PKB and p70S6K/mTOR pathways to regulate the expression of Id genes. Furthermore, IRS-1 is best known to be phosphorylated by insulin, IGF-1 and LIF (Argetsinger et al., 1995, Belletti et al., 2001). The mechanism by which IRS-1 is phosphorylated and aids the maintenance of self-renewal in mESCs is

yet to be defined. In the present study, insulin is demonstrated to activate both PKB and S6, downstream of PI3K in mESC, consistent with the signalling pathways defined by previous reports. Also, in agreement with the present study, in mouse $R^-$ fibroblasts (lacking IGF-1 receptors) LY294002 enhanced insulin-induced Erk phosphorylation. However, unlike the present study, LY294002 did not enhance Erk phosphorylation in unstimulated mouse $R^-$ fibroblasts cells (Choi and Sung, 2004).

High levels of phosphorylated Smad1 were detected in mESCs. Smad1 phosphorylation was unchanged by stimulation with BMP4 or withdrawal of BMP4 for up to 24 hours. These results are inconsistent with those reported by Ying et al., where over-night incubation in the absence of BMP4 resulted in undetectable Smad1 phosphorylation in unstimulated cells and Smad1 phosphorylation in BMP4-stimulated cells (Ying et al., 2003b). However this discrepancy might be explained by the use of a different mESC line. In the present study, the E14tg2A mESC line was used and cells were cultured in chemically defined media for at least 2 days prior to being used for experiments. However Ying et al., derived ESCs from blastocysts and cultured these cells directly in chemically defined media (Ying et al., 2003b). The different results may reflect the adaptation of mESCs to their routine culture media. Furthermore, Smad1, Smad5 and Smad8 are known to couple to BMP4 induced activation of the type 1 BMP receptor, BmprIa and type II receptor, BmprII, both of which are expressed in mESCs (Qi et al., 2004, Kishigami and Mishina, 2005). Since only antibodies detecting Smad1 were used in the present study, it is possible that Smad5 and/or Smad8 mediate the response to BMP4 in this mESC line.

## 3.6 Summary and discussion: The effect constitutively activating MAPK pathway signalling

Burdon et al., demonstrated that inhibition of MAPK pathway activity using the Mek inhibitor PD098059 resulted in enhanced self-renewal of mESCs and inhibition of differentiation in embryoid bodies (Burdon et al., 1999). Additionally, Kunath et al., have reported that FGF4-induced Erk activity was responsible for lineage commitment towards neural and mesodermal lineages although mESCs lacking FGF4-induced MAPK activity were still capable of undergoing differentiation (Kunath et al., 2007). The present study used mESC clones expressing constitutively active Mek to mimic the effect of activating the MAPK pathway.

The expression of constitutively active Mek* was confirmed upon withdrawal of tetracycline from 24h to 4 days. Furthermore, activation of the MAPK pathway was observed by induced expression of Mek*, in that Erk phosphorylation was enhanced. LIF-induced activation of Stat3 appeared normal when Mek* was expressed.

Constitutive activation of the MAPK pathway was not sufficient to induce a significant reduction in mESC self-renewal. A trend towards decreased alkaline phosphatase positive colonies was observed in the absence of tetracycline, when Mek* is expressed. Additionally, culture in the presence of excessive concentrations of LIF was not masking the effects of enhanced MAPK pathway activity. At sub-optimal LIF concentrations, colonies expressing Mek* exhibited a small, non-significant, but consistent reduction in alkaline phosphatase staining.

The media used for alkaline phosphatase assays supports the growth of both differentiated and undifferentiated cells when LIF is present. Culture in the absence of LIF or with low concentrations of LIF induced a reduction in alkaline phosphatase staining. Even in the presence of low concentrations of LIF, only a small decrease in self-renewal was observed when Mek* was expressed compared to cells expressing endogenous Mek alone. Taken together these results suggest that activation of the MAPK pathway can be overcome by compensatory mechanisms. Consistent with this notion, culture in routine mESC culture medium, optimised for self-renewal and undifferentiated growth of mESCs but supplemented with serum that allows the differentiation of mESCs, overcame these small decreases in alkaline phosphatase staining observed in the absence of tetracycline using typical alkaline phosphatase assay media. This indicates that factors present in routine culture medium can

overcome the small functional effects induced by enhanced MAPK pathway activity, in order to maintain self-renewal. The culture of Mek* mESC clones in chemically defined media may reveal more about the effect of factors present in culture media and the mechanisms involved in maintaining mESC self-renewal when the activity of the MAPK pathway is enhanced.

In conclusion, artificially activating the MAPK pathway leads to a minimal decrease in self-renewal. The effect of constitutive activation of the MAPK pathway might be overcome by compensatory mechanisms, possibly activated by factors present in serum or serum-replacement.

## 3.7 Summary and discussion: Inhibition of PI3K does not regulate lineage commitment

The present study has investigated the differentiation of mESCs in 2D monolayer culture when PI3Ks are inhibited. Inhibition of PI3Ks with LY294002 led to the differentiation of mESCs to cells types expressing a panel of markers representing all lineages. Previously the differentiation of mESCs in the form of 3D embryoid bodies had been described (Bone et al., 2007). Embryoid bodies have considerable morphological and gene expression pattern similarities to early embryos and therefore provide a useful 3D developmental model of early embryogenesis (Keller, 1995, Desbaillets et al., 2000). Treatment with the broad selectivity PI3K inhibitor LY294002 did not affect the number of embryoid bodies formed (Bone et al., 2007). However, embryoid bodies formed in the presence of LY294002 were found to be smaller compared to untreated embryoid bodies. A similar effect on embryoid body formation was observed in PDK-1 knockout mESCs (Bone et al., 2007). In terms of PI3K regulating differentiation towards a particular lineage, no difference was observed in the temporal expression of a number of early lineage marker genes in embryoid bodies formed from wild-type mESCs in the presence of LY294002 or in embryoid bodies formed from PDK-1 null mESCs. Quantitative expression analysis revealed a small enhancement in haematopoietic marker gene expression in embryoid bodies formed in the presence of LY294002, correlating to enhanced production of haematopoietic cell colonies. PDK-1 deficient cells were reported to form smaller colonies and fewer haematopoietic colony types, leading to the conclusion that PI3K was required for the proliferation of cells in order to form haematopoietic-type colonies as opposed to a restriction on differentiation (Bone et al., 2007). In contrast, the present study has demonstrated that differentiation of mESCs in 2D monolayer culture in the presence of LY294002 leads to a similar pattern of lineage marker expression to that observed in the absence of LIF.

Increased expression of GATA1 was observed on day 5 both in the absence of LIF and in the presence of LY294002, which is reminiscent of the up-regulation of GATA1 expression on days 4 and 5 with increasing expression towards day 6, observed in previous reports (Fujimoto et al., 2001, Robertson et al., 2000). In the presence of LY294002 and LIF, GATA6 expression was lower on days 3 and 4 compared to the presence of LIF alone, but comparable on days 5-7. This pattern suggests that the time-course involved in differentiation towards endodermal lineages is altered but not prevented by PI3K inhibition. Heterozygote GATA6/*lacZ* mouse embryos were used to demonstrate a double wave pattern of GATA6 expression during mouse embryo

development (Koutsourakis et al., 1999). The first wave of GATA6 expression was detected in blastocyst stage embryos (E3.5), specifically in the ICM and in the trophectoderm lining of the blastocoel cavity. The second wave of expression was detected at E7.0-E9.5 in parietal endoderm (derived from primitive endoderm, Figure 1.7), embryonic endoderm that forms the gut and embryonic mesoderm that forms the heart (Koutsourakis et al., 1999). If this same expression pattern is observed in mESC differentiation *in vitro*, cells entered into the protocol, used in the present study, on day 0 could be considered to represent E3.5. Therefore, cells differentiating in the presence of LIF appear to undergo this second wave of expression at day 6-7 correlating to E8.5-E9.5, consistent with Koutsourakis et al.,. Additionally, endoderm formation in the early stages of embryoid body differentiation was demonstrated to require GATA6 expression downstream of FGF signalling (Li et al., 2004). The altered expression pattern of GATA6 in differentiating mESCs in the presence of LY294002 observed here might be explained by the inhibition of PI3Ks preventing FGF signalling. Reduced FGF signalling may lead to a delay in the first wave of expression explaining the low expression of GATA6 on days 3 and 4 (Figure 3.13). Further investigation, covering more time-points, would be required to ascertain the normal expression pattern of GATA6 in differentiating mESCs in order to confirm a delay in expression when PI3K is inhibited and to investigate if this expression pattern was FGF-dependent.

Expression of Pax6 was detected in mESCs grown on feeder cells and in feeder-free culture conditions (Wiles and Johansson, 1999, Gordeeva et al., 2003). However, Pax6 expression was elevated in embryoid bodies compared to levels detected in mESCs (Gordeeva et al., 2003). Thus the expression of Pax6 in self-renewing mESCs after 3 days in the presence of LIF, as well as expression in differentiated cells after a number of days culture in the absence of LIF, or in the presence of LIF and LY294002, observed in the present study is consistent with previously reported expression patterns. Furthermore, the high levels of Pax6 expression might be explained by the positive autoregulation of Pax6 expression by Pax6 (Pinson et al., 2006).

In the ESC field, neural differentiation has often been predicted to be the default mode of differentiation of mESCs in the absence of cues directing differentiation towards other lineages or suppressing differentiation. It was reported that, in chemically defined serum, mESCs freely formed neural precursor cells in the absence of LIF, regardless of the presence or absence of N2 or B27 factors (Ying et al., 2003b). However, neural differentiation in chemically defined medium was reported to be dependent on endogenous FGF-induced signalling (Bouhon et al., 2005). FGF-induced PI3K/PKB signalling is dispensable for neural induction in mESC differentiation (Stavridis et al.,

2007). Therefore, inhibition of FGF signalling by PI3K inhibition with LY294002 does not explain reduced Pax6 expression observed in the present study.

Nodal expression, an indication of embryonic ectodermal and primitive endodermal lineages, was observed to be consistently reduced in the presence of LY294002 on day 3, which may also reflect a delay in expression as a result of PI3K inhibition. Nodal expression was reported to be detectable in embryoid bodies from day 2 onwards (Leahy et al., 1999), consistent with expression observed from day 3 onwards in the present study. Furthermore, FGF5, a marker of primitive ectoderm, was demonstrated to have a similar expression pattern to that observed for Nodal in differentiating mESCs, with low or undetectable expression in the presence of LY294002 on day 3. This provides further evidence for a short delay in differentiation towards ectodermal lineages when PI3K is inhibited. Consistent with the present findings, FGF5 was reported to have lower expression following 24, 48 and 72 hours incubation in the presence of LY294002 compared to the absence of LY294002 (Storm et al., 2009). Despite this apparent delay in expression, LY294002 treatment did not prevent or greatly enhance ectoderm marker expression compared to the presence or absence of LIF.

A consistent elevation in FGF5 expression was observed in the absence of LIF on days 4 and 5. Consistent with this, FGF5 expression is reported to be detectable in day 5 embryoid bodies but not detectable at day 10 and not detectable in self-renewing ESCs (other time points were not published) (Oka et al., 2002). However, FGF5 was detectable at very low levels in mESCs investigated in a separate study, nevertheless expression increased in embryoid bodies formed from aggregates of these cells (Cartwright et al., 2005). Moreover, FGF5 was detected from day 1 to day 10, with a peak in expression between days 2-5, in embryoid bodies formed by LIF withdrawal and culture in differentiation medium (Leahy et al., 1999). In a separate study, expression of FGF5 increased upon formation of embryoid bodies with a peak in expression on day 3 (Cartwright et al., 2005), slightly earlier than demonstrated in the present study.

In summary, mesodermal and endodermal differentiation, assessed with GATA1 and GATA6 respectively, was similar in cells incubated in the absence of LIF and cells treated with LY294002. However, inhibition of PI3K signalling appeared to cause a change in the expression of the ectodermal markers nodal and FGF5. Furthermore, an altered temporal expression pattern was observed for the neuroectoderm marker Pax6. Taken together, PI3K signalling does not appear to prevent or promote differentiation towards a specific lineage but does alter the temporal expression pattern of some lineage-associated markers.

# 4 Results: The role of Class I PI3K catalytic subunit isoforms in mESC self-renewal

## 4.1 Introduction and Aims

PI3Ks have been implicated in the regulation of both ESC proliferation and self-renewal (discussed in detail in Section 1.2.6). Indeed, class $I_A$ PI3Ks were specifically implicated in maintaining mESC self-renewal (Paling et al., 2004) and interestingly, this class of PI3Ks are known to be expressed from the one cell stage of mammalian embryo development (Riley et al., 2005, Riley et al., 2006).

One of the aims of this study was to investigate whether individual class $I_A$ PI3K catalytic subunit isoforms are responsible for regulating distinct aspects of mESC fate. In particular, regulation of self-renewal by PI3K catalytic subunit isoforms was of interest. Recently, a number of cell-permeable, small molecule inhibitors have been characterised regarding their selectivity for the class I PI3K family (the structures of these compounds are detailed in Table 2.3). Using a range of small molecule isoform specific inhibitors, complemented with gene silencing of individual isoforms, the roles of class $I_A$ PI3K catalytic subunit isoforms in regulating mESC self-renewal was assessed.

It has previously been reported that treatment of mESCs with the broad selectivity PI3K inhibitor LY294002, known to inhibit class I PI3Ks (Vlahos et al., 1994), reduces their capacity to self-renew (Paling et al, 2004). Recently, a new class I PI3K inhibitor, ZSTK474, has been described, which has fewer reported off-target effects on PI3K-related kinases (Kong and Yamori, 2007, Yaguchi et al., 2006) (for ZSTK474 structure see Table 2.3). The effect of this inhibitor was compared to the effects observed with LY294002 and results are presented in this chapter.

## 4.2 ZSTK474 induces a loss of mESC self-renewal

ZSTK474 is an s-triazine derivative that exhibits inhibition of tumour cell proliferation and is described as being more potent than wortmannin or LY294002 as an anti-tumour compound (Yaguchi et al., 2006). *Ex vivo*, ZSTK474 is reported to inhibit class $I_A$ PI3Ks in the nM range (Yaguchi et al., 2006) and to inhibit all isoforms of this class with similar selectivity (Kong and Yamori, 2007). It was the selectivity for class I PI3K isoforms, the class previously implicated in regulating mESC self-renewal (Paling et al., 2004), that warranted an investigation into the effect of treatment of mESCs with ZSTK474. Any affect of ZSTK474 on mESC self-renewal markers, such as alkaline phosphatase staining and Nanog expression, in comparison to the effects observed using LY294002 were of particular interest.

Self-renewing mESCs were treated for 4 days with vehicle, LY294002 or ZSTK474 and self-renewal assessed by alkaline phosphatase staining using a clonal assay. Treatment with vehicle, DMSO, did not alter the level of alkaline phosphatase staining when compared to untreated cells (Figure 4.1**A**). Treatment with the broad selectivity PI3K inhibitor LY294002 induced a significant reduction in the percentage of alkaline phosphatase positive, self-renewing mESC colonies, consistent with previous reports (Paling et al., 2004). Following four days incubation in the presence of ZSTK474, concentrations of 1µM or greater induced a significant decrease in the proportion of alkaline phosphatase positive colonies, indicating a loss of self-renewal. Considering the inhibition of class I PI3Ks by both ZSTK474 and LY294002, this similar reduction in the percentage of alkaline phosphatase positive colonies was predicted (Figure 4.1**A**).

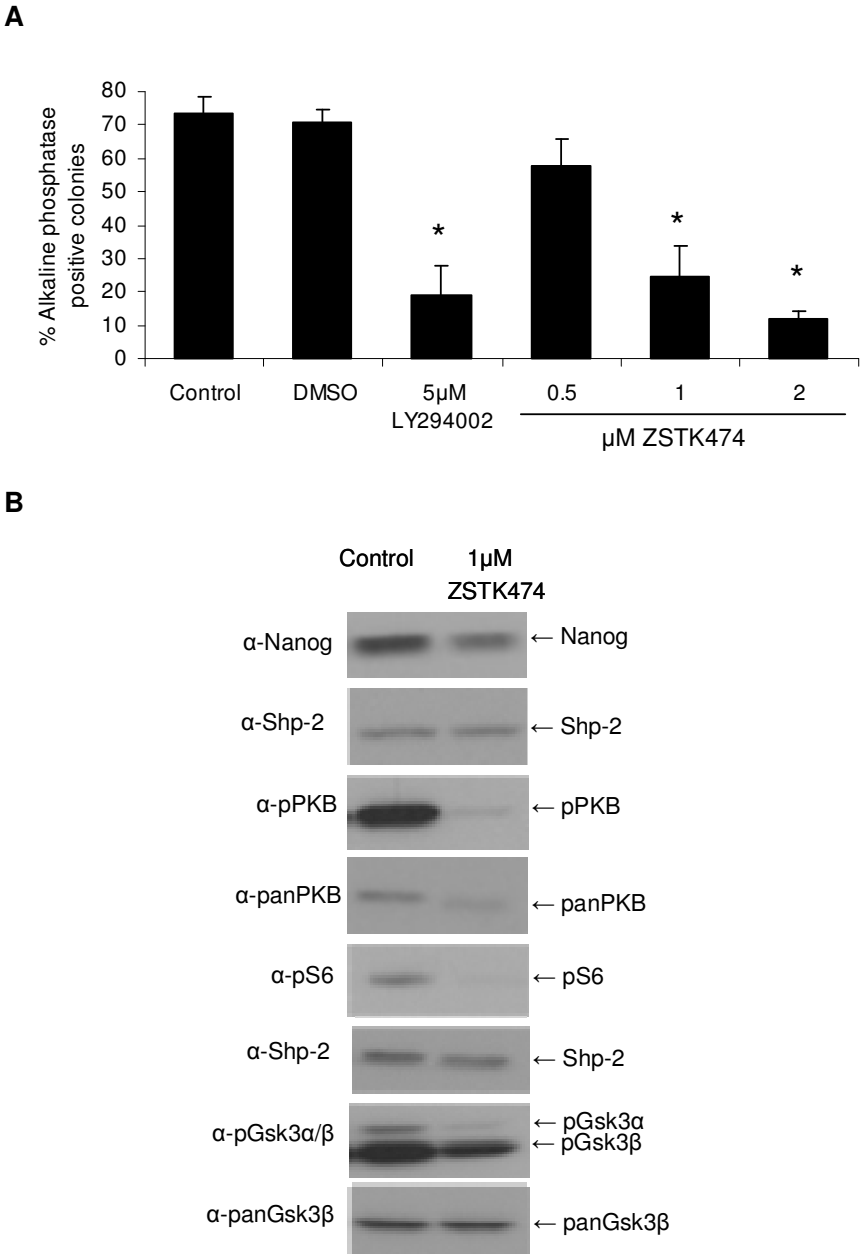



**Figure 4.1 ZSTK474 induces a loss of mESC self-renewal**

After 4 days incubation with the inhibitors indicated, colonies were stained for alkaline phosphatase activity (n=3) (**A**) or harvested for analysis of protein expression with the antibodies indicated (representative of two independent experiments) (**B**). Alkaline phosphatase positive colonies are represented as a percentage of all colonies for each treatment. Data were analysed by ANOVA and Dunnet's post hoc test, where * indicates $p<0.05$. Antibodies detecting Nanog, phosphorylated PKB (Ser473), S6 (Ser235/236) and Gsk-3 (Ser21/9) were used initially. Immunoblots were stripped and reprobed with antibodies detecting PKB, Shp-2 and Gsk-3 to assess equal loading.

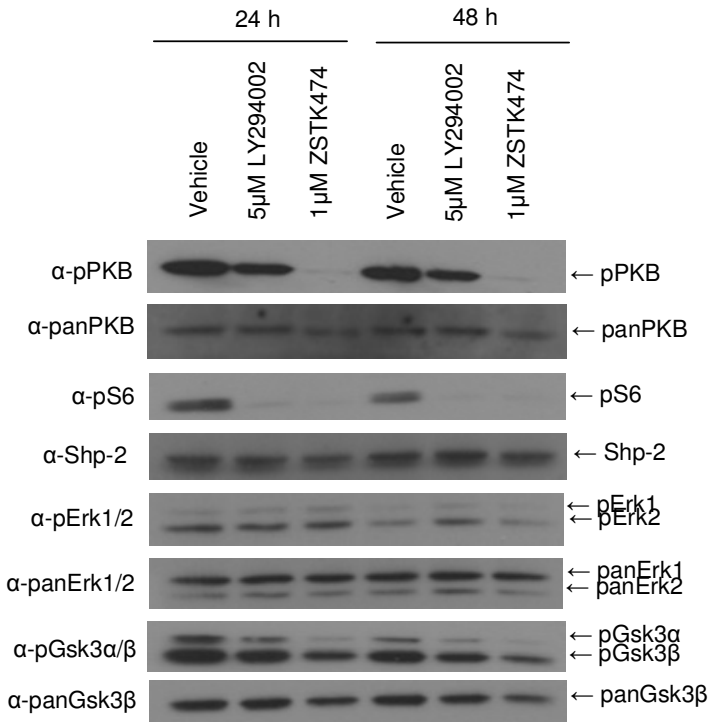
In order to assess the expression of the self-renewal marker Nanog, mESCs were treated for 4 days with ZSTK474, and then harvested for protein analysis by immunoblotting. Expression of Nanog protein was reduced in cells treated with ZSTK474 compared to levels observed in control cells after 4 days incubation, consistent with decreased alkaline phosphatase staining and a loss of self-renewal (Figure 4.1**B**). Furthermore, the activity of ZSTK474 was maintained for up to 4 days of incubation, judged by assessment of the phosphorylation states of signalling molecules downstream of PI3K. Phosphorylation of PKB, at Ser473, and S6, at Ser235/236, were both reduced in cells treated with ZSTK474 (Figure 4.1**B**). Typically, Gsk-3 activity is inhibited by PI3K-induced PKB activity (Cross et al., 1995), which involves phosphorylation at Ser21 and Ser9 of Gsk-3α and Gsk-3β respectively. Treatment of mESCs with ZSTK474 induced a reduction in Gsk-3 phosphorylation at these sites (Figure 4.1**B**), indicating reduced negative regulation of Gsk-3 and consistent with the observed reduction in PKB phosphorylation at sites important for PKB activity (Balendran et al., 1999, Williams et al., 2000).

As observed in the previous experiment, treatment with DMSO does not significantly alter the level of alkaline phosphatase staining when compared to untreated colonies (Figure 4.1**A**). Figures depicting alkaline phosphatase assay data from here on in will only show vehicle (DMSO) treatment, since DMSO is a suitable control.

Signalling downstream of PI3K was also investigated after 24 and 48 hours incubation in the presence of ZSTK474 or LY294002. Both inhibitors induced a decrease in levels of phosphorylated PKB at Ser473 but this was more pronounced in cells treated with ZSTK474 (Figure 4.2**A**). This may reflect the more potent inhibition of growth of tumour cells by ZSTK474 (Yaguchi et al., 2006) since growth and insulin signalling are often mediated via PI3K/PKB signalling. Consistent with this, a similar pattern of Gsk-3 phosphorylation at Ser9/21 was observed to that of PKB when LY294002 and ZSTK474 were compared. Unlike PKB and Gsk-3, the decrease observed in S6 phosphorylation was similar for both inhibitors at both time points (Figure 4.2**A**). At 24 hours, no alterations in the phosphorylation of Erk1 or Erk2 at Thr202/Tyr204 were observed with either inhibitor. At 48 hours a small enhancement in Erk2 phosphorylation was evident for LY294002 but not for ZSTK474. Enhancements in Erk phosphorylation by LY294002 are best observed following a period of serum starvation, whereas this experiment was designed to assess the effects on signalling during typical culture and incubation of mESCs.

A notable feature of decreased Nanog expression induced by LY294002 is the time-course for which Nanog protein expression is decreased. Nanog expression was shown to be decreased as early as 8 hours after application of LY294002 to mESCs, with a significant decrease reported at 24 and 48 hours (Storm et al., 2007). In the present study, small decreases in Nanog protein expression were observed at 24 and 48 hours for both LY294002 and ZSTK474 (Figure 4.2**B**) consistent with the early down-regulation of protein expression upon PI3K inhibition. During differentiation of mESCs, Oct4 protein expression typically takes longer to decrease than Nanog. Indeed Oct4 expression has been reported to be detected in differentiating mESCs up to 5 days after withdrawal from LIF (Sauter et al., 2005) and Oct4 protein and RNA levels were reported to not change significantly following PI3K inhibition with LY294002 (Storm et al., 2007). Consistent with these reports, treatment of mESCs with either LY294002 or ZSTK474 did not alter Oct4 expression at 24 or 48 hours (Figure 4.2**B**), although later time points were not investigated.

**A**




**B**

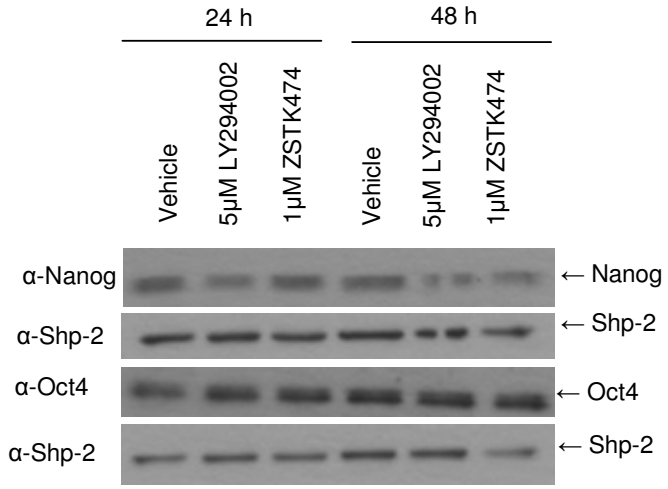



**Figure 4.2 Changes in signalling downstream of PI3Ks are similar following treatment with LY294002 or ZSTK474**

Murine ESCs were incubated for 24 or 48 hours with vehicle (DMSO), LY294002 or ZSTK474 and harvested for protein analysis by SDS-PAGE and immunoblotting. Antibodies detecting phosphorylated PKB (Ser473), S6 (Ser235/236), Erk1/2 (Thr202/Tyr204) and Gsk-3 (Ser21/9) were used to assess signalling (**A**) and antibodies detecting the self-renewal markers Nanog and Oct4 were used to assess self-renewal (**B**). Immunoblots were stripped and reprobed with the antibodies indicated to confirm equal loading. The data shown are representative of three independent experiments.

In addition to assessing the loss of self-renewal induced by ZSTK474 in comparison with LY294002, the effects of ZSTK474 on LIF-induced PI3K signalling were also compared to the effects seen using LY294002. In the absence of PI3K inhibitors, LIF stimulation induced an enhancement in PKB and S6 phosphorylation, at Ser473 and Ser235/236 respectively, and an increase in Erk1 phosphorylation (Figure 4.3). Inhibition of PI3Ks with LY294002 or ZSTK474, prior to LIF stimulation, resulted in reduced basal PKB and S6 phosphorylation. A decrease in LIF-induced PKB and S6 phosphorylation resulted following pre-treatment with LY294002. However, ZSTK474 pre-treatment resulted in a considerable inhibition of LIF-induced PKB and S6 phosphorylation. These differences in PKB and S6 phosphorylation observed with LY294002 and ZSTK474 upon LIF stimulation are similar to those observed when mESCs are incubated in the presence of these inhibitors alone (i.e. no prior serum starvation and no specific stimulation, as shown in Figure 4.2). A small enhancement in Erk2 phosphorylation was observed in LIF stimulated cells following LY294002 treatment. This enhancement in Erk phosphorylation with LY294002 is consistent with that demonstrated in mESCs cultured in chemically defined media using LY294002 and stimulation with LIF or BMP4 (Figure 3.2) and with previous reports (Paling et al., 2004). In contrast, ZSTK474 did not result in the enhancement of basal or LIF-stimulated Erk phosphorylation.

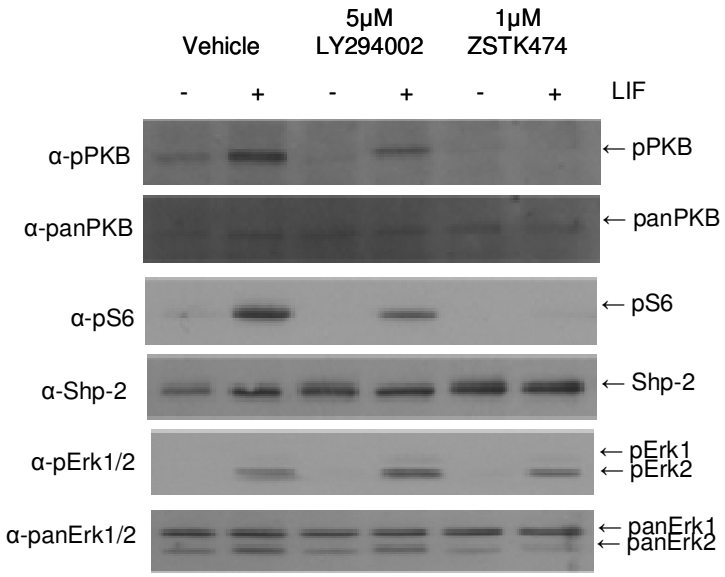



**Figure 4.3 Inhibition of PI3Ks with ZSTK474 prevents LIF-induced signalling downstream of PI3Ks**

Murine ESCs were serum and LIF starved for 4 hours and pre-treated with vehicle (DMSO), LY294002 or ZSTK474 for 30 minutes prior to stimulation. Stimulations were conducted with $10^3$ U/ml LIF for 10 minutes. Protein lysates were separated by SDS-PAGE and proteins detected by immunoblotting using the antibodies detecting phosphorylated PKB (Ser473), S6 (Ser235/236) and Erk1/2 (Thr202/Tyr204) as indicated. Immunoblots were stripped and reprobed with antibodies detecting PKB, Shp-2 and Erk to assess equal loading. The data shown are representative of two independent experiments.

## 4.3 PI3K isoform specific inhibitors are active in BaF/3 cells

Recently, small molecule inhibitors that display specificity over particular PI3K catalytic subunit isoforms have been synthesised and characterised. These selective inhibitors are useful tools, in comparison to the broad selectivity PI3K inhibitors, for dissecting the physiological processes that individual isoforms are involved in. BaF/3 cells, an immortalised murine bone marrow derived pro-B cell line with interleukin-3 (IL-3)-dependent proliferation (Palacios and Steinmetz, 1985), were used to assess the effect of a panel of selective and broad spectrum PI3K inhibitors on mouse cells known to express all class $I_A$ PI3K isoforms (Vanhaesebroeck et al., 1997).

In unstimulated BaF/3 cells, low basal levels of PKB phosphorylated at Ser473 were enhanced by stimulation with IL-3. The broad spectrum PI3K inhibitors, LY294002, wortmannin and ZSTK474, all inhibited IL-3-induced PKB phosphorylation (Figure 4.4**A**). BaF/3 cells are known to express all class I catalytic isoforms (Vanhaesebroeck et al., 1997). Accordingly, inhibitors with selectivity for p110α, p110β, p110δ (Figure 4.4**B**) and p110γ (Figure 4.4**A**) resulted in reduced IL-3-induced PKB phosphorylation compared to vehicle treated cells.

Selective inhibition of PI3K isoforms did not alter basal, unstimulated levels of PKB phosphorylation at Ser473 compared to vehicle pre-treatment alone (Figure 4.4**B**). Interestingly, the broad spectrum PI3K inhibitors induced a small enhancement in basal PKB phosphorylation (Figure 4.4**A**). In *Drosophila* and mammalian cell types, evidence exists demonstrating that the rictor-mTOR complex is capable of phosphorylating PKB at Ser473 (Radimerski et al., 2002, Sarbassov et al., 2005). Furthermore, knockdown of S6K activity has been shown to enhance rictor-mTOR-dependent PKB phosphorylation at this site (Harrington et al., 2004, Sarbassov et al., 2005). Thus, enhanced PKB phosphorylation, observed in the present study, might be the result of inhibition of PI3Ks and reduced downstream activation of S6K, leading to enhanced PKB phosphorylation via rictor/mTOR. Further investigation would be required to explore this possibility.

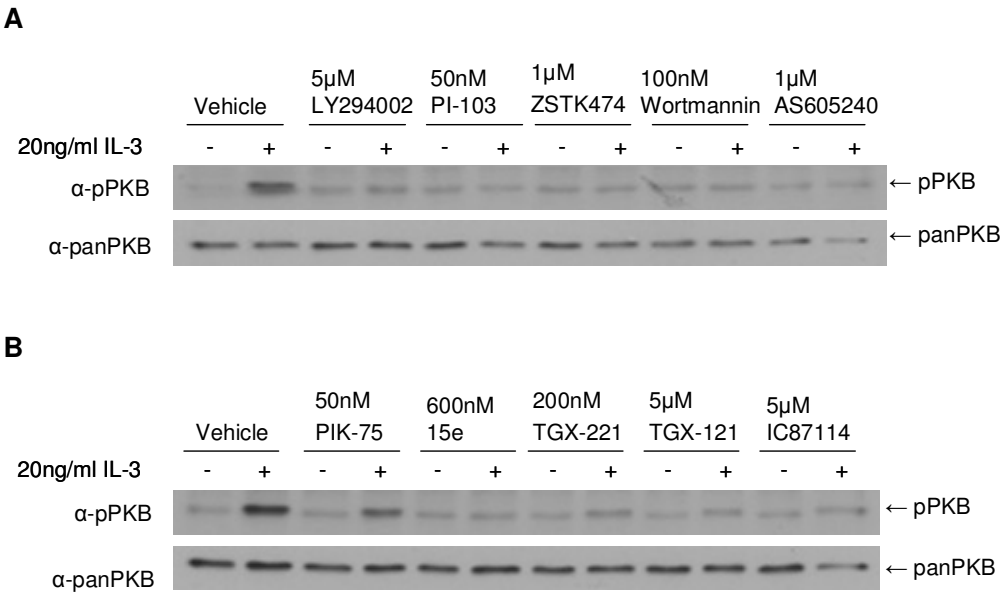



**Figure 4.4 Broad range and isoform selective inhibitors are active in BaF/3 cells**
BaF/3 cells were washed and resuspended in serum-free media in the absence of IL-3. Cells were pre-treated for 30 minutes with each of a panel of inhibitors. **A** PI3K broad spectrum inhibitors (LY294002, Wortmannin and ZSTK474), dual p110α and mTOR inhibitor (PI-103) and PI3K class $I_B$ p110γ selective inhibitor (AS605340). **B** PI3K class $I_A$ catalytic subunit isoform inhibitors selective for p110α (PIK-75 and compound 15e), p110β (TGX-221 and TGX-121) and p110δ (IC87114). Following pre-treatment with inhibitors, BaF/3 cells were stimulated with 20ng/ml IL-3 for 10 minutes. Cell lysates were resolved by SDS-PAGE and signalling downstream of PI3Ks assessed by immunoblotting using antibodies detecting PKB protein phosphorylated at Ser473. Blots were stripped and reprobed with antibodies detecting PKB to assess equal loading. The data shown are representative of three independent experiments.

Having ascertained that these inhibitors were active and could efficiently inhibit signalling downstream of PI3Ks, they were used to assess mESC fate governed by PI3K catalytic isoforms.

## 4.4 Investigating which p110 isoforms contribute to self-renewal

### *4.4.1 The role of p110β in mESC fate*

The role of the PI3K catalytic subunit isoform p110β in mESCs was of particular interest since mouse embryos homozygous for a partial deletion of *Pik3cb*, the gene encoding the p110β subunit, exhibit early embryonic lethality at E3.5 and ESC lines cannot be derived from these blastocysts (Bi et al., 2001). Of note here is that isolation of mESCs is achieved from wild-type blastocysts at E3.5 (Evans and Kaufman, 1981) the same time point at which embryonic lethality was reported in mice deficient in functional p110β (Bi et al., 2001). Both pharmacological inhibition and gene silencing techniques were used to determine if this isoform is involved in mESC self-renewal.

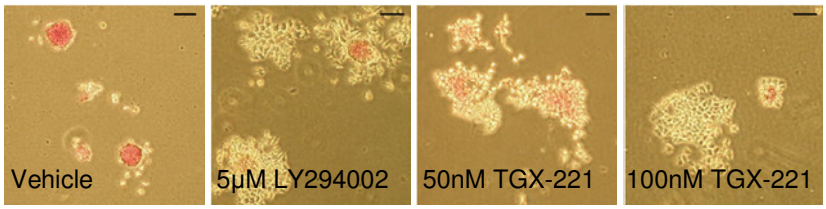
#### *4.4.1.1 Pharmacological inhibition of p110β leads to a loss of self-renewal*

In order to address the contribution of p110β to the maintenance of self-renewal, two isoform selective inhibitors of p110β, TGX-221 and TGX-121, the synthesis of which have been previously described (Jackson et al., 2004, Robertson et al., 2001), were used. TGX-221 is the more potent of these two p110β inhibitors and has been used effectively to assess p110β signalling and cellular function (Jackson et al., 2005).

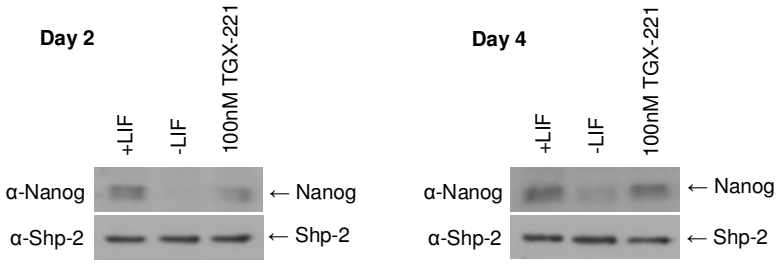
Murine ESCs incubated in the presence of vehicle for 4 days formed rounded colonies with smooth defined edges which stained strongly for alkaline phosphatase activity (Figure 4.5**A**). Treatment with LY294002 resulted in flattened colonies with rough borders which had weak staining or did not stain for alkaline phosphatase. This effect of broad spectrum inhibition of PI3Ks resulting in a loss of the self-renewal marker alkaline phosphatase is consistent with previous reports (Paling et al., 2004, Storm et al., 2007). Treatment of mESCs with the p110β selective inhibitor TGX-221 induced colonies to adopt a more differentiated morphology similar to colonies incubated with LY294002 (Figure 4.5**A**). Incubation in the absence of LIF resulted in a decrease in Nanog expression compared to the presence of LIF, on both day 2 and day 4, indicating a loss of self-renewal (Figure 4.5**B**). Murine ESCs incubated in the presence of TGX-221 and LIF expressed slightly lower levels of Nanog protein compared to cells incubated in the presence of LIF alone on day 2 (Figure 4.5**B**). When colonies were scored for alkaline phosphatase staining and results represented graphically, treatment with LY294002 typically reduced the percentage of alkaline phosphatase stained colonies to approximately 30% of all colonies present on a tissue culture dish, consistent with a

decrease in self-renewal. A significant reduction in alkaline phosphatase staining, to a level comparable with that attained in the presence of LY294002, was observed in the presence of 50nM TGX-221 (Figure 4.5**C**). Typically TGX-221 is used at 100nM, treatment of mESCs with 100nM or 1μM TGX-221 did not further reduce alkaline phosphatase staining compared to 50nM TGX-221 (Figure 4.5**D**). The less potent p110β selective inhibitor, TGX-121, also decreased alkaline phosphatase staining with a significant reduction in staining observed at concentrations greater than 5μM (Figure 4.5**E**).

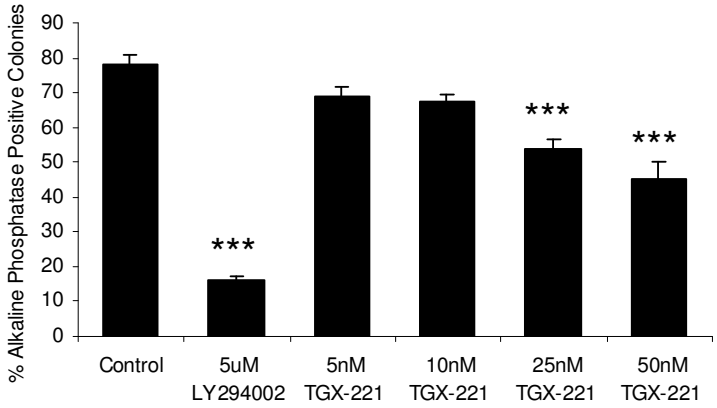
**A**



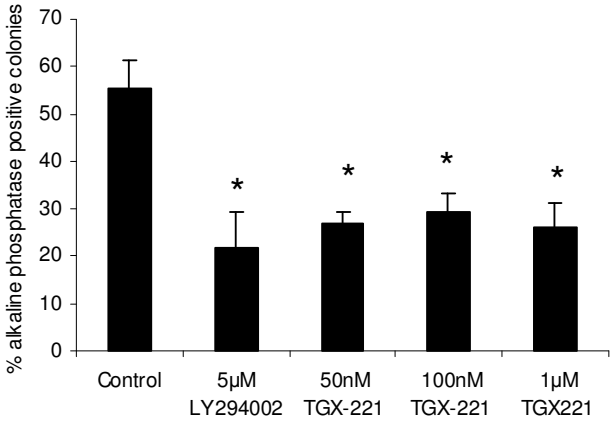

**B**




**C**



**D**




**E**

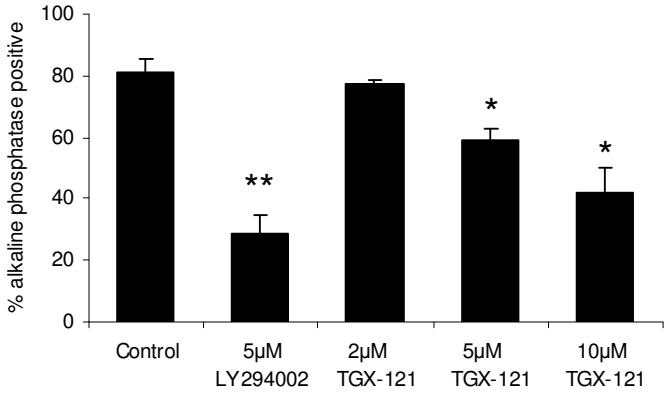
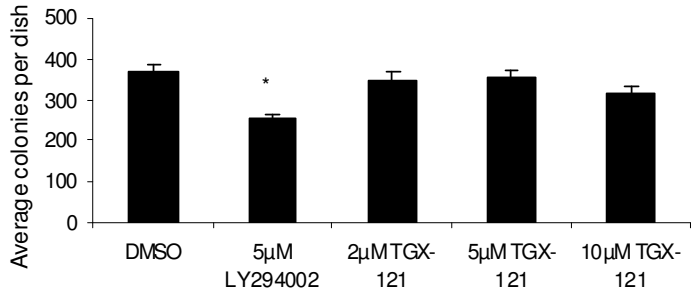



**Figure 4.5 Pharmacological inhibition of p110β leads to a decrease in self-renewal of mESCs**

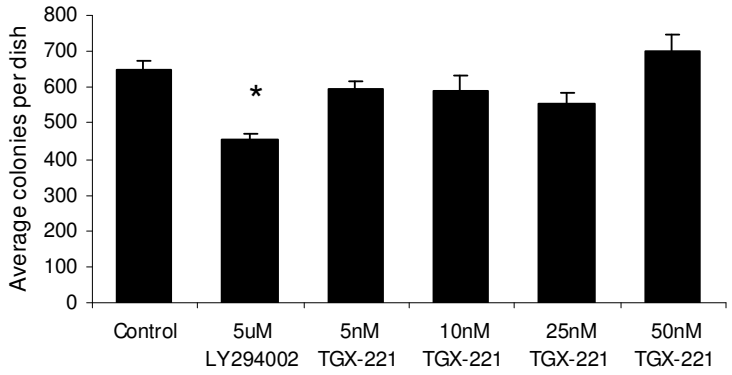
Murine ESCs were treated for 4 days with LY294002, TGX-221 or TGX-121 at the doses shown or with vehicle alone (DMSO). Self-renewal was assessed by staining for alkaline phosphatase activity. **A** Representative colonies are shown following treatment with LY294002 or TGX-221, scale bars=100μm. **B** Following incubation for 2 or 4 days in the presence or absence of LIF, or in the presence of LIF and TGX-221, mESCs were harvested and protein lysates analysed for the expression of Nanog by immunoblotting. Immunoblots were stripped and reprobed for Shp-2 to confirm equal loading. The average percentage of alkaline phosphatase positive, self-renewing colonies in each condition are shown with S.E.M. for TGX-221 treatment (n=3) **C** and (n=4) **D** and TGX-121 treatment (n=4) **E**. For each, ANOVA with Dunnet's post-hoc test was applied, where * indicates $p<0.05$ and ** indicates $p<0.01$.

The density at which mESCs are cultured is important for maintaining optimal self-renewal and for survival. It has previously been reported that mESCs display a dependency on autocrine signalling, thus fewer colonies may affect their ability to maintain self-renewal (Guo et al., 2006, Singla et al., 2008, Welham et al 2007). In order to verify that inhibition of p110β did not alter the number of colonies which may indirectly alter self-renewal, the total number of colonies per tissue culture dish for each treatment is represented graphically (Figure 4.6). Statistical analysis revealed that after 4 days incubation with neither TGX-121 (Figure 4.6**A**), nor treatment with TGX-221 (Figure 4.6**B** and **C**), gave a significant difference in the number of colonies per dish when compared with the number of colonies on vehicle treated dishes. Only LY294002 caused a decrease in the number of colonies when compared to vehicle treated colony numbers (Figure 4.6). This was only significant in panels **A** and **B**, but not panel **C** and might be explained by the inhibition of p110α by LY294002 (see Sections 4.4.5.3 and Figure 4.25) (Kingham and Welham, In Press).

**A**




**B**




**C**

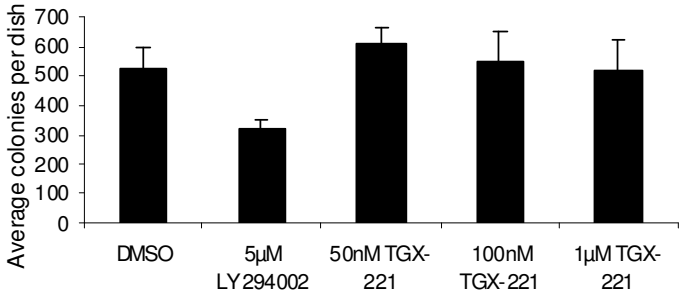



**Figure 4.6 Inhibition of p110β does not alter colony number**

The data obtained for Figure 4.5 is represented as the average number of colonies per dish shown with S.E.M. for TGX-121 (n=4) (**A**), TGX-221 (n=3) (**B**) and TGX-221 (n=4) (**C**). Statistical analysis using ANOVA and Dunnet's post hoc test were used to determine which treatments caused a significant change in the number of colonies compared to the number of colonies counted on vehicle (DMSO) treated control dishes.

The use of these two, structurally similar p110β isoform inhibitors has provided evidence which indicates that p110β activity is required in order to maintain optimal self-renewal.

#### *4.4.1.2 siRNA-mediated knockdown of p110β expression leads to a loss of self-renewal*

To further characterise mESC self-renewal regulation by p110β, and to support findings obtained by pharmacological inhibition of this isoform, short-interfering RNAs (siRNAs) were used to target *Pik3cb*, the gene encoding p110β.

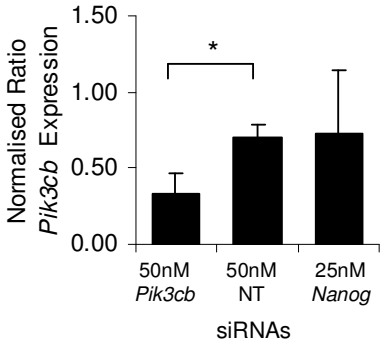
Targeting of *Pik3cb*, was conducted using two different commercially available siRNA products. Smartpool siRNA, supplied by Dharmacon, is a pool of four single siRNAs whereas Silencer Select siRNAs, supplied by Ambion, are individual siRNAs, two of which were used independently to knockdown the expression of p110β. Care was taken when choosing siRNA products so that in each case, siRNAs targeting different exons or exon boundaries were selected. The regions of *Pik3cb* targeted by Dharmacon Smartpool siRNAs and Ambion Silencer Select siRNAs are depicted in Figure 2.6. Off-target effects of using siRNAs include the binding of siRNA to mRNA with incomplete complementarity and subsequent silencing of non-target genes, which provides false data. Selecting siRNAs that have independent target regions within *Pik3cb* allowed such data to be more easily identified. In addition, the appropriate controls of non-targeting Dharmacon siRNA or negative control Ambion siRNA were selected and allowed siRNA toxicity effects, those not associated specifically with *Pik3cb* silencing, to be taken into account. The use of a pooled siRNA product, such as Dharmacon Smartpool siRNA, ensures efficient knock-down of the target gene. As a positive control, siRNAs targeting Nanog, the homeodomain transcription factor and key regulator of self-renewal (Chambers et al., 2003, Mitsui et al., 2003), were used.

##### *4.4.1.2.1 siRNA-mediated knockdown of p110β using Dharmacon Smartpool siRNA commercially available products*

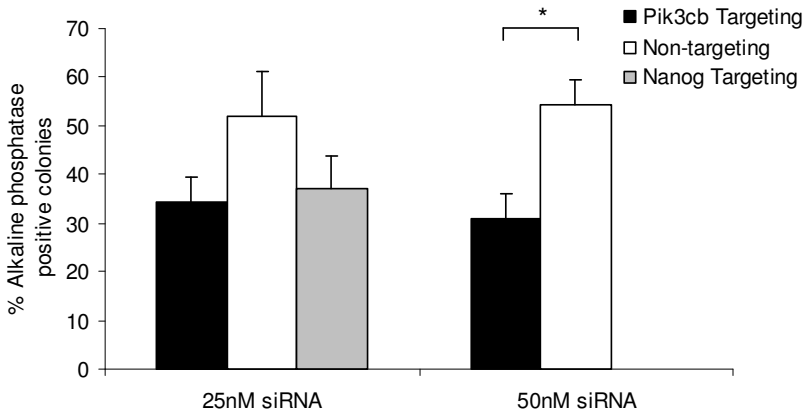
Using Dharmacon Smartpool siRNAs, which contain 4 specific gene targeting siRNAs, the effect of knockdown of *Pik3cb*, the gene encoding p110β, on self-renewal was assessed. Nanog targeting siRNA was used as a positive control. Quantitative PCR (QPCR) revealed a significant knock-down of *Pik3cb* RNA when using Dharmacon Smartpool *Pik3cb* targeting siRNAs (Figure 4.7**A**). Typically a 50-60% decrease in expression was achieved. No change in expression of *Pik3cb* was detected using *Nanog* siRNAs. On assessment of self-renewal, by alkaline phosphatase assay, knockdown of *Pik3cb* led to a reduction of approximately 50% in the proportion of alkaline phosphatase positive colonies (Figure 4.7**B**). A significant decrease in alkaline

phosphatase staining was observed following transfection with 50nM *Pik3cb* targeting siRNA. Transfection with *Nanog* targeting siRNAs decreased alkaline phosphatase staining, consistent with the requirement for Nanog protein expression to maintain self-renewal (Mitsui et al., 2003). Furthermore, a significant reduction in the proportion of pure self-renewing alkaline phosphatase colonies was observed with either *Nanog* or *Pik3cb* targeting siRNAs compared to non-targeting siRNAs (Figure 4.7**C**). Importantly, levels of self-renewal following siRNA-mediated knockdown of *Pik3cb* were comparable to pharmacological inhibition of p110β with TGX-221 (Figure 4.5) and with siRNA-mediated knockdown of *Nanog* (Figure 4.7**B**). Transfection with *Pik3cb* targeting siRNA did not significantly change the total number of colonies when compared to non-targeting control colony numbers (Figure 4.7**D**). Interestingly transfection with *Nanog* targeting siRNA induced a significant increase in the number of colonies per dish.

**A**




**B**



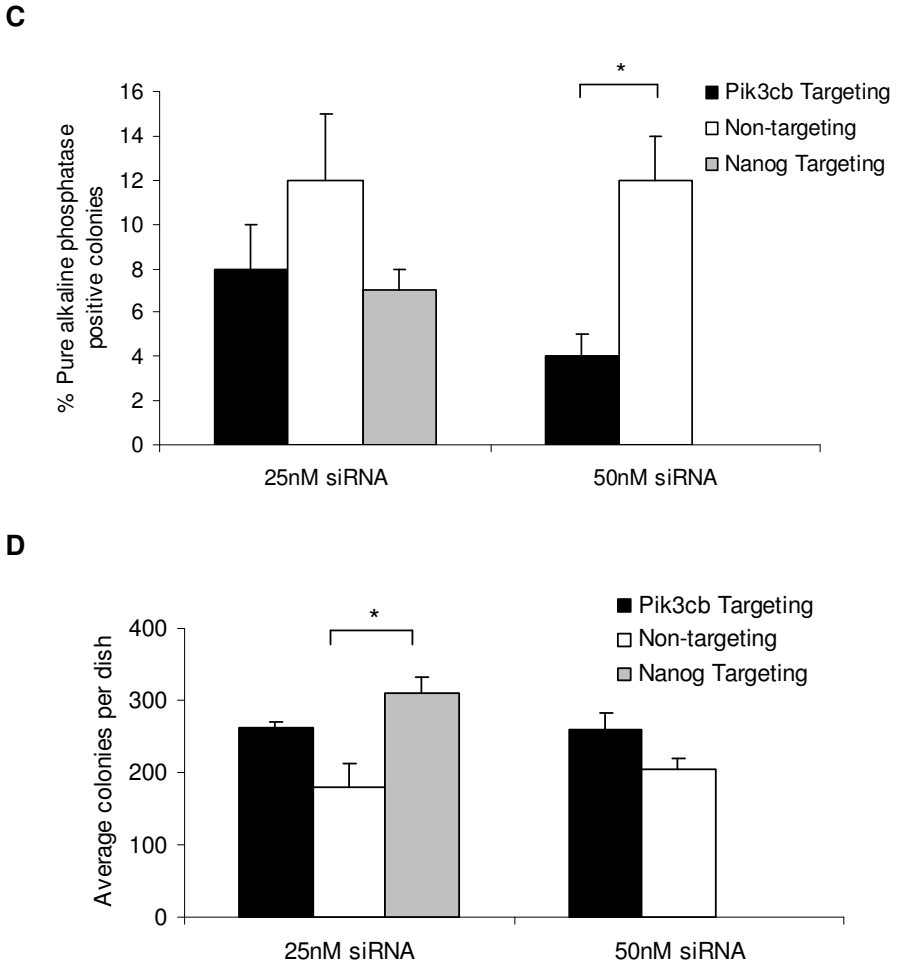



**Figure 4.7 Expression of p110β is required to maintain self-renewal**

Murine ESCs were transfected with Dharmacon *Pik3cb* or *Nanog* targeting siRNAs or non-targeting (NT) siRNAs. **A** QPCR was conducted in quadruplicate using samples of cDNA obtained by reverse transcribing RNA isolated from transfected cells. QPCR was used to measure knockdown of *Pik3cb* RNA, which was normalised relative to levels of *β-actin* RNA. A representative experiment of 4 independent experiments is shown with standard deviations. **B** Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline phosphatase staining. The average percentage of alkaline phosphatase positive, self-renewing colonies in each condition is shown with S.E.M (n=4). **C** Assessment of self-renewal by alkaline phosphatase staining is depicted by the percentage of pure alkaline phosphatase positive colonies (colonies that appear compact, round and stain bright red) of all colonies shown with S.E.M. (n=4). **D** From the self-renewal assay data, the total number of colonies per dish are shown with S.E.M. Statistical analysis was conducted using a Student's t-test to compare targeting siRNA data to non-targeting siRNA data, where * indicates $p<0.05$ and ** indicates $p<0.01$.

In addition to alkaline phosphatase staining, expression of Nanog protein and RNA is typically reduced following the induction of differentiation (Mitsui et al., 2003, Storm et al., 2007). As expected, a significant decrease in *Nanog* expression was observed following siRNA-mediated knockdown of *Nanog*, when assessed by QPCR (Figure 4.8**A**). A decrease in *Nanog* expression was also detected following transfection with *Pik3cb* targeting siRNAs, consistent with a decrease in alkaline phosphatase staining and indicating a loss of self-renewal. Semi-quantitative assessment of self-renewal markers, *Nanog* and *Rex1* was also conducted (Figure 4.8**B**). Knockdown of *Pik3cb* expression resulted in a modest decrease in *Nanog* expression and a very small decrease in *Rex1* expression. Knockdown of *Nanog* expression did not decrease *Pik3cb* expression and did not appear to alter *Rex1* expression. Consistent with a decrease in *Nanog* RNA, Nanog protein levels were reduced by knockdown of either *Nanog* or *Pik3cb* genes (Figure 4.8**C**). Levels of Oct4 protein expression appeared unchanged with 25nM *Pik3cb* siRNA when compared to 25nM non-targeting siRNA, however, a small decrease in Oct4 expression was observed with 50nM *Pik3cb* siRNA when compared to 50nM non-targeting siRNA.

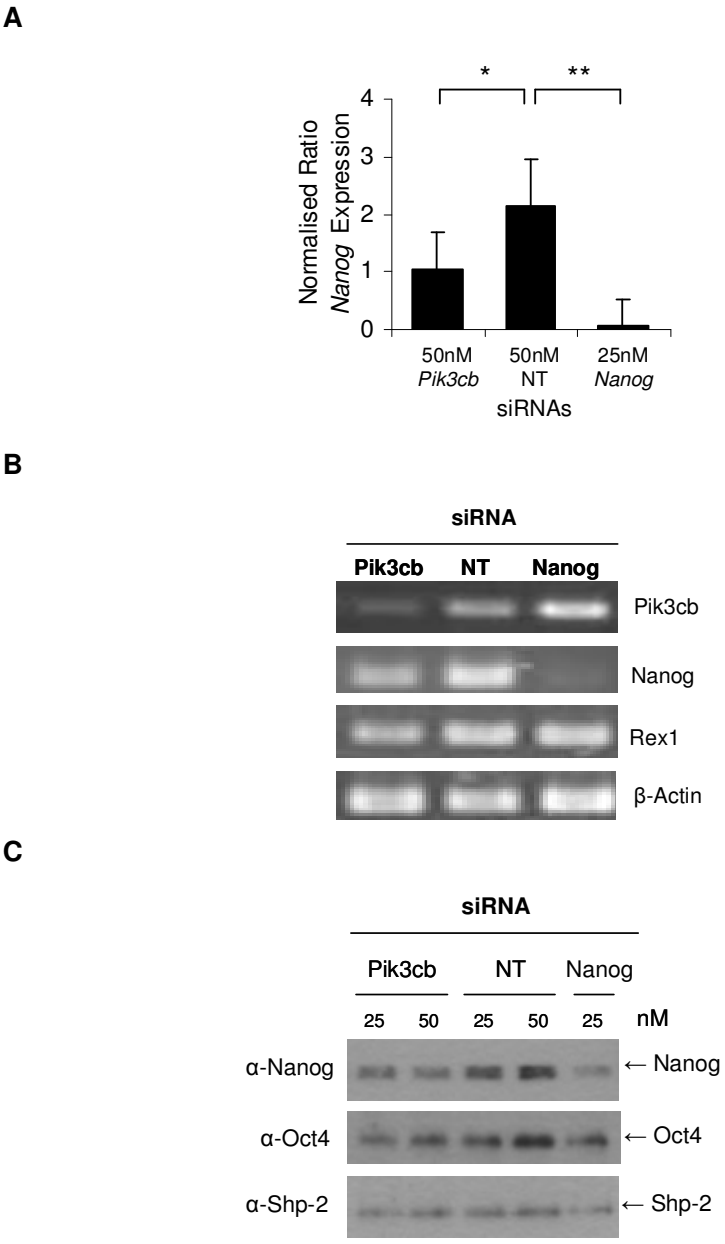



**Figure 4.8 Nanog expression is reduced following *Pik3cb* gene silencing**

**A** Following transfection with *Pik3cb*, *Nanog* or non-targeting (NT) siRNAs, QPCR was conducted on quadruplicate samples to measure knockdown of *Nanog* RNA. *Nanog* RNA levels were normalised relative to levels of *β-actin* RNA. The normalised ratio of *Nanog* expression is shown with standard deviations. A Student's t-test was conducted to compare gene targeting samples to non-targeting samples, where * indicates $p<0.05$ and ** indicates $p<0.01$. **B** Semi-quantitative PCR was conducted using primers detecting *Pik3cb*, *Nanog*, *Rex1* and *β-actin*. **C** Protein lysates were made from siRNA transfected cells and immunoblotting conducted using antibodies detecting Nanog and Oct4. Antibodies detecting Shp-2 were used to assess equal loading. In each case, the data shown are representative of four independent experiments.

#### *4.4.1.2.2 siRNA-mediated knockdown of p110β using Ambion Silencer Select siRNA commercially available products*

Two Ambion Silencer Select siRNAs, termed *Pik3cb* s93107 and *Pik3cb* s93108, were used independently to knockdown *Pik3cb* by targeting different regions of the gene to those targeted by Dharmacon Smartpool siRNAs (see Figure 2.6 for siRNA targeting regions of *Pik3cb*).

Using Ambion Silencer Select siRNAs to target *Pik3cb* resulted in a 40-60% knockdown of *Pik3cb* RNA expression (Figure 4.9**A**). Knockdown of *Pik3cb* significantly reduced the proportion of alkaline phosphatase positive colonies compared to negative control siRNA transfection (Figure 4.9**B**). The average number of colonies per dish was not significantly different for cells transfected with *Pik3cb* targeting siRNA compared to transfection with negative control siRNA (Figure 4.9**C**). Consistent with Dharmacon smartpool siRNA products, Ambion Silencer Select resulted in a similar level of *Pik3cb* knockdown, which in turn induced a reduction in alkaline phosphatase staining but did not affect colony number.

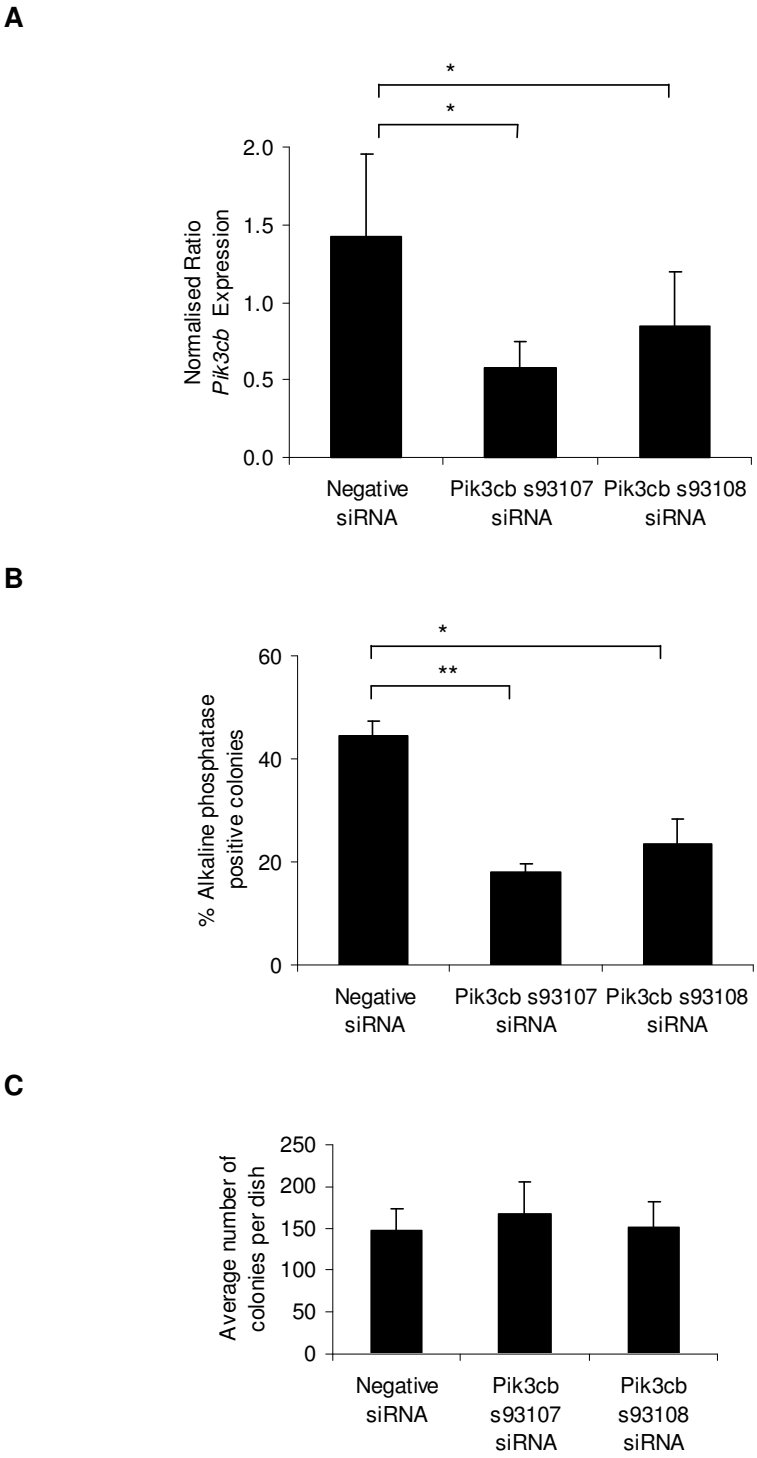
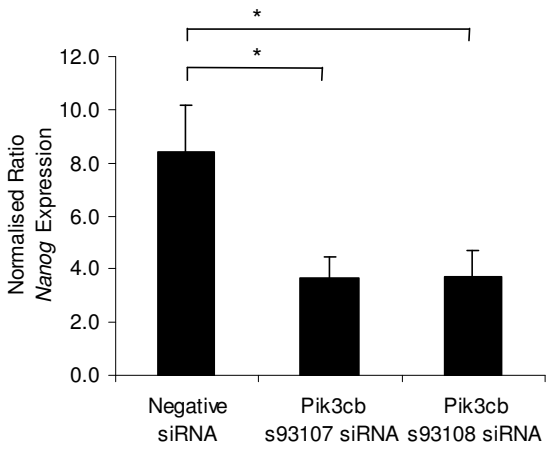



**Figure 4.9 siRNA-mediated knockdown of *Pik3cb* results in reduced self-renewal**
Murine ESCs were transfected with 10nM Ambion Silencer Select *Pik3cb* targeting siRNAs (s93107 or s93108) or with 10nM negative control Silencer Select siRNAs. **A** Following transfection, cells were plated for 4 days then RNA isolated and reverse transcribed. QPCR was conducted to detect knockdown of *Pik3cb* RNA normalised relative to levels of *β-actin* RNA. Representative data with standard deviations for

quadruplicate QPCR samples are shown. **B** Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline phosphatase staining. The average percentage of alkaline phosphatase positive colonies are shown (n=4), with S.E.M. **C** From the alkaline phosphatase data obtained, the average number of colonies per dish was plotted for each treatment with S.E.M. Statistical analysis was conducted using a Student's t-test to compare data for *Pik3cb* targeting siRNA compared to data for negative control siRNA where * indicates $p<0.05$ and ** indicates $p<0.01$. No significant difference was found in the number of colonies per dish for *Pik3cb* s093107 ($p=0.677$) or *Pik3cb* s093108 ($p=0.921$) siRNA treatment compared to Negative siRNA treatment.

Nanog RNA expression was reduced in cells transfected with either Pik3cb s93107 or Pik3cb s93108 compared to cells transfected with negative control siRNA (Figure 4.10A). On assessment of Nanog protein, targeting Pik3cb reduced Nanog protein expression when compared to Nanog protein in cells transfected with negative control siRNA (Figure 4.10**B**). Only modest decreases in Oct4 protein levels were observed following *Pik3cb* knockdown with *Pik3cb* s93107 or *Pik3cb* s93108 compared to negative control siRNA. Decreases in Nanog and Oct4 protein, along with a reduction in alkaline phosphatase staining are all consistent with a decrease in self-renewal of cells following knockdown of *Pik3cb*.

**A**




**B**

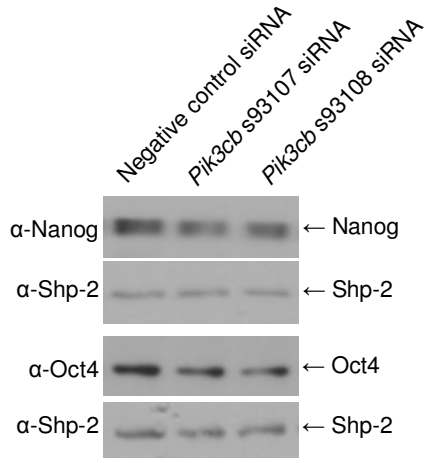



**Figure 4.10 Knockdown of *Pik3cb* RNA results in decreased Nanog RNA and protein expression**

Following transfection with 10nM *Pik3cb* targeting siRNAs (s93107 or s93108) or with negative control Silencer select siRNA, cells were plated for 4 days then harvested for RNA and protein expression analysis. **A** QPCR was conducted to detect *Nanog* RNA expression. *Nanog* RNA levels were normalised relative to levels of *β-actin* RNA. Representative data with standard deviations for quadruplicate QPCR samples are shown. A Student's t-test was conducted where * indicates $p<0.05$. **B** Immunoblotting was conducted using antibodies detecting Nanog and Oct4. Equal loading was assessed by stripping and reprobing immunoblots using antibodies detecting Shp-2.

In some instances, altering the expression of p110 isoforms has been shown to alter the expression of the regulatory subunit p85 or the expression of other p110 isoforms (Bi et al., 1999, Clayton et al., 2002, Fruman et al., 1999, Jou et al., 2002). In order to address this, p85 protein expression was assessed by immunoblotting and the expression of *Pik3ca* and *Pik3cd*, the genes encoding the catalytic subunit isoforms p110α and p110δ respectively, were investigated by QPCR. A combination of a low protein yield following siRNA-mediated gene silencing of mESCs and the low specificity and poor sensitivity of the antibodies available that detect p110α and p110δ meant that analysis of protein levels by immunoblotting could not be achieved for these isoforms. Following knockdown of *Pik3cb* by Dharmacon Smartpool siRNAs (Figure 4.11**A**) or by Ambion Silencer Select siRNAs (Figure 4.11**B**), p85 protein expression remained unchanged. Following knockdown of *Pik3cb* by Dharmacon Smartpool siRNAs, no significant or consistent change in the expression of either *Pik3ca* (Figure 4.11**C**) or *Pik3cd* (Figure 4.11**D**) were detected. Consistent with the data obtained using Dharmacon siRNAs, no significant changes in the expression of *Pik3ca* (Figure 4.11**E**) or *Pik3cd* (Figure 4.11**F**) were found following Ambion Silencer Select siRNA-mediated knockdown of *Pik3cb*.

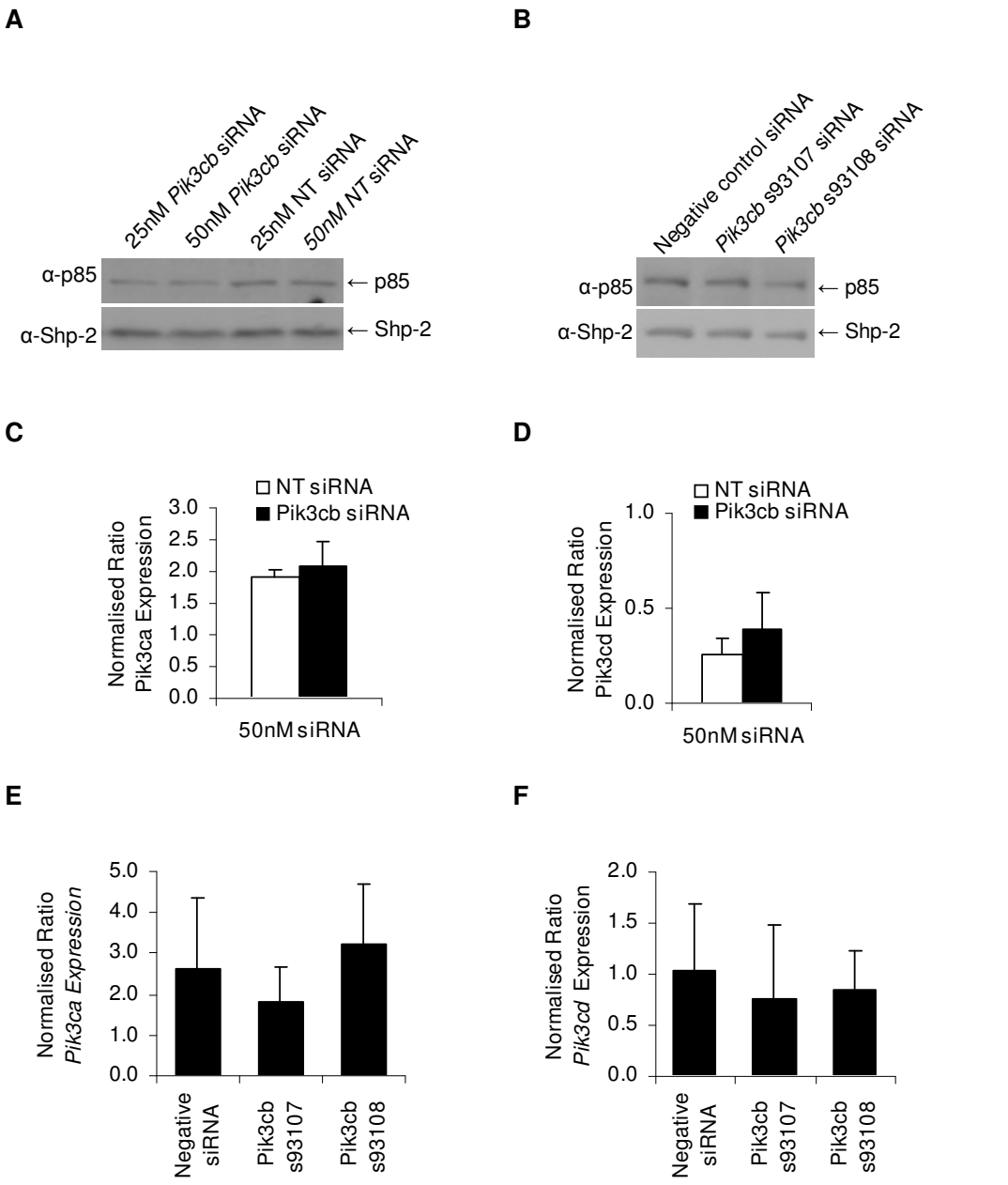



**Figure 4.11 Expression of other PI3K isoforms following p110β knockdown**

Immunoblotting was conducted using antibodies detecting p85 to assess protein expression following transfection with Dharmcon (**A**) or Ambion (**B**) siRNAs. Immunoblots were stripped and reprobed using antibodies detecting Shp-2 in order to assess equal loading. Samples were assessed in quadruplicate by QPCR for the expression of *Pik3ca* (**C**) and *Pik3cd* (**D**) following transfection with Dharmacon siRNA and expression of *Pik3ca* (**E**) and *Pik3cd* (**F**) following transfection with Ambion siRNA, normalised to relative levels of *β-actin*. All graphs show standard deviation and are representative of all experimental repeats (n=4).

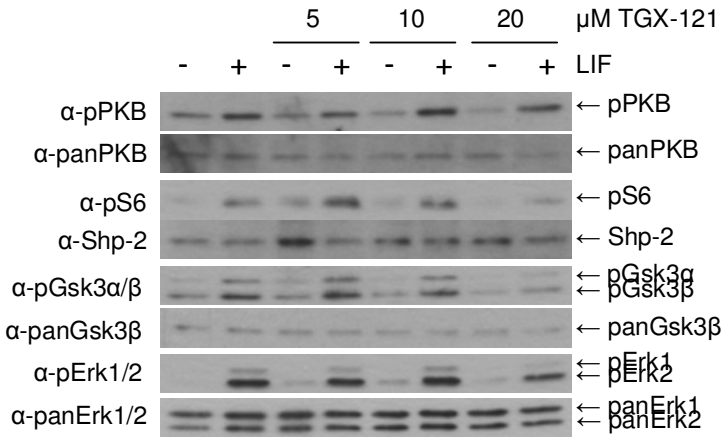
### *4.4.2 Addressing the signalling mediated by p110β in mESCs*

Signalling mediated by p110β following LIF stimulation was investigated using the p110β selective inhibitors, TGX-121 and TGX-221 (Figure 4.12). Basal and LIF-stimulated levels of PKB phosphorylation at Ser473, Gsk-3α/β phosphorylation at Ser20/9 and S6 phosphorylation at Ser235/236 were all unaffected by 5 or 10μM TGX-121 and 100, 200 or 1000nM TGX-221 (Figure 4.12). However, at the highest concentration of TGX-121 used, 20μM, a small decrease in LIF-induced PKB, S6 and Gsk-3 phosphorylation was observed. The selectivity of 20μM TGX-121 for targeting p110β alone could be questionable.

LIF-stimulated phosphorylation of Erk1 and Erk2 were also unaffected by pre-treatment with these p110β inhibitors although, basal Erk2 phosphorylation was modestly enhanced in unstimulated cells following incubation with either TGX-121 or TGX-221. This observation was noteworthy since an enhancement in Erk phosphorylation has been observed with LY294002 in mESCs (Paling et al., 2004) and was also observed in mESCs cultured and treated with LY294002 in chemically defined media (Figure 3.2). It appears that this phenomenon of inhibition of MAPK pathway activity by PI3K might be mediated by the p110β isoform since selective inhibition of p110β by either TGX-121, or the more potent TGX-221, enhanced basal Erk phosphorylation (Figure 4.12).

No alteration LIF-stimulated signalling was observed following treatment with typical doses of two p110β inhibitors despite the activity and expression of p110β being required for mESCs to maintain self-renewal (Figures 4.5, 4.7 and 4.9). This observation was surprising since LIF is the major extrinsic ligand known to promote mESCs self-renewal (Bard and Ross, 1991, Gough et al., 1998, Smith et al., 1988, Williams et al., 1988).

**A**

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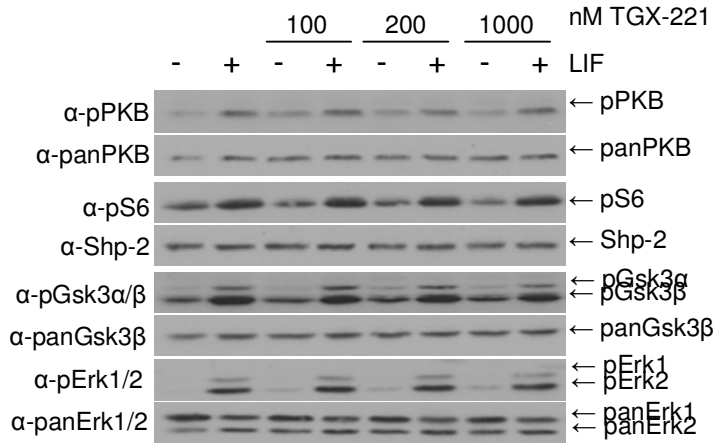



**Figure 4.12 LIF-induced signalling is not mediated by p110β**

Murine ESCs were treated with TGX-121 (**A**) or TGX-221 (**B**) for 30min prior to stimulation with $10^3$ U/ml LIF for 10min. Cell lysates were resolved by SDS-PAGE and immunoblotting carried out using the antibodies indicated to detect signalling downstream of PI3Ks. Equal loading was assessed by stripping and reprobing immunoblots with appropriate antibodies as indicated. The data shown are representative of three independent experiments.

#### *4.4.2.1 Investigating the activation of p110β by GPCR agonists in mESCs*

The activation of p110β by G proteins, the intracellular mediators of G protein coupled receptors (GPCRs), has been reported in a number of cell types (discussed in Section 1.1.2.1) (Ciraolo et al., 2008, Guillermet-Guibert et al., 2008, Maier et al., 1999, Murga et al., 2000, Roche et al., 1998). GPCR agonists are present in the serum and serum-replacement products used for the routine culture of mESCs. To investigate the possibility of these agonists activating p110β in mESCs, stimulation experiments were conducted using GPCR agonists following pre-treatment of mESCs with vehicle or p110β isoform inhibitors, TGX-121 and TGX-221. Serum contains a cocktail of ligands that can potentially activate a wide variety of receptors. However, boiling serum destroys many of these ligands with the exception of heat stable lysophospholipids, which typically activate GPCRs (personal communication Bart Vanhaesebroeck).

Knock-out Serum Replacement (KO SR) is used for the routine culture of mESCs and is optimised by the manufacturer for the culture of self-renewing mESCs. However, the components of this product are proprietary to the manufacturer, Invitrogen. Stimulation of mESCs with 10% (v/v) KO SR in GMEM would be expected to stimulate a variety of signalling pathways. Indeed 10% (v/v) KO SR in GMEM induced the phosphorylation of PKB at Ser473, Gsk3 at Ser20/9 and Erk1/2 at Thr202/Tyr204 (Figure 4.13). When boiled to denature heat unstable components, boiled 10% (v/v) KO SR in GMEM induced the phosphorylation of PKB and Gsk3 when compared to phosphorylated levels detected following stimulation with boiled GMEM alone. In addition, a small enhancement in Erk1/2 phosphorylation was also observed following stimulation with boiled 10% (v/v) KO SR in GMEM. In the presence of TGX-121 or TGX-221, no change in the phosphorylation states of PKB, Gsk3 or Erk1/2 was observed following stimulation with 10% (v/v) KO SR in GMEM. Interestingly, boiled 10% KO SR (v/v) in GMEM-induced stimulation of PKB phosphorylation was reduced following pre-treatment with either TGX-121 or TGX-221 (Figure 4.13). Additionally TGX-221 pre-treatment reduced the phosphorylation of Gsk3α/β and Erk1/2 induced by stimulation with boiled 10% (v/v) KO SR in GMEM. The results presented in Figure 4.13 indicate that components of KO SR, that are not denatured by boiling, stimulate signalling downstream of PI3Ks and that this is mediated, at least in part, by the p110β catalytic subunit isoform of PI3Ks. The activation of PKB following stimulation of GPCRs is consistent with a previous study which investigated the mechanism involved and demonstrated that activation of PKB in this manner occurred via interaction of p85 SH2 domains with tyrosine phosphorylated proteins and also required p85 interaction with p110β (Kubo et al., 2005).

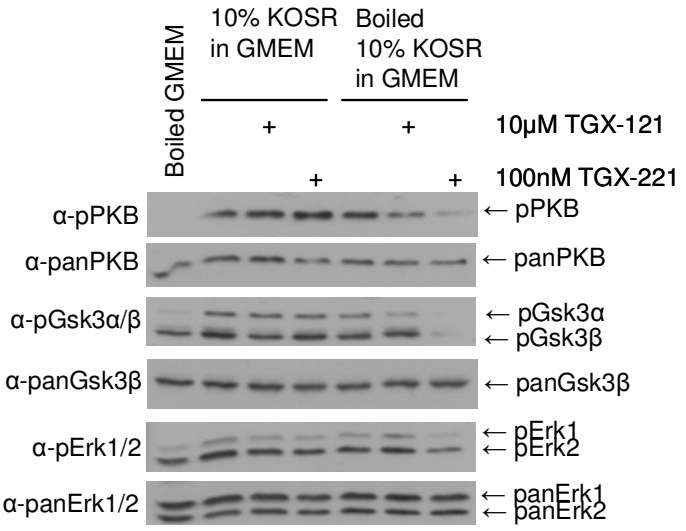



**Figure 4.13 Stimulation of GPCRs activated PKB via p110β in mESCs**

Murine ESCs were serum and LIF starved for 4 hours and pre-treated for 30 minutes with TGX-121 or TGX-221 where indicated. Stimulations were conducted for 5 minutes with boiled GMEM, 10% (v/v) Knock-out Serum Replacement (KO SR) in GMEM or boiled 10% (v/v) KO SR in GMEM. Boiling of GMEM and 10% (v/v) KO SR in GMEM was conducted for 5 minutes and the media was allowed to cool to room temperature prior to applying to mESCs. Protein lysates were analysed by immunoblotting using the antibodies indicated. Immunoblots were stripped and reprobed using appropriate antibodies, as indicated, to assess equal loading. The data presented here are representative of two independent experiments.

Two agonists in particular, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), are reported to activate PKB via p110β (Guillermet-Guibert et al., 2008, Roche et al., 1998, Yart et al., 2002). LPA and S1P receptors typically couple to heterotrimeric G proteins, including members of the $G_i$, $G_o$, $G_s$, $G_{12/13}$ and $G_q$ families of G proteins. Furthermore, LPA and S1P-induced signalling has been linked to cellular functions including cell migration, proliferation, cell-cell contacts and implantation (Meyer zu Heringdorf and Jakobs, 2007). Interestingly, S1P was reported to aid the maintenance of self renewal of human ESCs (hESCs) in the presence of mitomycin-treated mouse embryonic fibroblasts (MEFs) (feeder layer for the culture of undifferentiated hESCs) and platelet-derived growth factor (PDGF) (Pébay et al., 2005). The expression of G-protein-coupled lysophospholipid receptors in ESCs has not been fully investigated but reports do exist demonstrating the expression of LPA receptor $LPA_5$ and S1P receptors $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ in mESCs, and $LPA_1$, $LPA_2$, $LPA_3$, $S1P_1$, $S1P_2$, and

$S1P_3$ receptors in hESCs (Inniss and Moore, 2006, Kleger et al., 2007, Lee et al., 2006, Pébay et al., 2005). Additionally information gained from the NCBI database (http://www.ncbi.nlm.nih.gov/unigene accessed 04.12.08) suggests that $LPA_1$, $S1P_2$ and $S1P_5$ are expressed at the blastocyst stage of mouse embryo development. Furthermore, microarray data (Storm *et al.,* 2009) from the mESC line used in the present study (E14tg2A, clone R63) indicates that $LPA_2$, $LPA_3$, $S1P_2$, $S1P_3$ and $S1P_5$ receptor expression is detectable in these cells (Storm and Welham, unpublished data). It was therefore, of interest to examine the signalling induced following stimulation of these receptors with their agonists LPA and S1P.

LPA and S1P agonists of the GPCRs were used to stimulate mESCs and signalling downstream of PI3Ks, specifically mediated via the p110β isoform, was investigated. Pertussis toxin, an exotoxin produced by the bacterium *Bordetella pertussis*, which catalyses ADP ribosylation of $G_\alpha$ subunit of heterotrimeric G proteins $G_i$, $G_o$, and $G_t$, preventing these G proteins from interaction with GPCRs (Burns, 1988) was also used to assess GPCR coupling in mESCs.

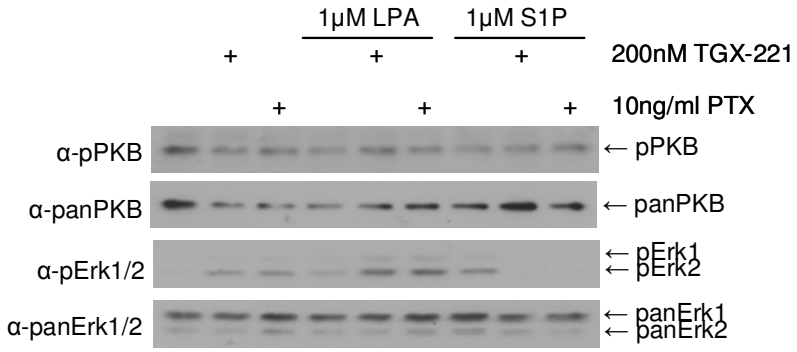



**Figure 4.14 Stimulation of mESCs with LPA and S1P**

Murine ESCs were serum and LIF starved for 4 hours and pre-treated for 30 minutes with TGX-221, or treated overnight with pertussis toxin (PTX). Stimulations were conducted for 5 minutes with lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) (n=1). Protein lysates were analysed by immunoblotting using antibodies detecting phosphorylated PKB (Ser473) and Erk (Thr202/Tyr204). Immunoblots were stripped and reprobed with antibodies detecting PKB and Erk to assess equal loading.

Stimulation of mESCs with LPA or S1P for 5 minutes failed to induce a change in the phosphorylation of PKB at Ser473 (Figure 4.14). However, GPCRs are known to be rapidly desensitised following stimulation, a process involving phosphorylation of activated receptors by GPCR kinases, subsequent arrestin binding and internalisation, which inhibits downstream signalling (Krupnick and Benovic, 1998, Zhang et al., 1997). Therefore, further investigation implementing shorter stimulation periods of mESCs with LPA or S1P might reveal GPCR-induced PKB phosphorylation. Levels of PKB phosphorylation were unaltered by the treatment of mESCs with pertussis toxin (Figure 4.14). Enhanced levels of basal Erk2 phosphorylation were observed following pre-treatment with TGX-221, as previously demonstrated (Figure 4.12), and also following pre-treatment with pertussis toxin (Figure 4.14). Stimulation with either LPA or S1P induced the phosphorylation of Erk2 when compared to basal, unstimulated levels (Figure 4.14). Pre-treatment with either TGX-221 or pertussis toxin appeared to further enhance LPA-induced Erk2 phosphorylation. Conversely, pre-treatment with either TGX-221 or pertussis toxin inhibited S1P-induced Erk2 phosphorylation.

The stimulation of Erk phosphorylation by LPA and S1P is consistent with the activation of ERK by $S1P_{1-4}$ and $LPA_{1-3}$ receptors (reviewed by Meyer zu Heringdorf and Jakobs 2007, Pébay et al., 2007). Furthermore, the use of pertussis toxin has implicated $G_i$, $G_o$ and $G_t$ G protein types, consistent with the coupling of LPA and S1P receptors to $G_i$ and $G_o$ G proteins (Meter zu Heringdorf and Jakobs, 2007, Pébay et al., 2005, Rosen and Goetzl, 2005). However, further investigation is required in order to determine if PI3K signalling is activated upon stimulation of mESCs with LPA or S1P.

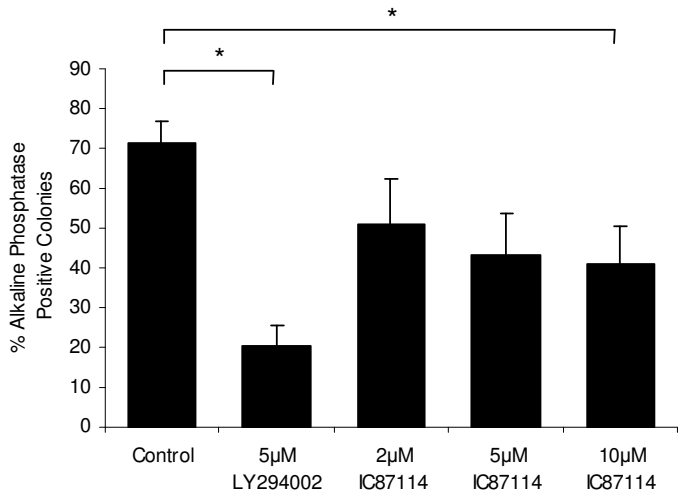
### *4.4.3 p110δ and the control of mESC fate*

In contrast to p110β, mice deficient in active p110δ are viable and fertile but are subject to impaired immune responses (Clayton et al., 2002, Jou et al., 2002). These reports are consistent with the expression of p110δ being largely restricted to cells of the immune system and a major role for p110δ in the lymphohaemopoietic system (Okkenhaug et al., 2002, Vanhaesebroeck et al., 1997). As with p110β, a role for p110δ in mESC self-renewal was investigated by both pharmacological inhibition and gene silencing techniques.

The isoform selective p110δ inhibitor, IC87114 (Sadhu et al., 2001) has been shown to inhibit p110δ in cells at doses of 5-10μM (Sadhu et al., 2003). To test the potential role of p110δ in mESCs, self-renewing cells were incubated with IC87114 for 4 days and the effects on self-renewal assessed by alkaline phosphatase assay.

Treatment of mESCs with 2 or 5μM IC87114 did not significantly reduce alkaline phosphatase staining (Figure 4.15**A**). However, treatment with 10μM IC87114 did induce a significant reduction in the percentage of alkaline phosphatase positive colonies when compared to control colonies. This decrease was not as great as that observed with the broad spectrum PI3K inhibitor LY294002 (Figure 4.15**A**) or that observed with the p110β inhibitors TGX-121 or TGX-221 (Figure 4.5**C**, **D** and **E**). Incubation of mESCs with IC87114 did not significantly alter the number of colonies on each dish compared to incubation with DMSO alone (Figure 4.15**B**).

**A**




**B**

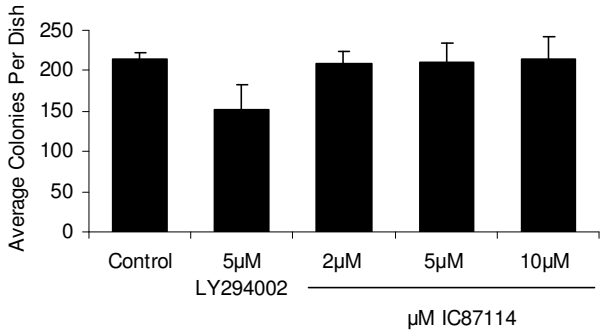
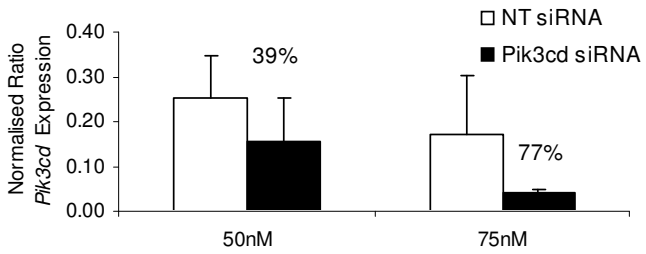



**Figure 4.15 Pharmacological inhibition of p110δ results in a modest decrease in alkaline phosphatase staining only at high doses**

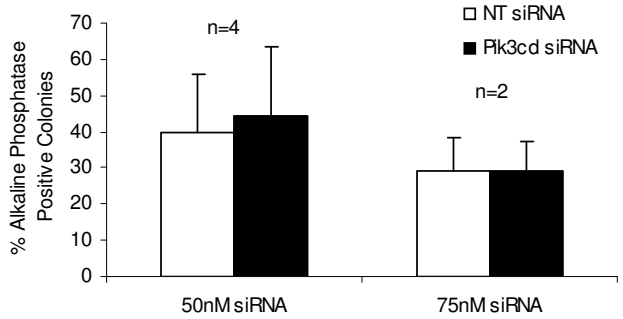
Murine ESCs were incubated with the p110δ inhibitor IC87114 or LY294002 for 4 days. Self-renewal was assessed by staining for alkaline phosphatase activity. Data are represented as the average percentage of alkaline phosphatase positive, self-renewing colonies in each condition (**A**) or as the average total number of colonies per dish for each treatment (**B**) both with S.E.M. (n=4). Data were analysed using ANOVA and, where significant, Dunnet's post hoc test was applied. * indicates $p<0.05$.

The role of p110δ in mESC self-renewal was further investigated using Dharmacon Smartpool siRNA targeting *Pik3cd* (Figure 4.16). When expression of *Pik3cd* RNA was assessed following siRNA-mediated knockdown, 50 and 75nM siRNA induced a 39% and 77% knockdown in *Pik3cd* expression respectively, when compared to non-targeting siRNA (Figure 4.16**A**). This knockdown followed a consistent trend, although not statistically significant. Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline phosphatase staining. No significant change in the proportion of cells staining positive for alkaline phosphatase activity was found (Figure 4.16**B**). The total number of colonies on dishes containing cells transfected with *Pik3cd* targeting siRNA was slightly greater than on dishes containing cells transfected with non-targeting siRNA, although this difference was not statistically significant (Figure 4.16**C**).

**A**




**B**




**C**

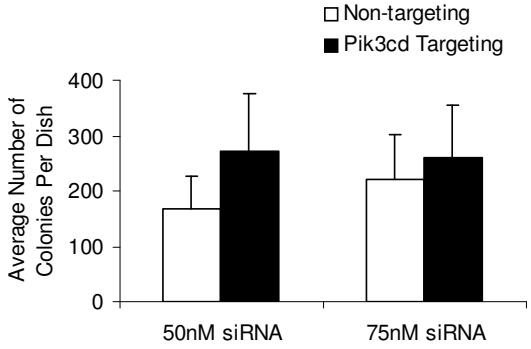
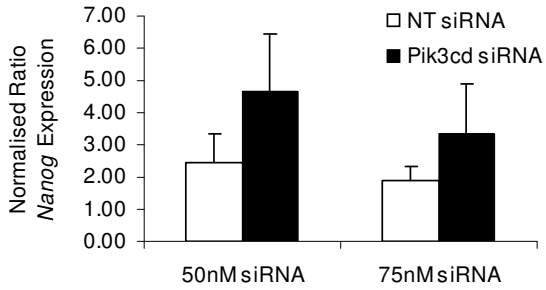



**Figure 4.16 Gene silencing of *Pik3cd* does not alter alkaline phosphatase staining**
Murine ESCs were transfected with Dharmacon *Pik3cd* targeting siRNAs or non-targeting (NT) siRNAs. **A** QPCR was conducted in quadruplicate to assess knockdown of *Pik3cd* RNA which was normalised relative to levels of *β-actin* RNA. A representative experiment of 4 independent experiments is shown with standard deviations. A Student's t-test was conducted to compare levels of *Pik3cd* expression following treatment with *Pik3cd* targeting siRNA and non-targeting (NT) siRNA. Although a consistent trend of reduced *Pik3cd* expression was detected with *Pik3cd* targeting siRNA, this decrease was not statistically significant. **B** Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline phosphatase staining. The average percentage of alkaline phosphatase positive, self-renewing colonies in each condition is shown with S.E.M (n=4). A Student's t-test revealed no statistical

significance. **C** The average total number of colonies per dish is shown with S.E.M. (n=4) and was analysed using a Student's t-test to confirm no statistical significance between the number of colonies on dishes incubated with *Pik3cd* targeting siRNA compared to dishes treated with non-targeting siRNA.

Consistent with no change in alkaline phosphatase activity, *Nanog* expression was not reduced by knockdown of *Pik3cd* (Figure 4.17**A**). In fact, expression of *Nanog* RNA appeared slightly enhanced in cells transfected with *Pik3cd* targeting siRNA compared to cells transfected with non-targeting siRNA. This enhancement in *Nanog* expression following gene silencing of *Pik3cd* was particularly noticeable using 50nM siRNA. Correlating with enhanced *Nanog* RNA expression, Nanog protein levels were also elevated following knockdown of *Pik3cd* (Figure 4.17**B**). Importantly, this enhancement in Nanog expression was not sufficient to alter alkaline phosphatase staining.

**A**




**B**

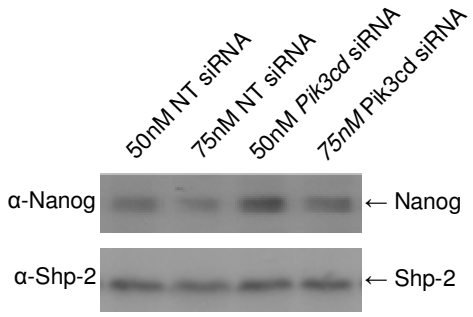



**Figure 4.17 Expression of Nanog RNA and protein is enhanced following knockdown of *Pik3cd***

**A** QPCR was conducted on samples from *Pik3cd* siRNA or non-targeting siRNA transfected cells for the expression of *Nanog* and normalised to relative levels of *β-actin* RNA. The data shown are representative of four independent experiments and is shown with standard deviations. **B** Protein lysates were analysed for the expression of Nanog and immunoblots were stripped and reprobed for Shp-2 to assess equal loading. The data shown are representative of four independent experiments.

The expression of p85 and other p110 isoforms were assessed following knockdown of *Pik3cd*. No change in the class $I_A$ PI3K regulatory subunit p85 protein expression was detected (Figure 4.18**A**). QPCR was used to assess the RNA expression of *Pik3ca* and *Pik3cb*, the genes encoding p110α and p110β respectively. Comparing 50nM *Pik3cd* targeting siRNA to 50nM non-targeting siRNA, no change in *Pik3ca* expression was observed (Figure 4.18**B**). At 75nM siRNA concentrations, *Pik3ca* expression was reduced although not significantly. This may reflect the high concentration of siRNA used since expression of *Pik3ca* was also decreased for 75nM non-targeting siRNA compared to 50nM non-targeting siRNA. No change in *Pik3cb* expression was detected using either 50 or 75nM *Pik3cd* targeting siRNA when compared to 50 or 75nM non-targeting siRNA respectively (Figure 4.18**C**).

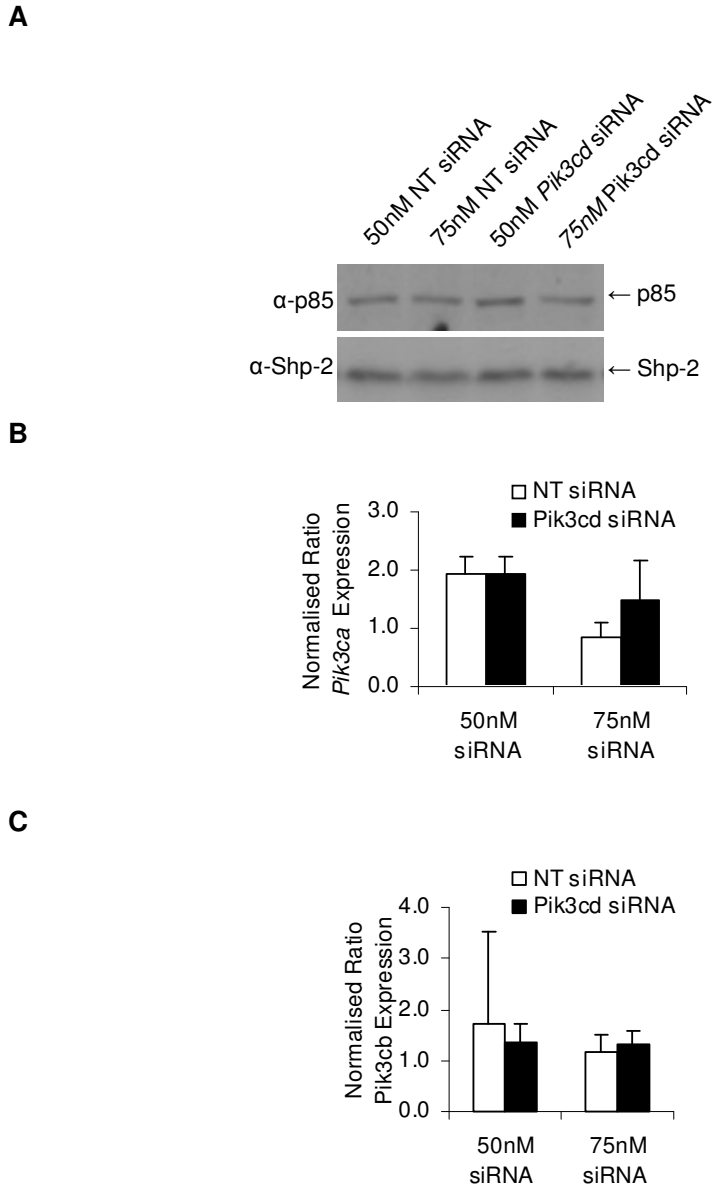



**Figure 4.18 Expression of other p110 isoform genes following knockdown of *Pik3cd***

**A** Protein lysates were obtained from cells following transfection with Dharmacon *Pik3cd* targeting siRNA. Immunoblotting was conducted and antibodies detecting p85 and Shp-2. Samples were assessed in quadruplicate by QPCR for the expression of *Pik3ca* (**B**) and *Pik3cb* (**C**) normalised to relative levels of *β-actin*. The data shown are representative of four independent experiments.

The incongruent results obtained by pharmacological inhibition of p110δ and siRNA-mediated knockdown of *Pik3cd*, the gene encoding p110δ, might reflect the fact that IC87114 shows some inhibition of p110β. In vitro assays demonstrated that inhibition of p110β by IC87114 occurs with an $IC_{50}$ of 16μM (Knight et al., 2006). Therefore, it is likely that, doses of 10μM IC87114 could inhibit p110β as well as p110δ. This certainly seems plausible given that knockdown of *Pik3cd* did not alter the expression of other isoforms and did not induce a decrease in self-renewal. Additionally, these decreases in alkaline phosphatase staining observed following pharmacological inhibition or gene silencing of p110β (Figures 4.5-4.10) were greater than those observed with IC87114 at the highest concentrations used. Therefore, changes in alkaline phosphatase staining with IC87114 may not be directly attributable to the inhibition of p110δ but could be the result of inhibition of p110β by IC87114. To investigate this possibility, IC87114 and TGX-121 or TGX-221 were applied simultaneously and self-renewal analysed by alkaline phosphatase assay.

As demonstrated previously, treatment of mESCs with 5μM LY294002 for 4 days results in a significant reduction in the percentage of alkaline phosphatase positive, self-renewing mESC colonies compared to vehicle treatment (Figure 4.19) (Paling et al., 2004, Storm et al., 2007). In the presence of 5μM IC87114, a small but not significant decrease in alkaline phosphatase positive colonies was also observed. Treatment with 5 or 10μM TGX-221 resulted in a significant reduction in stained colonies compared to vehicle treatment (Figure 4.19), consistent with data presented previously (Figures 4.5 and 4.15). Incubation in the presence of both 5μM TGX-121 and 5μM IC87114 resulted in fewer alkaline phosphatase positive colonies when compared with 5μM TGX-121 alone or 5μM IC87114 alone, although this reduction was not significant. Interestingly, 10μM TGX-121 and 5μM IC87114 resulted in a smaller decrease when compared to 10μM TGX-121 alone. Therefore, a plateau is reached at the highest concentration of TGX-121 used, where the addition of IC87114 can not further reduce alkaline phosphatase staining. If both p110β and p110δ regulated self-renewal, then a further decrease in alkaline phosphatase staining would be expected when both p110β and p110δ were inhibited compared to inhibition of p110β alone. Additionally, IC87114 induces a significant decrease in alkaline phosphatase staining only at the highest concentrations used (Figure 4.15**A**). Taken together, these results indicate that IC87114 may exhibit inhibition of p110β, which would account for a small decrease in self-renewal following treatment with IC87114.

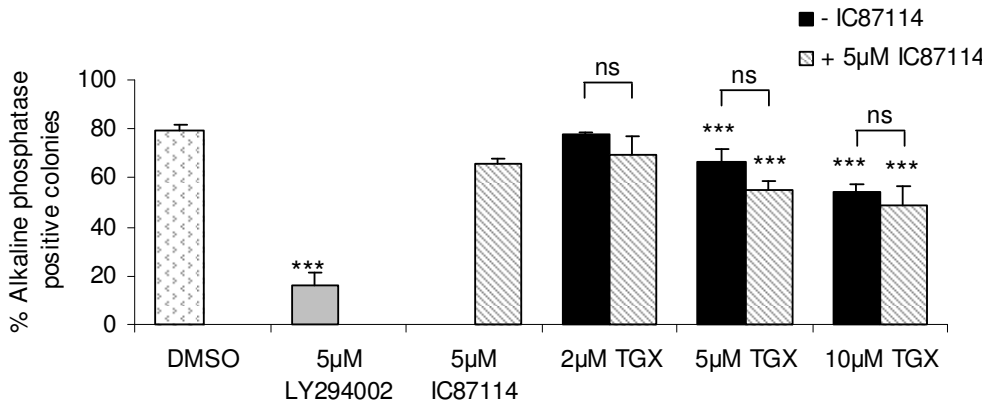



**Figure 4.19 Simultaneous inhibition of p110β and p110δ**

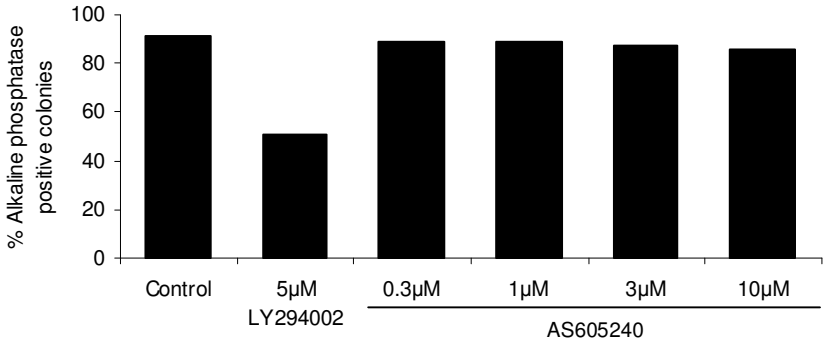
Murine ESCs were incubated in the presence or absence of IC87114 and TGX-121 and self-renewal was assessed by alkaline phosphatase staining after 4 days. Data are represented as the mean percentage of alkaline phosphatase positive colonies with S.E.M. (n=3). Data were analysed by ANOVA and Fisher's post hoc test applied comparing the percentage of alkaline phosphatase positive colonies for each treatment, where *** indicates $p<0.005$ when compared to DMSO control and ns (not significant) indicates no significant difference between the treatments indicated.

### *4.4.4 Treatment with p110γ selective inhibitor does not alter self-renewal*

The expression of p110γ in blastocyst stage mouse embryos is undetectable (NCBI virtual Northern http://www.ncbi.nlm.nih.gov/unigene accessed 25.05.07) and microarray data obtained from samples of the mESC line used in the present study indicates that p110γ expression is extremely low if present at all (Storm and Welham, unpublished data). Regardless of reported undetectable expression, and for completeness of the investigation into class I PI3K isoforms in the regulation of mESC self-renewal, any contribution of this isoform towards maintaining self-renewal was explored using a selective inhibitor, AS605240. This inhibitor has been shown to target the p110γ isoform with sufficient selectivity in mouse models to inhibit rheumatoid arthritis joint inflammation and to prolong the survival of mice with systemic lupus erythematosus (Barber et al., 2005, Camps et al., 2005).

When mESCs were treated with AS605240 for 4 days, no changes in the percentage of alkaline phosphatase positive colonies were detected (Figure 4.20**A**). For this experiment, AS605240 was used at 0.3-10μM, sufficient to cause inhibition of p110γ (Camps et al., 2005). Additionally, treatment with AS605240 did not alter the number of colonies present compared to control colony counts (Figure 4.20**B)**.

**A**




**B**

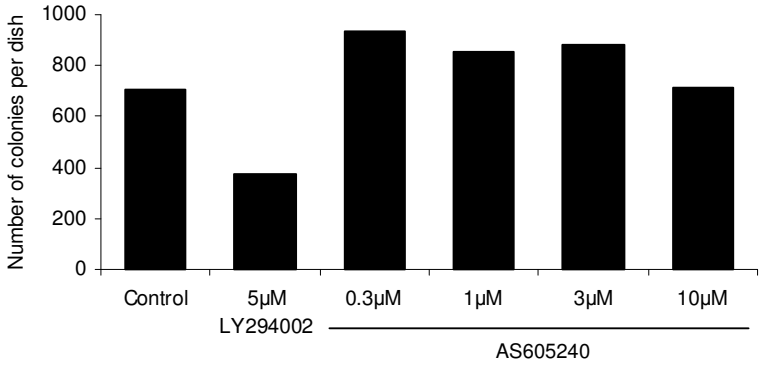



**Figure 4.20 The p110γ inhibitor AS605240 does not alter alkaline phosphatase staining**

Murine ESCs were plated at clonal density and treated for 4 days with vehicle (DMSO) or AS605240, a selective p110γ isoform inhibitor. Self-renewal was assessed by alkaline phosphatase staining. The percentage of alkaline phosphatase positive colonies (**A**) and the total number of colonies per treatment dish (**B**) are shown (n=1).

Having found no change in alkaline phosphatase staining and observed no effect of AS605240 on colony morphology or number, in addition to reported undetectable expression of p110γ in mESCs, a role for p110γ in mESC fate was not further investigated.

### *4.4.5 p110α does not regulate mESC self-renewal*

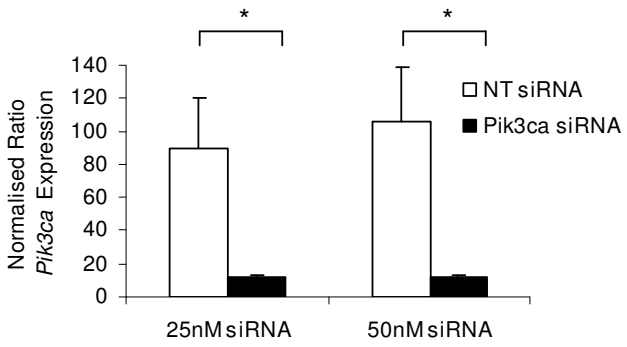
To investigate if mESCs require the activity or expression of p110α in order to maintain self-renewal, both pharmacological inhibition, with two structurally different inhibitors, and siRNA gene silencing approaches were implemented.

#### *4.4.5.1 siRNA-mediated knockdown of p110α does not alter self-renewal*

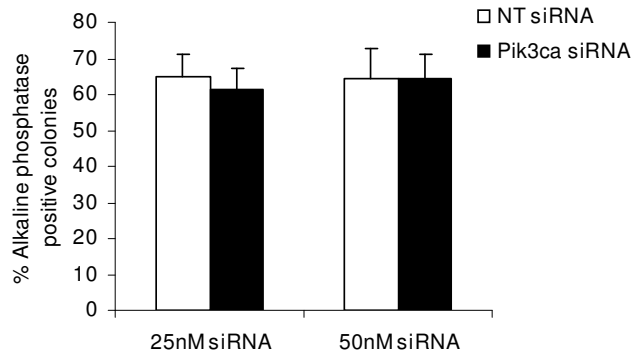
To investigate a role for PI3K p110α in maintaining mESC self-renewal, Dharmacon Smartpool siRNAs targeting *Pik3ca*, the gene encoding p110α, were used.

Targeting *Pik3ca* with siRNA led to a significant reduction in the expression of *Pik3ca*, typically 88% reduction in *Pik3ca* expression was achieved (Figure 4.21**A**). Despite such a high level of knockdown, on assessment of self-renewal by alkaline phosphatase staining, no change in the percentage of alkaline phosphatase positive colonies was found in cells treated with *Pik3ca* targeting siRNAs compared to cells treated with non-targeting siRNAs (Figure 4.21**B**). Cells transfected with *Pik3ca* targeting siRNAs formed similar colony numbers to those transfected with non-targeting siRNAs (Figure 4.21**C**).

**A**




**B**




**C**

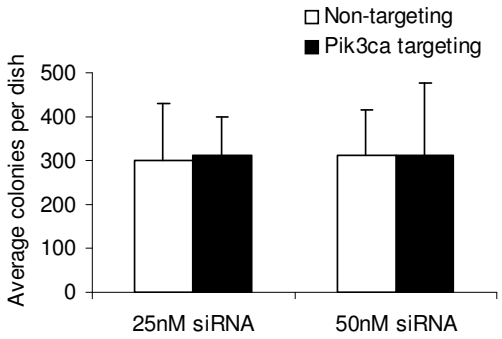
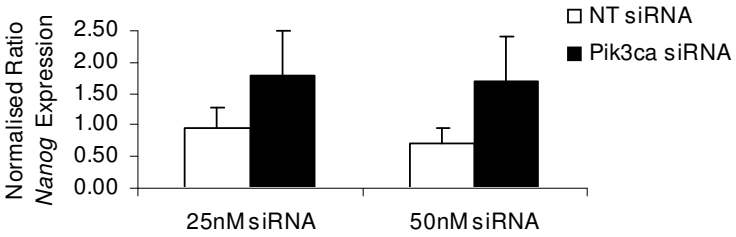


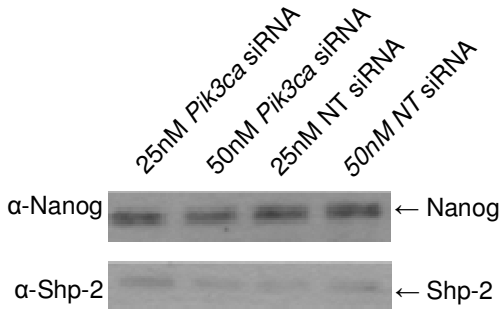

**Figure 4.21 Expression of *Pik3ca* is not required to maintain mESC self-renewal**
Murine ESCs were transfected with *Pik3ca* or non-targeting Dharmacon Smartpool siRNAs. **A** QPCR of quadruplicate samples was conducted to detect knockdown of *Pik3ca* RNA. Mean values are given with standard deviation and are representative of four independent experiments. **B** Following transfection cells were plated for 4 days and self-renewal assessed by alkaline phosphatase staining (n=4). The average percentage of alkaline phosphatase positive, self-renewing colonies in each condition are shown with S.E.M. No statistical significance was found. **C** The average total number of colonies on each dish is shown with S.E.M. A Student's t-test was conducted comparing non-targeting and *Pik3ca* targeting and no statistical significance was found.

Having observed no change in alkaline phosphatase staining following gene silencing of *Pik3ca*, the expression of other self-renewal markers were investigated. Using QPCR to detect *Nanog* expression revealed that expression was slightly elevated following *Pik3ca* knockdown compared to levels in control, non-targeting siRNA treated cells (Figure 4.22**A**). However, statistically, there was no significant difference in *Nanog* RNA expression following treatment with *Pik3ca* targeting siRNAs compared to non-targeting siRNAs. Importantly, despite the changes observed in *Nanog* expression, Nanog protein levels appeared unchanged following *Pik3ca* knockdown (Figure 4.22**B**). Consistent with QPCR data (Figure 4.21**A**), semi-quantitative PCR using primers detecting *Pik3ca* demonstrated a notable knockdown of expression following the use of *Pik3ca* targeting siRNAs compared to non-targeting siRNAs (Figure 4.22**C**). Consistent with no change in alkaline phosphatase staining, no detectable change in the expression of the self-renewal markers *Nanog* or *Rex1* was found upon semi-quantitative PCR analysis (Figure 4.22**C**). These data, assessing a number of self-renewal markers, strongly indicate that expression of *Pik3ca*, the gene encoding p110α, is not required in order for mESCs to self-renew.

**A**




**B**




**C**

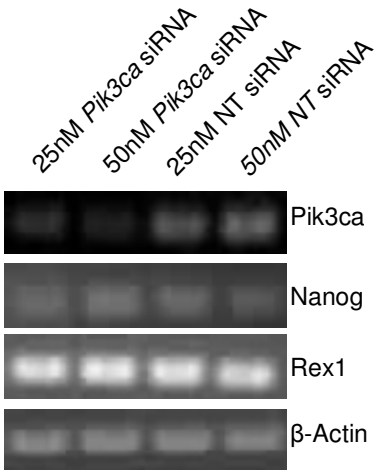


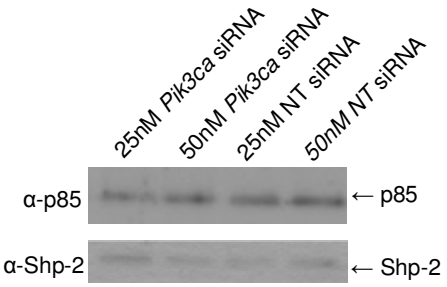

**Figure 4.22 p110α does not regulate mESC self-renewal**

RNA was isolated from mESCs transfected with *Pik3ca* targeting or non targeting siRNAs and reverse transcribed. The data presented here are representative of four independent experiments. **A** QPCR was used to assess changes in *Nanog* expression. Samples were assessed in quadruplicate. The mean expression is shown normalised to *β-actin* expression, with standard deviations. **B** Nanog protein expression was assessed by immunoblotting. Immunoblots were stripped and reprobed with antibodies detecting Shp-2 in order to assess equal loading, then reprobed with antibodies detecting p85 to

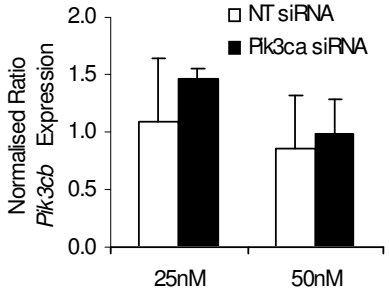
detect levels of expression. **C** Semi-quantitative PCR was conducted using primers indicated to assess knockdown of *Pik3ca* and expression of *Nanog* and *Rex1* self-renewal markers. The data shown are representative of 4 independent experiments.

In order to confirm that knockdown of *Pik3ca* by siRNA did not affect the expression of p85 or the expression of other p110 isoforms, immunoblotting and QPCR was conducted (Figure 4.23). Following knockdown of *Pik3ca,* mESCs exhibited similar levels of p85 protein when compared to cells treated with non-targeting siRNA (Figure 4.23**A**). *Pik3cb*, encoding p110β, showed no significant change in expression following knockdown of *Pik3ca* (Figure 4.23**B**). Additionally, expression of *Pik3cd*, encoding p110δ, was also unaffected by knockdown of *Pik3ca* (Figure 4.23**C**).

**A**




**B**




**C**

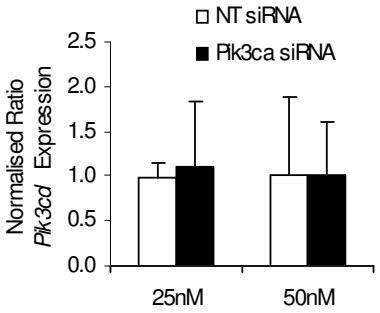



**Figure 4.23 Expression of *Pik3cb* and *Pik3cd* remains unchanged by *Pik3ca* knockdown**

Following siRNA-mediated knockdown of *Pik3ca*, p85 protein levels were assessed by immunoblotting using antibodies detecting p85. Blots were stripped and reprobed using antibodies detecting Shp-2 to assess equal loading. The effect of siRNA knockdown of *Pik3ca* on the expression of *Pik3cb* (**A**) and *Pik3cd* (**B**) was assessed by QPCR. Expression of *Pik3cb* or *Pik3cd* was assessed in quadruplicate and expression was normalised to *β-actin* expression. Mean data are shown with standard deviations. A representative experiment from four independent experiments is shown.

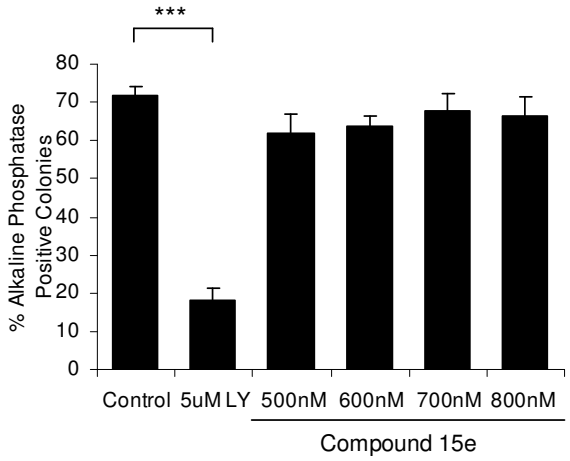
#### *4.4.5.2 Pharmacological inhibition of p110α does not lead to a loss of self-renewal*

Two structurally distinct p110α inhibitors were also employed to investigate a possible role for p110α in the regulation of mESC self-renewal and to complement the studies performed with siRNA-mediated gene silencing of this isoform. Following its synthesis (Hayakawa et al., 2001a) PIK-75 has been fully characterised as a selective p110α inhibitor. In kinase assays, PIK-75 was reported to inhibit p110α with an $IC_{50}$ of 5.8nM (Knight et al., 2006). Additionally, $IC_{50}$ values for the inhibition of mTORC1 and mTORC2 by PIK-75 were 1 and 10μM respectively, indicating that at concentrations of PIK-75 selective for p110α inhibition, mTOR is unlikely to be targeted. Another selective p110α inhibitor, compound 15e, was synthesised and identified as a potential anti-tumour compound since it was effective at inhibiting A375 cell proliferation with an $IC_{50}$ of 580nM (Hayakawa et al., 2006). The inhibition of mTOR or other PI3K related kinases by compound 15e has not yet been described.

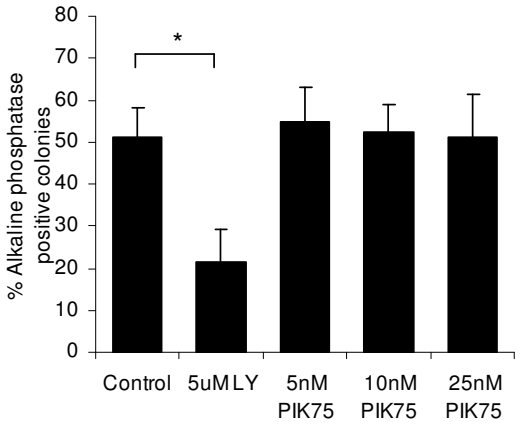
Treatment of mESCs with compound 15e resulted in no significant change in the levels of alkaline phosphatase staining (Figure 4.24**A**). Similarly, treatment with PIK-75 did not alter the percentage of alkaline phosphatase positive colonies when compared to control, vehicle treated colonies (Figure 4.24**B**). Therefore, inhibition of p110α with either compound 15e or PIK-75 did not result in a change in alkaline phosphatase staining, which is consistent with the effects observed following knockdown of *Pik3ca*, where no loss of self-renewal was evident (Figures 4.21 and 4.22). In each case, LY294002 was used as a positive control to demonstrate a significant reduction in alkaline phosphatase staining, representative of a loss of self-renewal (Figure 4.24).

Following incubation with p110α inhibitors for 4 days, the expression of Nanog was assessed. In the absence of LIF, Nanog protein expression was decreased consistent with a loss of self-renewal (Figure 4.24**C**). Treatment of mESCs with PIK-75 in the presence of LIF did not alter the level of Nanog expression when compared with mESCs incubated in the presence of LIF alone (Figure 4.24**C**). In the presence of LIF and compound 15e, Nanog levels appeared to be enhanced compared to the presence of LIF alone. This slight enhancement in Nanog expression did not increase alkaline phosphatase staining (Figure 4.24**A** and Figure 4.24**B**) and is similar to the enhanced expression of *Nanog* RNA observed following knockdown of *Pik3ca* by siRNA (Figure 4.22**A** and **B**), which also did not enhance alkaline phosphatase staining (Figure 4.21**B**).

**A**




**B**




**C**

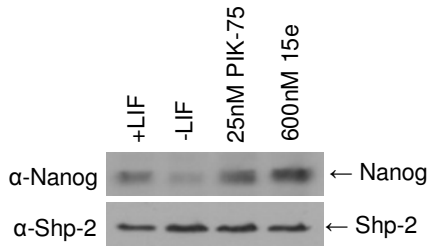



**Figure 4.24 Pharmacological inhibition of p110α with two structurally distinct inhibitors does not alter self-renewal**

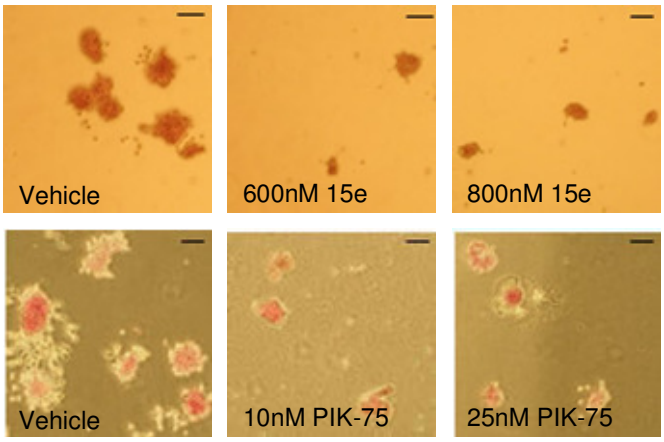
Murine ESCs were incubated with vehicle (DMSO), LY294002, compound 15e (**A**) or PIK-75 (**B**) for 4 days. Self-renewal was assessed by staining for alkaline phosphatase activity. Colonies were scored for alkaline phosphatase staining and alkaline phosphatase positive colonies represented as average percentage of total colonies present on a culture dish, n=5 for compound 15e, n=3 for PIK-75. Statistical analysis using ANOVA was conducted and Dunnet's post hoc test applied to compare inhibitor

treatments with vehicle treatment, where * indicates $p<0.05$ and *** indicates $p<0.005$. **C** Following 4 days incubation in the absence of LIF or in the presence of LIF with vehicle (DMSO), PIK-75 or 15e mESCs were harvested and protein lysates analysed for the expression of Nanog and Shp-2 using appropriate antibodies.

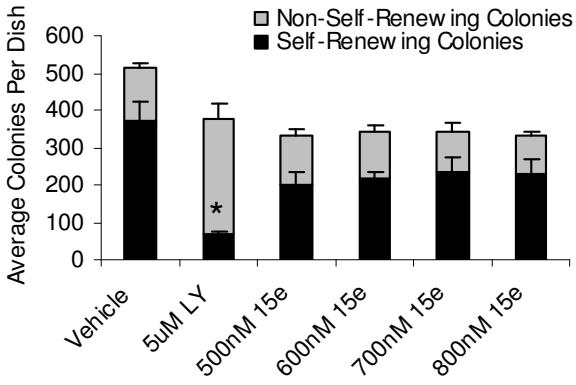
#### *4.4.5.3 Incubation with selective p110α inhibitors produces smaller and fewer mESC colonies*

Although alkaline phosphatase staining was unchanged following treatment with p110α inhibitors, it was observed that colonies treated with these inhibitors were smaller in diameter compared to vehicle treated colonies (Figure 4.25**A**). In addition, fewer colonies were present after 4 days treatment with compound 15e (Figure 4.25**B**). Increasing the dose of PIK-75 produced fewer colonies after 4 days (Figure 4.25**C**). Importantly, the proportion of alkaline phosphatase positive colonies (represented by dark bars) of the total number of colonies remained unchanged by pharmacological inhibition of p110α with either compound 15e or PIK-75 (Figure 4.25**B** and **C**).

**A**




**B**




**C**

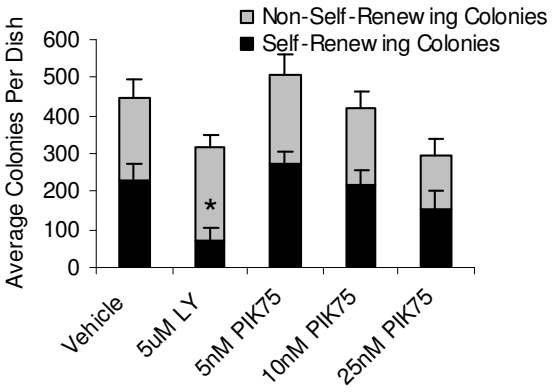



**Figure 4.25 Inhibition of p110α results in smaller and fewer colonies**

Murine ESCs were treated with DMSO, LY294002, compound 15e or PIK-75 for 4 days as described in Figure 4.24. Representative colonies are depicted following staining for alkaline phosphatase activity (scale bar = 100 µm) (**A**). Data obtained from scoring alkaline phosphatase staining of mESC colonies are represented as the average number of self-renewing alkaline phosphatase positive colonies (black histogram bars) and the average number of non-self-renewing, alkaline phosphatase negative colonies

(grey histogram bars) with S.E.M. for treatment with compound 15e (n=5) (**B**) and PIK-75 (n=3) (**C**). Statistical analysis was conducted using ANOVA and Dunnet's post hoc test, where * indicates $p<0.05$.

A reduction in colony size and number is indicative of either reduced proliferation or enhanced apoptosis. Subsequently, the regulation of mESC proliferation and metabolism by PI3K catalytic subunit isoforms, particularly by p110α, was investigated and is reported and discussed in detail in Chapter 5. Furthermore, a direct method of assessing apoptosis was employed to address this matter following mESC treatment with p110α inhibitors (Section 5.22).

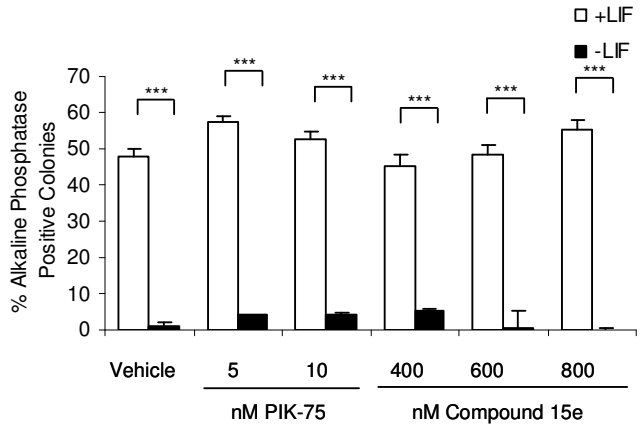
#### *4.4.5.4 Inhibition of p110α does not prevent differentiation*

Inhibition of p110α does not appear to lead to a decrease in mESC self-renewal suggesting that p110α does not regulate self-renewal. Alternatively, inhibition of p110α could aid the maintenance of self-renewal which might explain the lack of a reduction in self-renewal following inhibition. Another possibility is that since p110α is reported to be important for proliferation and insulin signalling (Bi et al., 1999, Foukas et al., 2006, Knight et al., 2006), inhibition of this isoform could result in apoptosis of differentiated cells, which may explain reduced colony numbers following treatment with p110α inhibitors.

In order to investigate whether inhibition of p110α affects mESC differentiation or leads to apoptosis of differentiated derivatives, mESCs were treated with compound 15e or PIK-75 and incubated in the presence or absence of LIF. LIF is required to maintain self-renewal of mESCs in culture, therefore incubation in the absence of LIF results in spontaneous differentiation to precursors of multiple cell lineages (Boeuf et al., 1997, Cartwright et al., 2005, Niwa et al., 2000), resulting in a significant reduction in the proportion of alkaline phosphatase positive colonies (Figure 4.26**A**). Colonies formed from mESCs incubated in the absence of LIF, but in the presence of PIK-75 or compound 15e, formed differentiated colonies that were negative for alkaline phosphatase activity (Figure 4.26**A**). These results suggest that in the absence of LIF inhibition of p110α cannot maintain self-renewal and also that inhibition of p110α does not prevent the spontaneous differentiation of mESCs observed upon LIF withdrawal. However, treatment with compound 15e or PIK-75 resulted in the formation of fewer

colonies following 4 days incubation (Figure 4.26**B**). In the absence of LIF, but presence of p110α inhibitors, the majority of colonies were differentiated, arguing against the selective apoptosis of differentiated colonies when p110α is inhibited. However, it is important to investigate apoptosis in a more direct manner; this was conducted and is reported in Section 5.2.2.

**A**




**B**

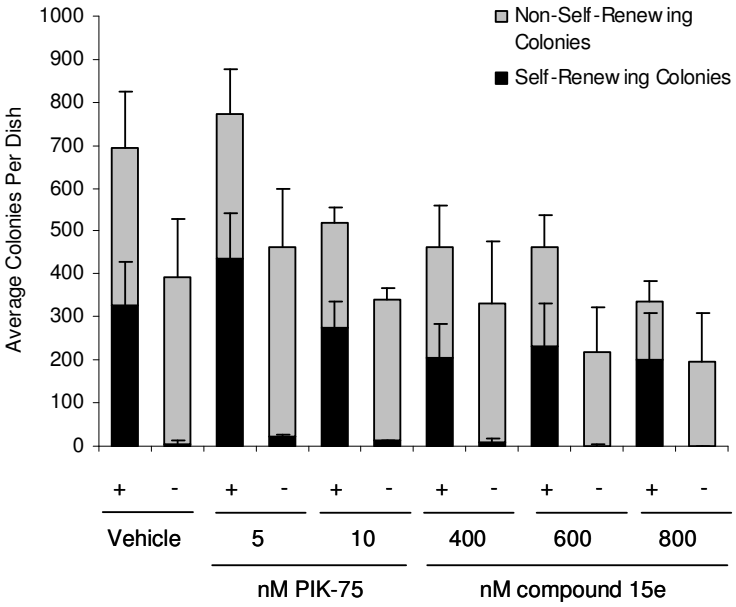



**Figure 4.26 Inhibition of p110α does not prevent differentiation**

Murine ESCs were treated with p110α inhibitors compound 15e or PIK-75 for 4 days, at the doses indicated, in the presence or absence of LIF. Self-renewal was assessed by alkaline phosphatase staining (n=3). Data are represented as the average percentage of self-renewing colonies of the total number of colonies on each culture dish (**A**) or as the average number of self-renewing, alkaline phosphatase positive colonies (black histogram bars) and the average number of non-self-renewing alkaline phosphatase negative colonies (grey histogram bars) (**B**) with S.E.M.. Statistical analysis was conducted using ANOVA and Fisher's post hoc test where *** indicates $p<0.005$.

## 4.5 Summary and discussion: Comparison of LY294002 and ZSTK474 induced loss of self-renewal in mESCs

Inhibition of PI3Ks with the broad selectivity PI3K inhibitor LY294002 has been shown to induce a loss of mESC self-renewal, indicated by a decrease in alkaline phosphatase staining and a reduction in Nanog protein and RNA expression (Paling et al., 2004, Storm et al., 2007). The effects mediated by LY294002 were compared to those induced by the recently described class I selective PI3K inhibitor ZSTK474 (Kong and Yamori, 2007, Yaguchi et al., 2006).

ZSTK474 was demonstrated to induce a reduction in alkaline phosphatase staining, to a level similar to that induced by incubation of mESCs with LY294002. In addition, ZSTK474 produced similar temporal changes in Nanog protein expression to those observed with LY294002 after 24 hours and 48 hours incubation, and up to 4 days incubation (Figures 4.2 and 4.1 respectively). Interestingly, ZSTK474 did not induce an enhancement in basal or stimulated Erk phosphorylation as is often observed with LY294002. Maybe subtle differences in the inhibition of PI3K classes, or related protein kinases (discussed in Sections 1.1.3 and 4.2) can account for the observed disparity in Erk phosphorylation. It is still unknown how PI3Ks might exert a dampening effect on MAPK signalling but this phenomenon has been reported in other cell types (discussed in Section 3.2.4) (Kanasaki et al., 2008, Laprise et al., 2004, Romano et al., 2006).

Taken together, these results suggest that inhibition of PI3Ks by LY29402 or by ZSTK474 result in similar responses despite the differences in inhibitor profiles. Furthermore, the use of an alternative inhibitor to LY294002, one which selectively inhibits class I PI3Ks (Kong and Yamori, 2007, Yaguchi et al., 2006), confirms the involvement of this class of PI3Ks in maintaining mESC self-renewal (Paling et al., 2004).

## 4.6 Summary and discussion: The roles of PI3K class I isoforms in the regulation of mESC self-renewal

The activity of broad spectrum and isoform selective PI3K inhibitors used in this study were first confirmed in BaF/3 pro-B cell line. The broad spectrum PI3K inhibitors, LY294002, wortmannin and ZSTK474, inhibited IL-3-induced PKB phosphorylation in BaF/3 cells. Additionally, selective inhibitors of p110α, PIK-75 and compound 15e, p110β, TGX-121 and TGX-221, p110δ, IC87114, and p110γ, AS605240, reduced IL-3-induced PKB phosphorylation. These results verified the activity of these inhibitors in a mouse cell line which is known to express all class I PI3K isoforms (Vanhaesebroeck et al., 1997). Subsequently, these inhibitors were used to investigate the roles of PI3K catalytic isoforms in regulating mESC self-renewal.

Using siRNA-mediated knockdown and pharmacological inhibitors, the expression of *Pik3cb* and the activity of the protein encoded by this gene, p110β, were demonstrated to be required for mESCs to maintain optimal self-renewal. Importantly, consistent results implicating p110β in mESC self-renewal were obtained using two different p110β inhibitors and three different siRNAs. Interestingly, levels of self-renewal following p110β inhibition or *Pik3cb* knockdown were comparable to levels observed with LY294002 treatment or *Nanog* knockdown. These observations suggest that p110β is the predominant isoform implicated in maintaining mESC self-renewal.

The time-course with which inhibition of p110β induces a reduction in Nanog protein expression is similar to that previously reported for LY294002 (Paling et al., 2004), in that expression of Nanog is reduced early, by 48 hours for TGX-221 (Figure 4.5), and before a change in colony morphology is observed. Therefore, reduced Nanog expression is likely to be a more direct result of inhibition of p110β, rather than a secondary response that follows inhibition of p110β inducing a loss of self-renewal.

The principle role of p110β in maintaining self-renewal of mESCs, defined in the present study, is consistent with, and supports the requirement for, the expression of p110β in blastocyst stage mouse embryos (Bi et al., 2001). In the ICM, self-renewal of cells is important for the establishment and development of the embryo proper (Figure 1.6 and Figure 1.7) thus explaining the requirement of p110β activity for the survival of embryos beyond the blastocyst stage (Bi et al., 2001). Interestingly, the incomplete penetrance of embryonic lethality reported in mice expressing a catalytically inactive knock-in of p110β (Ciraolo et al., 2008, Guillermet-Guibert et al., 2008) was proposed to be the result of

non-catalytic functions mediated by p110β (Christoforidis et al., 1999, Ciraolo et al., 2008). This reasoning also explains the embryonic lethality of mouse embryos completely lacking p110β expression (Bi et al., 2001). In mESCs the requirement for both expression and activity of p110β was demonstrated to maintain optimal self-renewal, suggesting that a non-catalytic role for p110β in maintaining self-renewal was not essential. However, a non-catalytic role for p110β in mESC fate cannot be disregarded based on the results presented in the present study alone.

In mESCs, p110β did not appear to be activated by LIF stimulation, certainly inhibition of p110β did not alter LIF-induced signalling downstream of PI3Ks. This was surprising given that LIF is an important extrinsic factor necessary for the maintenance of mESC self-renewal in culture (Bard and Ross, 1991, Gough et al., 1989, Smith et al., 1988, Williams et al., 1988). A number of reports indicate the activation of p110β via G proteins following the activation of GPCRs (Ciraolo et al., 2008, Guillermet-Guibert et al., 2008, Kubo et al., 2005, Maier et al., 1999, Murga et al., 2000, Roche et al., 1998) (discussed in Section 1.1.2.1). The activation of p110β by GPCRs in mESCs is certainly plausible given the low or undetectable levels of expression of p110γ in mESCs (Storm and Welham, unpublished data) and the lack of effect on alkaline phosphatase staining observed using AS605240, a selective p110γ inhibitor, leaving p110β as the only PI3K isoform capable of coupling to GPCRs in mESCs. The crude method of boiling serum to denature ligands other than heat stable lysophospholipids, agonists of GPCRs, followed by stimulation of mESCs, revealed enhanced PKB phosphorylation that could be inhibited with TGX-121 or TGX-221, inhibitors of p110β. This indicates that p110β may well couple to GPCRs and G proteins in mESCs, as reported previously in other cell types (Ciraolo et al., 2008, Guillermet-Guibert et al., 2008). However, the physiological relevance of this coupling requires further investigation.

In contrast to the results observed with boiled serum, neither LPA nor S1P induced the same response in terms of PKB phosphorylation. LPA and S1P did stimulate Erk phosphorylation which, following the inhibition of p110β by TGX-221 or inhibition of $G_i$ or $G_o$ G proteins by pertussis toxin, could be enhanced for LPA and decreased for S1P. These results are consistent with an enhancement in Erk phosphorylation known to be induced by activation of LPA and S1P receptors via $G_i$ (Meyer zu Heringdorf and Jakobs, 2007, Pébay et al., 2005, Rosen and Goetzl, 2005). Furthermore, activation of the MAPK pathway, known to promote differentiation (Kunath et al., 2007, Stavridis et al., 2007), by LPA and S1P is consistent with reports indicating that lysophospholipids can induce differentiation. Indeed, S1P is reported to induce cardiac differentiation in embryoid bodies formed from mESCs (Sachinidis et al., 2003) and

sphingosylphosphorylcholine, which can stimulate S1P receptors as well as specific receptors such as GPR4 expressed in mESCs, was shown to induce cardiac and neural differentiation of mESCs (Kleger et al., 2007). As with GPCR activation coupling to PI3K signalling, further investigation would be required to fully characterise GPCR agonist-induced differentiation and the signalling pathways underlying this cellular response.

The enhancement in Erk phosphorylation, often observed following mESC incubation with LY294002 (Paling et al., 2004) (Figure 3.2), was also observed with TGX-121 and TGX-221 (Figure 4.12), thereby implicating p110β in the dampening effect exerted by PI3Ks on MAPK pathway activity under normal mESC culture conditions. A number of reports demonstrate this effect in other cell types (Kanasaki et al., 2008, Laprise et al., 2004, Romano et al., 2006) and suggest that this phenomenon could be mediated by the regulation of Raf-1 phosphorylation by PKB (Rommel et al., 1999) but these reports did not link this observation to a specific PI3K isoform. Interestingly, an enhancement in Erk phosphorylation was observed in MEFs derived from p110β kinase-dead knock-in mice (Ciraolo et al., 2008), consistent with the implication of this isoform in regulating MAPK pathway activity in mESCs, demonstrated in the present study (Kingham and Welham, In Press). Furthermore, cross-talk between PI3K and MAPK signalling may provide another mechanism for p110β to contribute to the maintenance of mESC self-renewal, since it has previously been reported that inhibition of the MAPK pathway enhances self-renewal (Burdon et al., 1999). In chapter 3, it was demonstrated that an enhancement in MAPK pathway activity induced only a small decrease in the capacity of mESCs to maintain self-renewal. Inhibition of p110β induced an enhancement in Erk phosphorylation, indicating an enhancement in the activity of the MAPK pathway, but the loss of self-renewal following p110β inhibition (Figure 4.5) was much more pronounced than that observed following induction of enhanced MAPK pathway activation (Figure 3.10). Thus, the differentiation of mESCs induced by p110β inhibition might be aided by, but can not be fully accounted for, by enhanced MAPK pathway activation and is therefore likely to involve other pathways.

Based on the studies reported here, a working model to explain how p110β controls self-renewal is presented in Figure 4.27. Activation of p110β may occur via typical tyrosine kinase receptor activation or via GPCR activation by factors present in serum or serum replacement used for routine culture. Following activation, p110β may contribute to the maintenance of self-renewal through an undefined pathway involving the preservation of expression of the master regulator of self-renewal, Nanog. Additionally, p110β may

regulate MAPK pathway signalling upstream of Erk, preventing differentiation induced by MAPK activity.

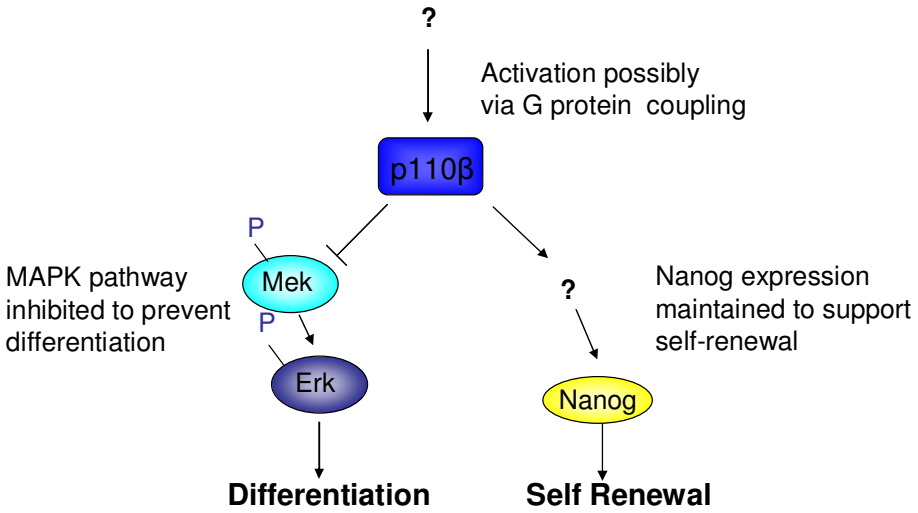



**Figure 4.27 Model of the regulation of mESC self-renewal by p110β**

Evidence presented in the present study indicates that PI3K p110β is the predominant isoform to maintain mESC self-renewal and might do so by inhibiting the activity of the MAPK pathway and supporting the maintenance of Nanog expression, a master regulator of self-renewal. Additionally, other pathways involving p110β, including non-catalytic functions, may contribute to maintaining an undifferentiated state.

The expression levels of markers of self-renewal are used to assess the self-renewal state of ESCs for investigation purposes. In the present study alkaline phosphatase and Nanog markers were used. In the stem cell field, Oct4 expression is frequently presented in conjunction with Nanog expression data. Little or no change in Oct4 expression was observed during the present study, even when significant changes were observed for Nanog and alkaline phosphatase expression, consistent with the observations of others in our research group (Paling et al., 2004, Storm et al., 2007). Interestingly, Oct 4 expression levels in self-renewing mESCs cultured on a feeder layer, self-renewing mESCs in feeder-free culture and in embryoid bodies have been described to be 'indistinguishable' (Gordeeva et al., 2003). In support of this, the time-course for a decrease in Oct4 expression following the induction of differentiation is somewhat delayed in comparison to Nanog. Oct4 is reported to be detected in day 5 embryoid bodies at similar levels to those detected in ESCs used to form such embryoid bodies (Oka et al., 2002). Furthermore, a different research group report Oct4

expression to be maintained in mESCs cultured in the absence of LIF for up to 5 days (no longer time points were published) and in embryoid bodies up to day 16 (no longer time points were published) (Sauter et al., 2005). These reports indicate that Oct4 expression may not best represent the self-renewal state of mESCs, particularly when early differentiation or triggers of loss of self-renewal are of interest, as in the present study. In the present study, Rex1 expression was used to demonstrate a loss of self-renewal induced by knockdown of *Pik3cb*. Rex1 is expressed specifically in preimplantation embryos, trophoblast and spermatocytes but importantly, expression in mESCs is reduced following induction of differentiation (Rogers et al., 1991). Rex1 expression is reported to be detectable in mESCs but undetectable in day 5 embryoid bodies (Oka et al., 2002) and was notably reduced in embryoid bodies by day 3.25 (Robertson et al., 2000) making it a suitable marker for self-renewal and a loss of expression was detectable when mESCs are in the early stages of differentiation.

No role for p110α in the maintenance of self-renewal was observed following pharmacological inhibition with two structurally distinct selective inhibitors or by siRNA-mediated knockdown of *Pik3ca*, the gene encoding p110α. These findings suggest that neither the expression nor the activity of p110α is required in order for mESC self-renewal to be maintained. Furthermore, inhibition of p110α was demonstrated not to prevent mESC differentiation and not to induce cell death, which would have resulted in a lack of differentiated colony types following a period of incubation in the absence of LIF. Furthermore, an enhancement in *Nanog* expression was observed at a transcriptional level only following *Pik3ca* knockdown but was enhanced at a protein level following inhibition of p110α with compound 15e. Importantly, this small enhancement in Nanog expression was not sufficient to enhance alkaline phosphatase staining in the presence of LIF and did not prevent differentiation in the absence of LIF. Furthermore, enhanced Nanog protein was not observed following gene silencing of *Pik3ca* or following inhibition of p110α by PIK-75. Smaller colonies, which were fewer in number, were observed following incubation of mESCs with p110α inhibitors, indicating an effect on mESC proliferation. This was further investigated and is reported and discussed in detail in chapter 5.

Knockdown of p110δ had no effect on self-renewal and the reduction in self-renewal observed using the highest concentrations of the p110δ inhibitor, IC87114, are best explained by the inhibition of p110β, which would be predicted at these concentrations (Knight et al., 2006). Interestingly, knockdown of *Pik3cd*, encoding p110δ, led to enhanced expression of Nanog protein and RNA. In microarray data from samples comparing self-renewing mESCs to those undergoing differentiation, the expression of *Pik3cd* was enhanced following induction of differentiation (Storm and Welham, unpublished data). One explanation for enhanced Nanog expression is that by knocking down expression of *Pik3cd*, under a protocol where transfection is conducted over a period of 3 days, cells may be restricted from differentiating. Consistent with this, mESCs transfected with non-targeting siRNA would continue to undergo low levels of spontaneous differentiation, a feature of ESCs (Cheng et al., 1998, Ying et al., 2003a, Ying et al., 2003b) and would result in lower Nanog expression when compared to mESCs transfected with *Pik3cd* siRNA, which might be subject to restricted spontaneous differentiation and therefore maintain the Nanog expression levels possessed on commencement of the transfection protocol.

In summary, p110β is the predominant class $I_A$ PI3K catalytic isoform that regulates mESC self-renewal, whereas p110α or p110δ do not directly or independently regulate self-renewal.

# 5 Results: The regulation of mESC proliferation by PI3K catalytic subunit p110α and a link between proliferation and the regulation of self-renewal

## 5.1 Introduction and Aims

ESCs exhibit a continual proliferative capacity, which has been likened to the perpetual proliferation of tumour cells. PI3Ks have been implicated in regulating ESC growth and proliferation in a number of published reports. In mESCs, deletion or pharmacological inhibition of mammalian target of rapamycin (mTOR), a downstream effector of PI3K signalling, results in proliferative defects (Murakami et al., 2004). Experiments involving the broad selectivity PI3K inhibitor LY294002 give disparate results largely owing to the concentrations used. For example, doses of 10μM LY294002 or above were reported to inhibit the proliferation of mESCs but lower doses, that did not affect cell viability or proliferation, induced differentiation (Paling et al., 2004). In agreement with this, 20-40μM LY294002 led to a reduction in cell growth after 48 hours (Lianguzova et al., 2007) and 25μM LY294002 led to an increase in the number of mESCs in $G_1$ phase after 24 hours, with a dramatic reduction in cell growth being reported after 4 days (Jirmanova et al., 2002). However, inhibition of mTOR is possible at 1-30μM LY294002 (Brunn et al., 1996) and, since mTOR is also reported to be important for mESC proliferation (Murakami et al., 2004), the effects seen with LY294002 might reflect inhibition of mTOR rather than PI3K directly.

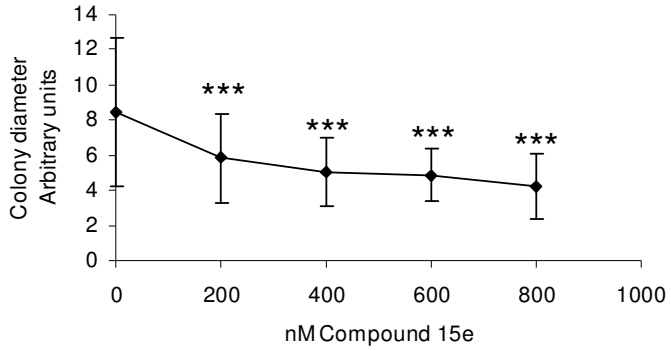
In chapter 4, an assessment was made as to which class I PI3K isoforms contribute to the regulation of self-renewal of mESCs (Section 4.4). The catalytic p110β isoform was implicated as the major contributor to the maintenance of mESC self-renewal (Kingham and Welham, In Press). However, when catalytic isoform p110α was targeted by the selective inhibitors compound 15e and PIK-75, colonies were smaller and fewer in number. These observations suggested that p110α might regulate mESC growth or proliferation. In order to realise the aim of identifying how p110 catalytic isoforms contribute to mESC fate, p110α, p110β and p110δ, were investigated for their participation in proliferation, metabolism and insulin signalling.

Self-renewal is often defined as the proliferation of ESCs whilst suppressing differentiation (Burdon et al., 2002, Burdon et al., 1999, Savatier et al., 1994, Smith, 2001). Having identified which PI3K catalytic isoforms regulate the cellular functions of self-renewal and proliferation, the possibility that these two features are in someway linked in ESCs was explored.

## 5.2 p110α regulates mESC proliferation

When the effect of inhibition of p110α on self-renewal was assessed, treatment with selective inhibitors for 4 days produced smaller colonies that were fewer in number (Figure 4.25). In fact, with PIK-75 at concentrations greater than 25nM, few colonies were visible after 4 days incubation and were too small to score for alkaline phosphatase staining. To demonstrate the change in size of colonies following treatment with p110α inhibitors, compound 15e and PIK-75, the diameters of fixed and alkaline phosphatase stained colonies were measured using a graticule. Treatment with 200nM compound 15e induced a significant reduction in colony diameter and 800nM compound 15e resulted in colonies that were approximately half the diameter of colonies formed from cells incubated in the absence of inhibitor (Figure 5.1**A**). Treatment with 5nM PIK-75 or greater concentrations also led to significantly smaller colonies after 4 days incubation (Figure 5.1**B**). Indeed 25nM PIK-75 produced colonies that were approximately one third the diameter of colonies formed from cells incubated in the absence of PIK-75.

**A**




**B**

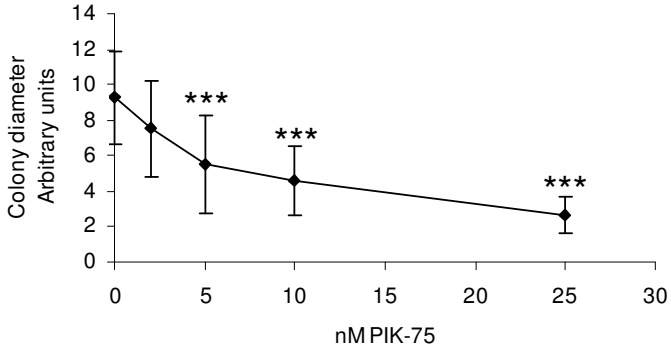



**Figure 5.1 Compound 15e and PIK-75 lead to the formation of smaller mESC colonies**

Murine ESCs were plated at clonal density and incubated for 4 days with compound 15 (**A**) or PIK-75 (**B**) at the doses indicated. Colonies were fixed and stained for alkaline phosphatase. Under a microscope, colony diameters were measured (n=30), for each treatment. The graphs shown represent the average colony diameter for each treatment with standard deviations. Statistical analysis was conducted using ANOVA and Dunnet's post hoc test, where *** indicates $p<0.001$ A representative experiment from n=5 and n=3 independent experiments for compound 15e and PIK-75 respectively, was chosen for quantitative assessment of colony diameters.

The smaller colony sizes observed following inhibition of p110α could be explained by reduced cell proliferation which would lead to reduced colony growth. Alternatively, inhibition of p110α could lead to the induction of programmed cell death. To explore these two possibilities, cell counts to assess cell proliferation and an investigation into the early stages of apoptosis were both conducted.

### *5.2.1 Incubation with p110α inhibitors results in reduced cell numbers*

To assess cell proliferation, mESCs were incubated with LY294002, compound 15e or PIK-75 and harvested after 1-4 days. Harvested cells were counted using the vital dye, trypan blue, in order to assess proliferation. After 24 and 48 hours incubation with LY294002, numbers of cells retrieved from LY294002 treatment dishes were similar to numbers of cells harvested from vehicle treatment dishes (Figure 5.2), consistent with a previous report from this research group (Paling et al., 2004). However, in the present study, fewer cells were counted from dishes treated with LY294002 on days 3 and 4 when compared to vehicle treatments at these time points. This observation might be explained by differentiated cells, which would be present at this later time point, becoming sensitive to LY294002 resulting in reduced proliferation and therefore a reduction in the number of cells retrieved. After 24 hours of incubation in the presence of compound 15e or PIK-75, a similar number of cells were retrieved from these plates as those counted from dishes treated with vehicle (DMSO) alone (Figure 5.2). In the presence of compound 15e, dramatically fewer cells were retrieved on days 2-4 than those counted from vehicle treated plates. Furthermore, PIK-75 treatment also resulted in much lower numbers of cells being retrieved from dishes incubated with this inhibitor compared to vehicle alone on days 2-4 (Figure 5.2). Incubation of mESCs with compound 15e or PIK-75 resulted in fewer cells being harvested in comparison to the number of cells harvested following incubation with LY294002.

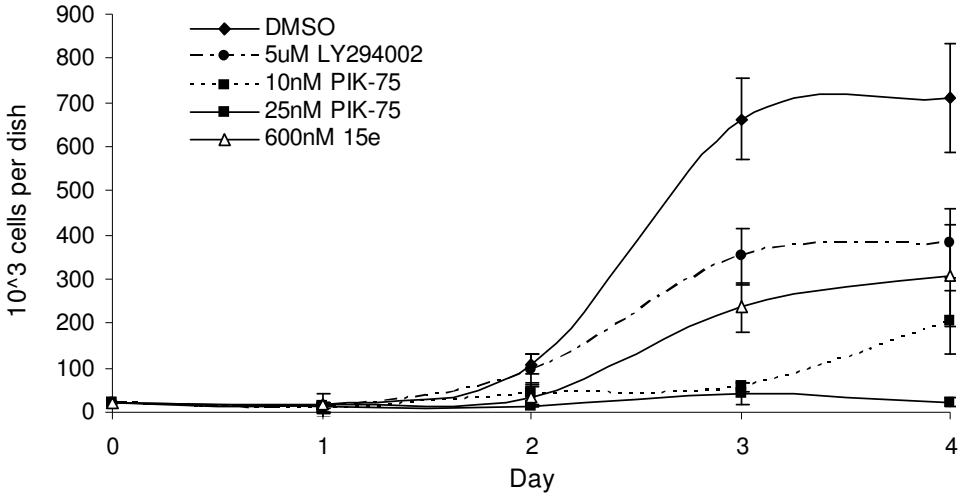



**Figure 5.2 p110α is coupled to mESC proliferation**

To assess cell proliferation, viable cell numbers were determined at 24 hour intervals for up to 4 days. Murine ESCs were treated with vehicle (DMSO), 5μM LY294002, 10nM PIK-75, 25nM PIK-75 or 600nM 15e, dishes were set up in triplicate for each treatment. The average number of cells harvested from each culture dish (counted in triplicate, using a haemocytometer) is shown with standard deviations. The experiment shown is representative of three independent experiments.

A viable cell count following a period of incubation is one way of assessing cell proliferation. However, since this method only takes into account viable, adherent cells, it is possible that reduced cell numbers are the result of apoptosis rather than reduced proliferation.

### *5.2.2 Inhibitors of p110α do not induce apoptosis*

The reduction in cell number observed following incubation with p110α inhibition could arise due to apoptosis, rather than a reduction in proliferation. To investigate this possibility directly, mitochondrial membrane permeability transition, a hallmark of and an irreversible point in programmed cell death (Rottenberg and Wu, 1998), was examined following inhibition of PI3Ks. The green fluorescent dye, 3,3'–dihexyloxacarbocyanide iodide ($DiOC_6$), stains the mitochondrial membrane of live cells but not mitochondria that have become permeabilised, a feature of apoptotic cells (explained in more detail in Section 2.4.3). $DiOC_6$ fluorescence can be measured by flow cytometry, therefore it was

important to first establish that p110α inhibitors do not exhibit fluorescence. The fluorescence intensity profiles of cells treated with DMSO, LY294002, compound 15e and PIK-75 (green histogram traces) were similar to that for untreated cells (purple histogram) (Figure 5.3) indicating that these inhibitors are not fluorescent and, therefore, are suitable for use with this method.

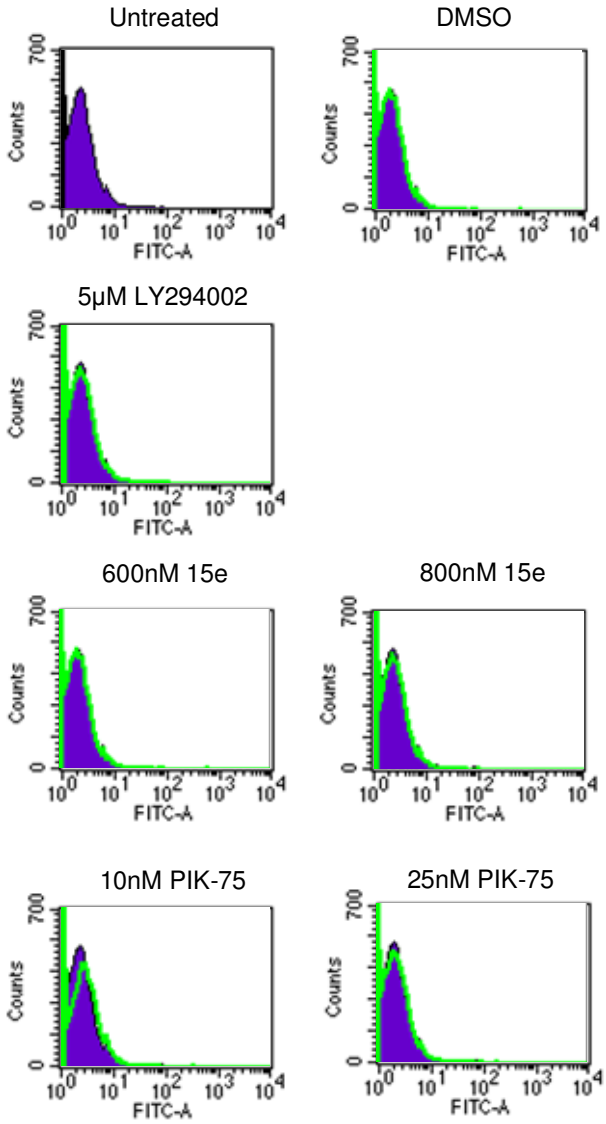



**Figure 5.3 Inhibitors of p110α do not exhibit fluorescence**

Murine ESCs were incubated for 18h in the absence of any treatment or in the presence of vehicle (DMSO), LY294002, compound 15e or PIK-75 at the concentrations indicated. Data are represented as a histogram of cell counts vs. fluorescence intensity, where untreated cells are represented by a purple trace and inhibitor treatments overlaid in green.

An example of the fluorescence profile exhibited by untreated control mESCs is represented by the purple trace in Figure 5.4**A**. When mESCs are stained with $DiOC_6$, a positive shift in fluorescence intensity is observed (green trace) (Figure 5.4**A**). During the early stages of apoptosis, the mitochondrial membrane becomes permeable and the proton gradient is lost (Petit et al., 1995, Rottenberg and Wu, 1998), thus cells undergoing apoptosis exhibit reduced fluorescence, evident by a negative shift of the histogram trace. It is known that $DiOC_6$ stains the mitochondrial membrane of live cells, but at higher concentrations, non-specific staining of the endoplasmic reticulum also occurs (Terasaki, 1989). Since staining of the endoplasmic reticulum is unaffected in the early stages of apoptosis, non-specific staining can mask the detection of loss of mitochondrial membrane integrity by keeping fluorescence high. Therefore care was taken to select a dose where only specific staining occurred. In most cell types, less than 10nM $DiOC_6$ typically gives specific mitochondrial membrane staining, for mESCs, 1nM $DiOC_6$ was found to be suitable. As a positive control, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupling agent, was used to induce apoptosis. DMSO treated cells were used as a negative control (purple trace) to compare the fluorescence profiles of cells treated with inhibitors (Figure 5.4**B**). When mESCs were treated with CCCP (green trace) prior to $DiOC_6$ staining, a negative shift in fluorescence intensity was observed compared to DMSO treated cells (purple trace). This negative shift indicates reduced $DiOC_6$ staining consistent with the presence of apoptotic cells. An alternative way of analysing this flow cytometry data is to assess the percentage of cells with lower fluorescence intensity in each treatment. In DMSO treated cells, 32.8% of cells were unstained (Figure 5.4) whereas treatment of mESCs with CCCP induced apoptosis so that 77.3% of cells were found to have lower fluorescence intensity. This confirmed that 1nM $DiOC_6$ was suitable for detecting apoptotic cells and that CCCP was a satisfactory positive control.

When comparing treatment of mESCs with 5μM LY294002 (green trace) and treatment with DMSO alone (purple trace), no change in fluorescence was visible indicating that cells were not apoptotic (Figure 5.4**B**). A small enhancement in the percentage of unstained cells (of 7%) was observed with LY294002, similar to that observed previously in similar conditions (Paling et al., 2004). At 600nM compound 15e, fluorescence was similar to that of DMSO treated cells. However, 800nM compound 15e induced a small shift in fluorescence with 45% of cells adopting lower fluorescence intensity, an increase of ~12% when compared to DMSO treated cells. This indicates that at this concentration of compound 15e, a proportion of cells enter the early stages of apoptosis. Importantly, at 600nM compound 15e, viable cell counts were reduced compared to DMSO treatment (Figure 5.2) but this dose did not induce apoptosis (Figure 5.4) therefore the alteration in viable cell counts is most likely to arise predominantly from reduced cell division. Treatment of mESCs with 10 or 25nM PIK-75 failed to alter $DiOC_6$ staining compared to treatment of mESCs with DMSO alone (Figure 5.4) indicating that apoptosis is not induced by PIK-75 at these concentrations. Reduced cell numbers were detected following incubation with 10 or 25nM PIK-75 (Figure 5.2), which is not due to apoptosis but likely due to reduced cell division.

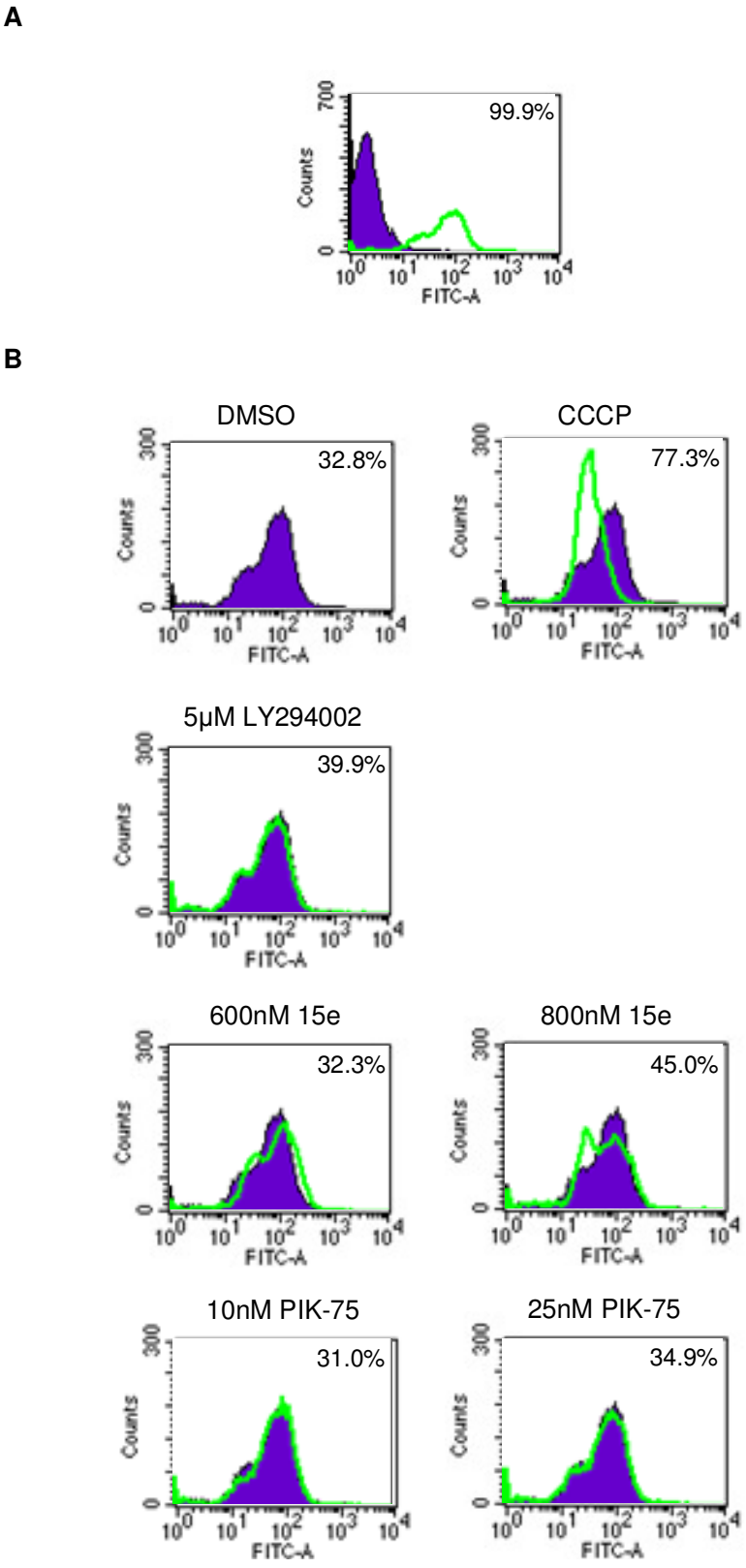



**Figure 5.4 DiOC6 staining of mESCs following p110α inhibitor treatment**

Murine ESCs were incubated for 18h in the absence of any treatment or in the presence of vehicle (DMSO), LY294002, compound 15e or PIK-75 at the concentrations indicated.

Data are represented as a histogram of cell counts vs. fluorescence intensity. **A** Untreated cells were divided into two samples. One sample was treated with 1nM $DiOC_6$ for 30 minutes in dark conditions. Both samples were analysed by flow cytometry. Unstained cells are represented by a purple trace and $DiOC_6$ stained cells shown by an overlaid green trace. **B** A sample of untreated cells were incubated with 100μM CCCP for 20 minutes and used as a positive control. Samples were treated with 1nM $DiOC_6$ for 30 minutes in dark conditions and fluorescence measured by flow cytometry. DMSO treated cells were used as a representative negative control shown by a purple trace and inhibitor treatments represented as a green trace. $DiOC_6$ staining data were analysed using Cell Quest flow cytometry software (BD). The percentage of cells exhibiting lower fluorescence intensity (cells potentially in early apoptosis) for each treatment is indicated.

Taking into account the results of viable cell counts and $DiOC_6$ staining following inhibition of p110α with selective inhibitors, the reduction in mESC cell numbers observed following inhibition of p110α PI3Ks appears most likely to arise as a result of reduced cell division, rather than a significant increase in apoptosis. Therefore, these studies support a role for p110α coupled to mESC proliferation, consistent with previous reports highlighting a proliferative defect and embryonic lethality, but no abnormal apoptosis, in p110α knock-out mouse embryos (Bi et al., 1999).

## 5.3 p110α is implicated in insulin and LIF signal transduction in mESCs

Insulin was shown to activate signalling downstream of PI3Ks in mESCs cultured in a chemically defined media (Figure 3.3) (Welham et al., 2007). This media, of known components, contains the supplements N2 and B27, both of which contain insulin (Table 2.1) and insulin is also a likely component of undefined serum replacement, used for the routine culture of mESCs. PI3K activity is reported to be essential for glucose metabolism and the survival of blastocyst stage embryos (Riley et al., 2006). Since mESCs are derived from blastocysts, identification of the isoform or isoforms mediating insulin signalling in mESCs was of interest.

Insulin target tissues typically express p110α and p110β as a result of their ubiquitous expression, whereas the expression of p110δ is largely restricted to cells of the immune system (Vanhaesebroeck et al., 1997). Predictably, both p110α and p110β have been implicated in insulin signalling. Studies of knock-in mice expressing a catalytically inactive form of p110α and investigations using selective p110α inhibitors have mapped out a predominant role in insulin signalling and in functional responses to insulin for p110α (Foukas et al., 2006, Knight et al., 2006). However, p110β has also been reported to mediate insulin signalling in certain cell types (Asano et al., 2000, Hooshmand-Rad et al., 2000, Roche et al., 1994).

Selective p110 inhibitors were used to identify which isoform or isoforms participate in insulin-induced signalling in mESCs. In vehicle (DMSO) pre-treated cells, incubation with insulin led to enhanced PKB phosphorylation at Ser473 (Figure 5.5**A**), consistent with the activation of the PI3K-PKB pathway by insulin in other cell types (Cross et al., 1995) and in mESCs (Figure 3.3) (Welham et al., 2007). Basal PKB phosphorylation was decreased by pre-treatment with the broad selectivity PI3K inhibitors LY294002 and wortmannin, and by the dual p110α/mTOR inhibitor PI-103. Inhibition of insulin-induced PKB phosphorylation by PI-103 is consistent with observed effects of this inhibitor in Chinese hamster ovary cells expressing human insulin receptor (CHO-IR cells) (Chaussade et al., 2007). Insulin-stimulated PKB phosphorylation was not visibly prevented by pre-treatment with LY294002, TGX-121 or IC87114, indicating that p110β and p110δ are unlikely to be involved in insulin signalling in mESCs. Conversely, pre-treatment with PI-103 the dual p110α/mTOR inhibitor revealed a decrease in insulin-induced PKB phosphorylation (Figure 5.5**A**).

The involvement of p110α in insulin-induced signalling was further investigated using the selective p110α inhibitors, compound 15e and PIK-75. Treatment with compound 15e reduced basal PKB phosphorylation at Ser473 in unstimulated cells (Figure 5.5**B**). Compound 15e pre-treated cells also had reduced levels of PKB phosphorylation following insulin stimulation compared to vehicle pre-treated insulin-stimulated cells. Furthermore, increasing concentrations of compound 15e resulted in decreasing insulin-stimulated PKB phosphorylation. Similarly, both basal and insulin-induced S6 phosphorylation were reduced by compound 15e pre-treatment (Figure 5.5**B**). PIK-75 pre-treatment induced a concentration-dependent decrease in basal levels of PKB phosphorylation in unstimulated cells (Figure 5.5**C**). A pronounced decrease in insulin-induced PKB phosphorylation was observed in cells pre-treated with PIK-75. As with compound 15e, an inverse relationship between PIK-75 concentration and levels of insulin-induced PKB phosphorylation were observed. Additionally, both basal and insulin-stimulated S6 phosphorylation were reduced by PIK-75 pre-treatment. The present findings are consistent with reports of the involvement of p110α in insulin signal transduction in other cell types (Foukas et al., 2006, Knight et al., 2006).

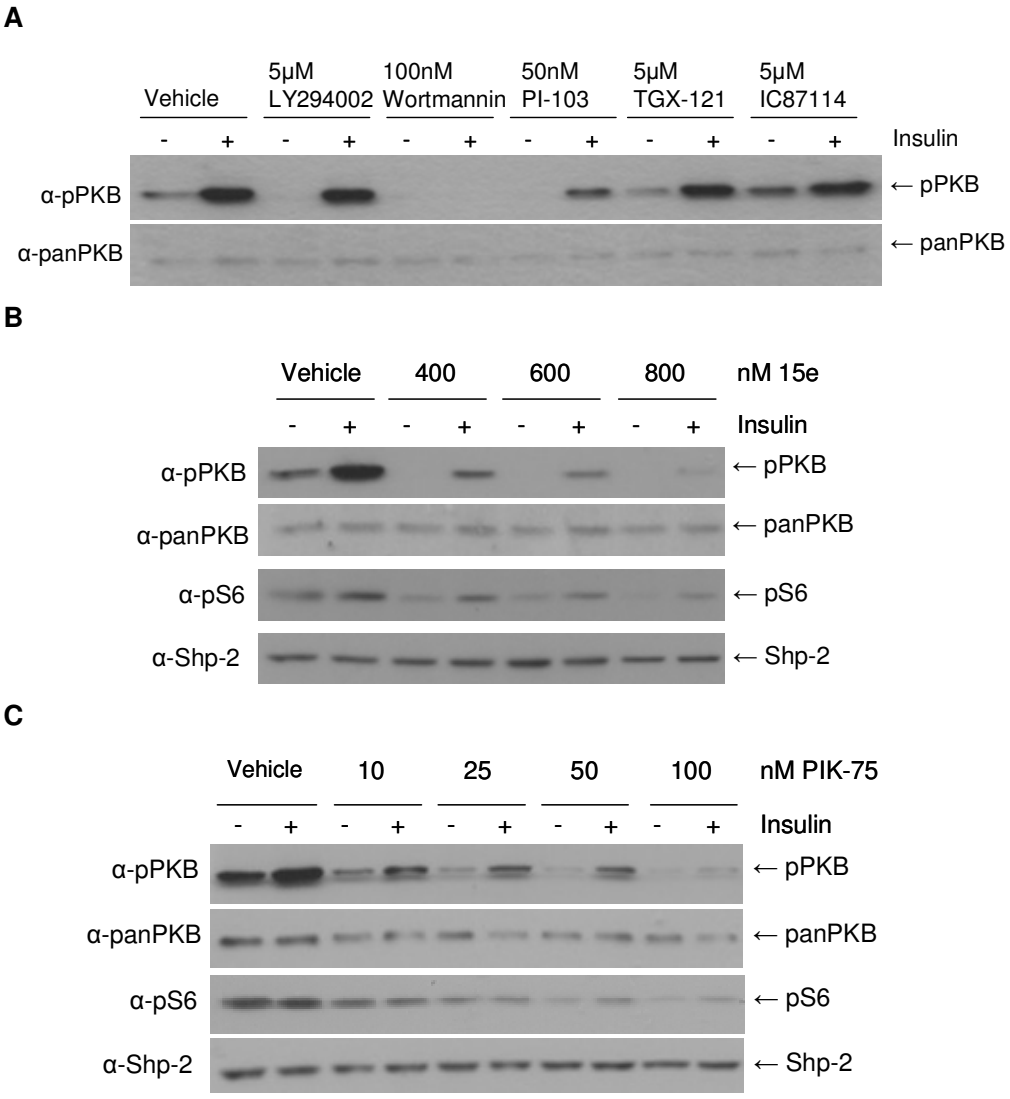



**Figure 5.5 p110α mediated insulin induced signalling**

Murine ESCs were pre-treated with vehicle (DMSO), LY294002, Wortmannin, PI-103, TGX-121 or IC87114 (**A**), compound 15e (**B**) or PIK-75 (**C**) at the concentrations indicated. Following pre-treatment, mESCs were stimulated with 10μg/ml insulin for 5 min. The signalling downstream of PI3Ks was assessed using the antibodies indicated and equal loading was assessed by stripping and reprobing blots with appropriate antibodies. The data shown are representative of three independent experiments for each panel.

More recently, redundant functionality for PI3K catalytic isoforms has been reported. Knockdown of p110α or p110β with short-hairpin RNA in CHO-IR cells did not affect insulin-induced PKB phosphorylation (Kubo et al., 2005). Additionally, in a report using selective isoform inhibitors, independent inhibition of p110α, p110β or p110δ had little effect on insulin-induced PKB phosphorylation in a human hepatoma (HepG2) cell line (Chaussade et al., 2007). However, inhibition of p110α and p110β or p110α and p110δ simultaneously resulted in a significant attenuation in insulin signalling via PKB, suggesting functional redundancy of these isoforms (Chaussade et al., 2007). Of interest, neither p110α ($p110\alpha^{+/-}$) nor p110β ($p110\beta^{+/-}$) heterozygote mice are insulin resistant but combined heterozygote mice ($p110\alpha^{+/-}$ $p110\beta^{+/-}$) exhibit slight glucose intolerance (Brachmann et al., 2005). It has been suggested that these findings demonstrate functional redundancy *in vivo*, however, a ~50% reduction in p85 expression was reported in insulin-sensitive tissues of $p110\alpha^{+/-}$ $p110\beta^{+/-}$ mice (Brachmann et al., 2005), which could explain the observed phenotype.

Having already demonstrated the participation of p110α in mESC insulin signalling, the possibility of functional redundancy of p110β and p110δ in insulin signalling of mESCs was explored.

In mESCs pre-treated with the p110β inhibitors, TGX-121 or TGX-221, alone or the p110δ inhibitor, IC87114, alone, insulin-induced PKB phosphorylation at Ser473 was unaffected compared to untreated cells (Figure 5.6). When p110β and p110δ were inhibited simultaneously, no change in PKB phosphorylation was observed suggesting that p110β and p110δ do not exhibit redundancy in terms of insulin signalling and confirming that these isoforms do not participate in insulin signalling in mESCs.

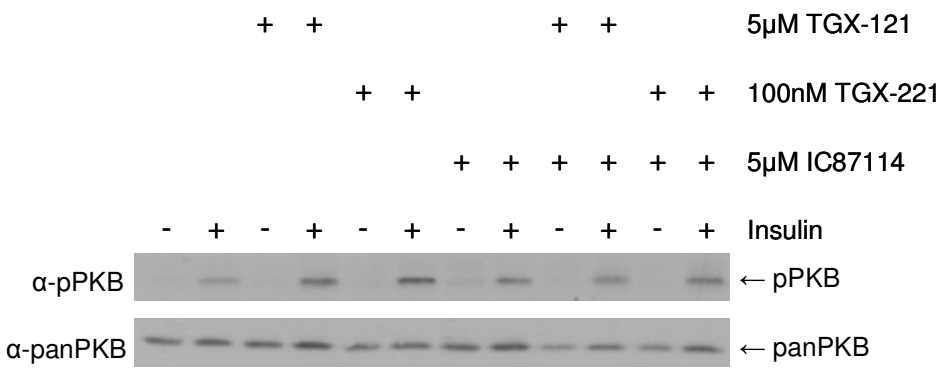



**Figure 5.6 p110β and p110δ do not exhibit functional redundancy in mESC insulin signalling**

Murine ESCs were pre-treated with 5μM TGX-121, 100nM TGX-221 or 5μM IC87114 alone and in combination as indicated. Cells were stimulated with 10μg/ml insulin for 5 min. Following SDS-PAGE and immunoblotting, PKB phosphorylation at Ser473 was detected with appropriate antibodies. Immunoblots were stripped and reprobed using antibodies detecting PKB to assess equal loading. The example shown is representative of two independent experiments.

### *5.3.1 LIF-induced activation of signalling downstream of PI3K is mediated by p110α*

LIF-mediated signalling was shown to be unperturbed by the inhibition of p110β (Figure 4.12) despite a major role for this isoform in regulating self-renewal of mESCs. To that end, evidence presented in the previous chapter suggests that p110β might be activated via GPCRs (Figure 4.13). However, LIF was shown to activate signalling downstream of PI3Ks in mESCs (Paling et al., 2004) (also see section 3.2). To test if p110α mediates LIF-induced signalling the selective inhibitors compound 15e and PIK-75 were used.

In the absence of inhibitors, LIF stimulation of mESCs induced an enhancement in the phosphorylation of PKB at Ser473 and S6 at Ser235/236 (Figure 5.7**A** and **B**) Following pre-treatment with compound 15e (Figure 5.7**A**) or PIK-75 (Figure 5.7**B**), basal unstimulated levels of PKB and S6 phosphorylation were reduced when compared to unstimulated, vehicle pre-treatment alone. Pre-treatment with compound 15e resulted in the inhibition of LIF-induced PKB phosphorylation but only a small reduction in S6 phosphorylation (Figure 5.7**A**). Similarly, pre-treatment with PIK-75 prevented LIF-

induced PKB phosphorylation but no change in LIF-induced S6 phosphorylation (Figure 5.7**B**). This observation is interesting since LIF is the predominant extrinsic factor known to promote self-renewal and is required to maintain undifferentiated mESCs in culture (Bard and Ross, 1991, Gough et al., 1989, Smith et al., 1988, Williams et al., 1988) but p110α was demonstrated not to couple to the maintenance of self-renewal (Section 4.4.5).

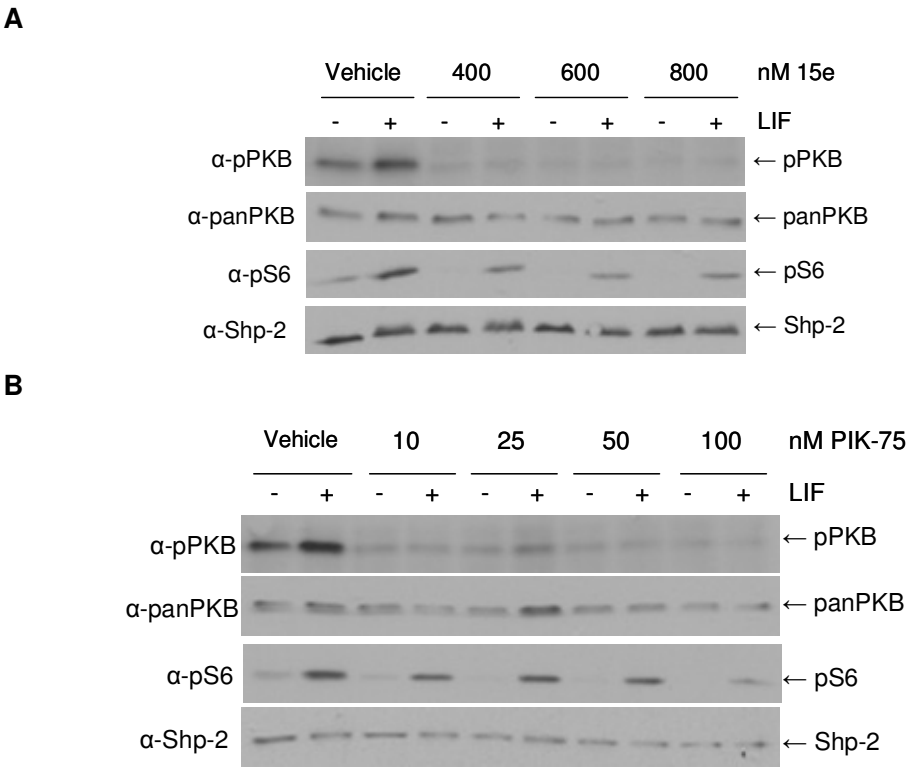



**Figure 5.7 Inhibition of p110α prevents LIF-mediated signalling**

Murine ESCs were treated with compound 15e (**A**) or PIK-75 (**B**) for 30min prior to stimulation with $10^3$ U/ml LIF for 10min. Cell lysates were resolved by SDS-PAGE and immunoblotting carried out using the antibodies indicated to detect signalling downstream of PI3K.

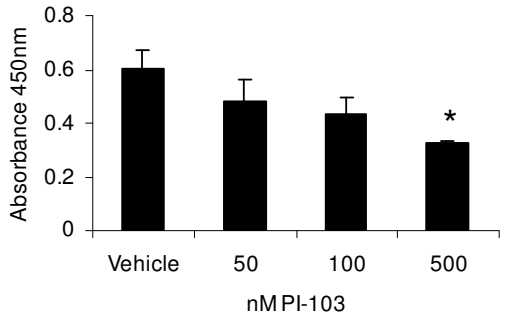
## 5.4 p110α-mediated signalling regulates mESC metabolism

Having identified a role for p110α in proliferation and insulin signalling in mESCs, it seemed plausible that p110α may also couple to the regulation of mESC metabolism. An assessment of mESC metabolism was made following incubation with selective p110 isoform inhibitors.

An XTT-based metabolism assay relies on the cleavage of the yellow tetrazolium salt XTT by mitochondrial dehydrogenases, present in metabolically active cells, to produce an orange, water-soluble, formazan dye product (Roehm et al., 1991, Scudiero et al., 1988). The bioreduction of XTT is potentiated by the addition of PMS, an electron coupling agent. When the assay has developed, over a period of 4 hours, optical density is measured at 450nm. Lower absorbance at 450nm, when compared to vehicle treatment, indicates a reduced amount of mitochondrial dehydrogenase activity consistent with reduced metabolic activity.

Initially, the effect of a number of p110 selective inhibitors on metabolic activity was assessed using the mouse pro-B cell line, BaF/3. Following 48 hour incubation with PI-103, BaF/3 cells displayed reduced absorbance at 450nm indicating a reduced level of metabolic activity compared to vehicle treated BaF/3 cells (Figure 5.8**A**). A statistically significant reduction in metabolic activity was detected using 500nM PI-103. In contrast to PI-103, neither TGX-121 (Figure 5.8**B**) nor IC87114 (Figure 5.8**C**) altered relative metabolic activity of BaF/3 cells.

**A**

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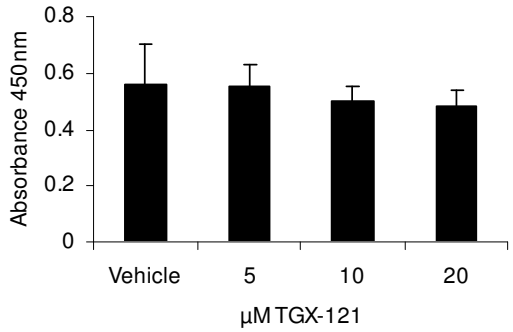



**C**

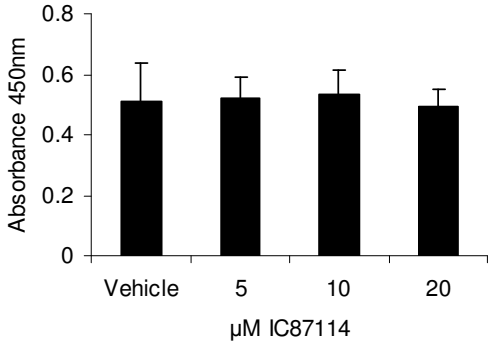
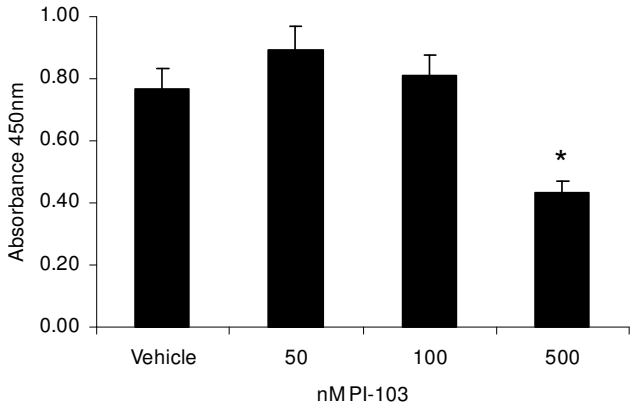



**Figure 5.8 Effect of selective isoform inhibitors on the metabolism of BaF/3 cells**
BaF/3 cells were plated in 96 well plates and vehicle (DMSO), PI-103, TGX-121 or IC87114 added at the concentrations indicated. After 48 hours incubation, XTT-PMS solution was added to each well and incubated for a further 4 hours. Plates were read for the absorbance of light at a wavelength of 450nm. Data are the average of six replicates for each treatment. Data were analysed by ANOVA and Dunnet's post-hoc test applied, where * indicates $p<0.05$. Experiments shown are representative of two independent experiments.

Next, the effect of the selective p110α isoform inhibitors on the metabolic activity of mESCs was investigated by XTT assay. Following 48 hours incubation in the presence of 50 or 100nM PI-103, absorbance at 450nm was similar to that for vehicle treated cells, indicating no change in relative metabolic activity (Figure 5.9**A**). At 500nM PI-103 produced a significant decrease in relative metabolic activity compared to vehicle treated mESCs, similar to that found with BaF/3 cells (Figure 5.8). No significant reduction in relative metabolic activity of mESCs was detected following 48 hours incubation with 100nM PIK-75 (Figure 5.9**B**). PIK-75 is known to inhibit mTOR at higher concentrations, but not in the range of selective inhibition of p110α (5-10nM) used for these studies (Knight et al., 2006). Therefore a significant reduction in metabolic activity of mESCs at 150nM could arise as the result of mTOR inhibition. Interestingly, the concentrations of PI-103 or PIK-75 that induce a significant reduction in relative metabolic activity are higher than concentrations typically used and higher than concentrations required to inhibit PI3K activity (Fan et al., 2006, Knight et al., 2006). The effects of compound 15e on mTOR have not been reported but compound 15e is typically used at 600nM in the present study, a concentration shown to inhibit insulin signalling via p110α (Figure 5.5**C**). Treatment of mESCs with compound 15e produced a significant reduction in metabolic activity at 500nM and above (Figure 5.9**C**).

**A**

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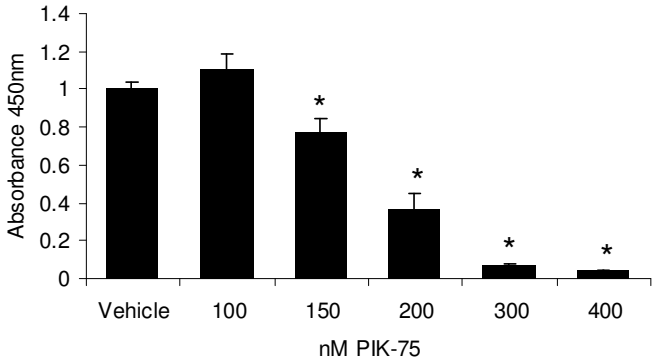


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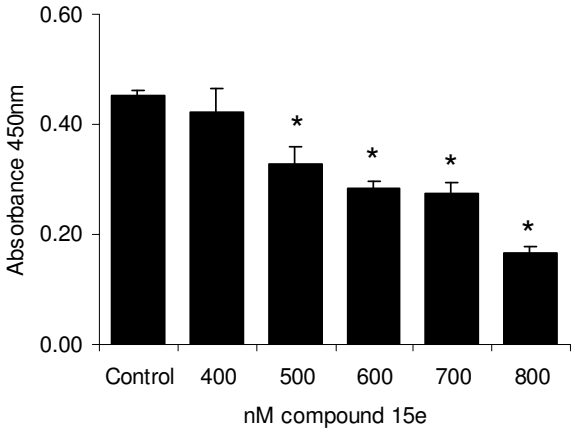



**Figure 5.9 Effect of p110α isoform inhibitors on the metabolism of mESCs**

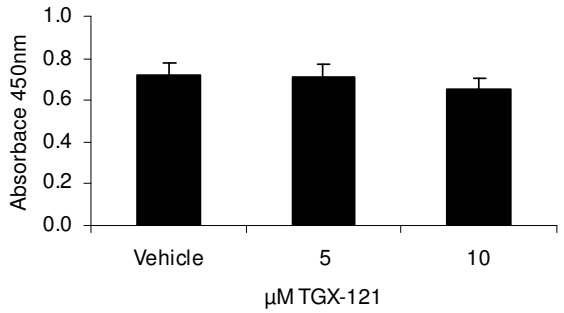
Murine ESCs were plated in 96 well plates and vehicle (DMSO), PI-103 (**A**), PIK-75 (**B**) or compound 15e (**C**) added at the concentrations indicated. After 48 hours incubation, XTT-PMS solution was added to each well and incubated for a further 4 hours. Plates were read for the absorbance of light at a wavelength of 450nm. Data are the average of six replicates for each treatment and was analysed by ANOVA and Dunnet's post-hoc

test, where * indicates $p<0.05$. Experiments shown are representative of three independent experiments.

These three inhibitors, with selectivity for p110α, appear to produce conflicting results. It is known that mTOR is important for the proliferation of mESCs (Murakami et al., 2004) and that PIK-75 displays off-target inhibition of mTOR at μM concentrations. However, if off-target inhibition was suspected, only higher concentrations of PIK-75 would induce a significant decrease in metabolic activity. Additionally, given the dual inhibition of mTOR and p110α by PI-103 (Fan et al., 2006, Knight et al., 2006) a decrease in metabolic activity would have been predicted at nM concentrations, previously shown to inhibit signalling downstream of mTOR (Fan et al., 2006). Thus, typical doses of PI-103 and PIK-75 did not induce reduced metabolic activity. In contrast, compound 15e reduced metabolic activity at concentrations typically used (600nM) although no evidence exits for the inhibition of mTOR by compound 15e, this may explain a decrease in metabolic activity. In any case, the implications of mTOR inhibition on mESC fate cannot be ignored and are addressed in Sections 5.5.1 and 5.5.2.

The effect of inhibition of p110β and p110δ on mESC metabolic activity was also investigated. Treatment of mESCs with the less potent p110β inhibitor, TGX-121, gave no change in relative metabolic activity compared to vehicle treated cells (Figure 5.10**A**). Importantly the concentrations used to assess metabolic activity, 5 and 10μM, do cause a notable and statistically significant reduction in self-renewal (Figure 4.5). Consistent with these findings, the more potent p110β inhibitor, TGX-221, produced no change in relative metabolic activity up to 500nM (Figure 5.10**B**), whereas 100nM TGX-221 produces a significant decrease in self-renewal (Figure 4.5). IC87114, the selective p110δ inhibitor, also failed to induce a change in metabolic activity of mESCs when compared to vehicle treated mESCs (Figure 5.10**C**).

**A**

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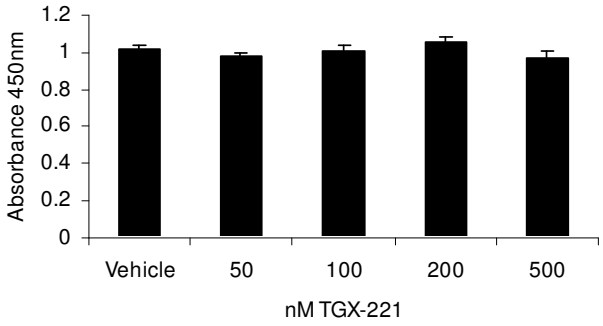



**C**

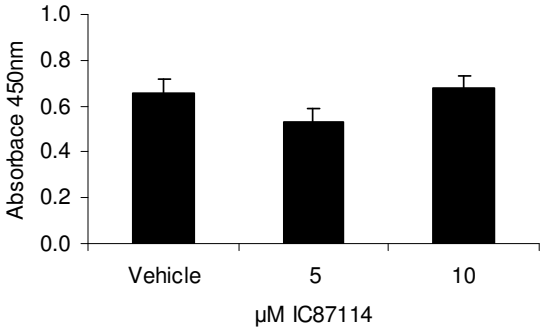



**Figure 5.10 Inhibition of p110β and p110δ does not decrease mESC metabolic activity**

Murine ESCs were plated in 96 well plates and vehicle (DMSO), TGX-121 (**A**), TGX-221 (**B**) or IC87114 (**C**) added at the concentrations indicated. After 48 hours incubation, XTT-PMS solution was added to each well and incubated for a further 4 hours. Plates were read for the absorbance of light at a wavelength of 450nm. Data are the average of six replicates for each treatment and was analysed by ANOVA and no statistical significance found. Experiments shown are representative of three independent experiments.

For completeness, the effect of ZSTK474, a selective class I PI3K inhibitor (Dan et al., 2008, Kong and Yamori, 2007, Yaguchi et al., 2006), on mESC metabolic activity was also addressed by XTT assay. ZSTK474 induced a decrease in the absorbance of light of wavelength 450nm, indicating a decrease in metabolic activity of mESCs (Figure 5.11). Concentrations of 1μM ZSTK474 induced a significant decrease in relative metabolic activity when compared to vehicle-treated control cells (Figure 5.11). LY294002 was not shown to result in reduced metabolic activity of mESCs following 48 hours incubation, measured by XTT assay (Paling et al., 2004). This difference in ZSTK474 and LY294002 is likely the result of different inhibitor profiles and possibly due to the inhibition of other classes of PI3K by LY294002. Incidentally, the decreased metabolism detected in mESCs following incubation with ZSTK474 is consistent with previous findings demonstrating reduced growth and proliferation of tumour cells in mice following ZSTK474 administration (Yaguchi et al., 2006) and with reduced proliferation, but not apoptosis, of human cancer xenografts (Dan et al., 2008).

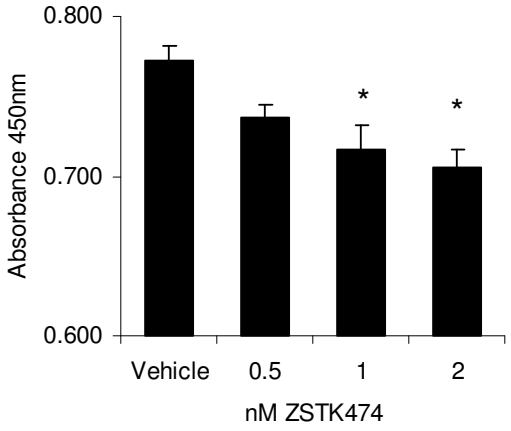



**Figure 5.11 ZSTK474 induces a reduced metabolic activity of mESCs**

Murine ESCs were plated in a 96 well plate in the presence of vehicle (DMSO) or ZSTK474 at the concentrations indicated. After 48 hours incubation, XTT-PMS solution was added to each well and incubated for a further 4 hours. The plate was read for the absorbance of light at a wavelength of 450nm. Data are the average of six replicates for each treatment and was analysed by ANOVA and Dunnet's post-hoc test, where * indicates $p<0.05$. The experiment shown is representative of two independent experiments.

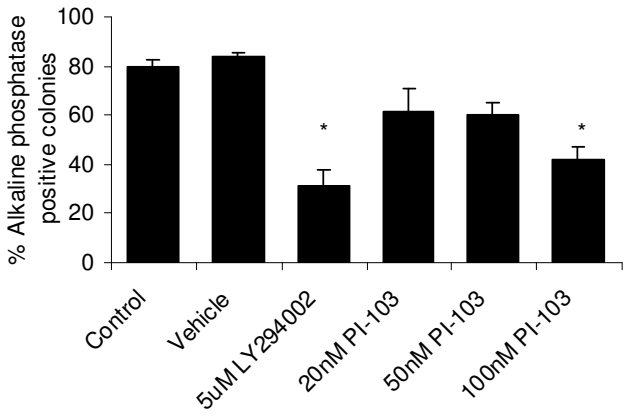
## 5.5 Are proliferation and self-renewal linked?

Self-renewal is frequently defined as proliferation with the suppression of differentiation. However, no reports exist that investigate these two cellular processes as a linked function in mESCs. The present study has defined distinct roles for p110α in regulating mESC proliferation and insulin signalling and p110β in maintaining mESC self-renewal. Furthermore, at concentrations of the selective p110β inhibitors, TGX-121 and TGX-221, which induce a loss of self-renewal (Figure 4.5), no significant alterations in metabolic activity are detected (Figure 5.10**A** and **B**) indicating that differentiation can occur without detectable and significant changes in metabolism. Conversely, inhibition of p110α reduces mESC proliferation (Figure 5.2), metabolism (Figure 5.9**B** and **C**) and insulin signalling (Figure 5.5**B** and **C**), with no change in self-renewal (Figure 4.25), although differentiation is not restricted in mESCs with reduced proliferation (Figure 4.26), indicating that these events are likely to be regulated independently.

### *5.5.1 Inhibition of mTOR and p110α by PI-103 results in a decrease in self-renewal*

The notion that proliferation and self-renewal are linked was initially explored using PI-103, an inhibitor that is commonly described as a dual mTOR and p110α inhibitor (Fan et al., 2006, Hayakawa et al., 2001, Knight et al., 2006). As demonstrated previously, treatment of mESCs with vehicle does not significantly alter alkaline phosphatase staining, indicating no change in self-renewal (Figure 5.12**A**). Treatment with LY294002 results in a significant decrease in alkaline phosphatase staining, typically reduced to approximately 30% alkaline phosphatase positive colonies of all colonies present after 4 days incubation. Incubation of cells with PI-103 also resulted in a decrease in alkaline phosphatase staining (Figure 5.12**A**). At 100nM PI-103, a significant decrease in alkaline phosphatase positive colonies was observed. Furthermore, at 50nM PI-103, no change in Nanog expression was observed (Figure 5.12**B**). However, 100nM PI-103 induced a decrease in the expression of Nanog after 4 days incubation, which was comparable to the expression of Nanog in mESCs incubated in the absence of LIF (Figure 5.12**B**). A decrease in alkaline phosphatase staining and a reduction in Nanog expression are symptomatic of a loss of self-renewal but only occur at the highest concentration of PI-103 used in the present study.

**A**

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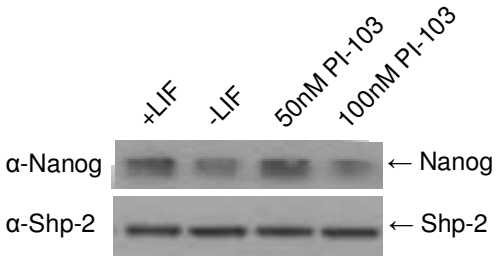



**Figure 5.12 PI-103 induces a reduction in self-renewal**

**A** Murine ESCs were plated at clonal density and incubated in the presence of PI-103. After 4 days self-renewal was assessed by alkaline phosphatase staining and colonies were scored for staining and colony morphology. Data are represented as the mean percentage of alkaline phosphatase positive, self-renewing colonies with S.E.M. (n=6). Data were analysed by ANOVA and Dunnet's post hoc test used to determine which treatments were significant compared to vehicle treatment. **B** mESCs were incubated in the presence or absence of LIF and in the presence of LIF plus PI-103 at the concentrations indicated for 4 days. Protein lysates were analysed for the expression of Nanog immunoblotting with the antibodies indicated. Immunoblots were stripped and reprobed using antibodies detecting Shp-2 to assess equal loading.

PI-103 is reported to inhibit p110α with an $IC_{50}$ value of 8nM and rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 with $IC_{50}$ values of 20 and 83nM respectively (Knight et al., 2006). However, PI-103 is only slightly more selective for p110α than the other p110 isoforms which are reported to have $IC_{50}$ values of 88, 48, 150nM for p110β, p110δ and p110γ respectively (Knight et al., 2006). Additionally, in

Chapter 4, it was demonstrated that selective inhibition or knockdown of p110α did not alter self-renewal where as, a predominant role for p110β in regulating mESC self-renewal was defined (Kingham and Welham, In Press). Therefore, the loss in self-renewal exhibited by mESCs following incubation with PI-103 is likely to arise as a result of inhibition of p110β and is certainly plausible given the inhibition profile of PI-103 and the concentrations used to induce this effect. However, it is difficult to compare functional effects with $IC_{50}$ values obtained from in vitro assays since factors such as cell permeability, stability of inhibitors under culture conditions and incubation periods can alter the concentration of inhibitor available to inhibit target proteins.

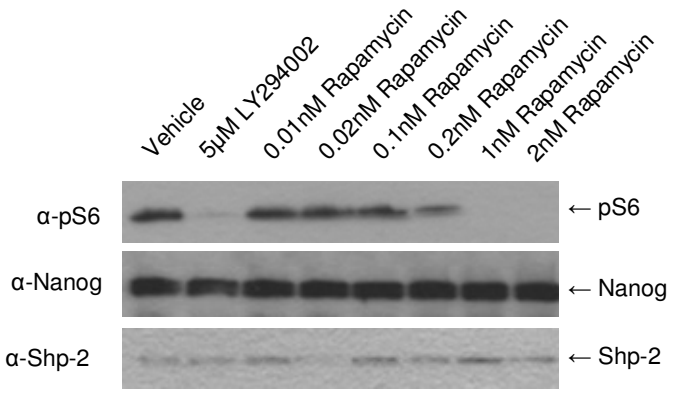
### *5.5.2 Partial inhibition of mTOR reveals a synergistic relationship between self-renewal and proliferation*

As a point of interest, inhibition of PI3Ks with LY294002 decreases mESC alkaline phosphatase staining to a similar, but consistently lower, level than that observed using inhibitors of p110β. One explanation for this might be the simultaneous inhibition of PI3Ks and mTOR, since LY294002 is reported to also target mTOR (Brunn et al., 1996, Knight et al., 2006) and mTOR activity and expression is reported to be required for mESC proliferation (Murakami et al., 2004), symptomatic of a synergistic relationship between self-renewal and proliferation.

In order to investigate possible synergy between self-renewal and proliferation, it was necessary to be able to manipulate proliferation in a manner that allowed a loss of self-renewal to be induced and subsequently quantified. It has previously been reported that mTOR activity is an important regulator of mESC proliferation in studies which involved the use of the specific mTORC1 inhibitor, rapamycin, amongst other methods (Murakami et al., 2004). Therefore, a range of concentrations of rapamycin were tested to find a suitable concentration that resulted in partial inhibition of mTOR and a reduction, but not complete suppression, of proliferation. After 24 hours incubation, analysis of S6 phosphorylation, downstream of mTOR, indicated that 1nM rapamycin inhibited mTOR sufficiently to reduce S6 phosphorylation (Figure 5.13**A**). In contrast, no change in S6 phosphorylation, compared to vehicle treated mESCs, was detected using 0.01 or 0.02nM rapamycin (Figure 5.13**A**). This indicates that a suitable, partial inhibition of mTOR can be achieved between 0.1 and 0.2nM. In addition, no change in Nanog expression was detected using rapamycin. Following 48 hours incubation with rapamycin, an XTT assay was used to assess relative metabolic activity of mESCs. As

expected, rapamycin decreased relative metabolic activity (Figure 5.13**B**), consistent with a reported role for mTOR in mESC proliferation (Murakami et al., 2004). At concentrations of 0.25nM and greater, the decrease in relative metabolic activity induced by rapamycin was significantly different from vehicle treated mESCs (Figure 5.13**B**). Therefore, 0.1nM appeared to be a suitable concentration of rapamycin to use to assess a possible relationship between proliferation and self-renewal since it resulted in partial inhibition of signalling downstream of mTOR and reduced metabolic activity.

**A**




**B**

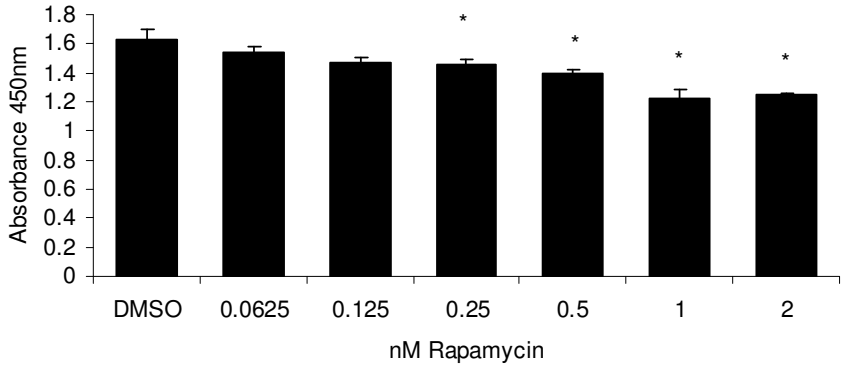
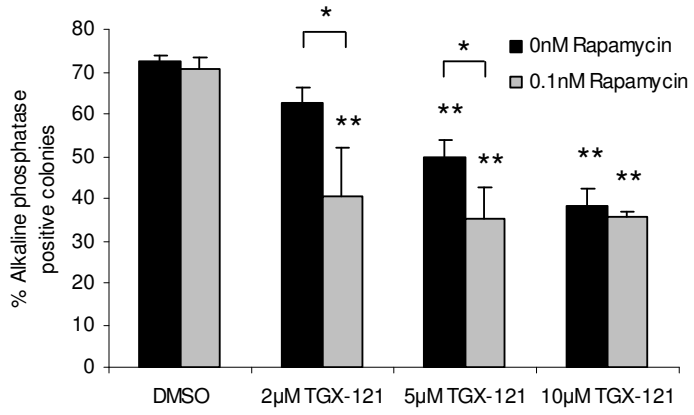



**Figure 5.13 Identifying a concentration of rapamycin that partially inhibits mTOR**
Murine ESCs were incubated with vehicle (DMSO), LY294002 or rapamycin at the concentrations indicated. **A** After 24 hours cells were harvested. Protein lysates were analysed for S6 phosphorylation by immunoblotting. Immunoblots were stripped and reprobed to assess Nanog expression, and to assess equal loading, antibodies detecting Shp-2 were used. The data shown are representative of two independent experiments. **B** After 48 hours an XTT assay was conducted to assess relative metabolic activity (see Sections 2.4.1 and 5.4 for details). Mean absorbance at 450nm of 6 replicates is shown with standard deviations (n=1). Statistical analysis was conducted

using AVOVA and Dunnet's post hoc test to compare each concentration of rapamycin to vehicle treatment, where * indicates $p<0.05$.

To test the hypothesis that proliferation and self-renewal were linked, mESCs with reduced proliferation, due to partial mTOR inhibition, were induced to differentiate, by inhibition of p110β, and changes in self-renewal assessed by alkaline phosphatase assay. Treatment with 0.1nM rapamycin did not significantly alter the percentage of alkaline phosphatase positive colonies after 4 days incubation when compared to control colonies (Figure 5.14**A**). As demonstrated previously (Figure 4.5**E**), treatment with TGX-121 induces a significant reduction in alkaline phosphatase positive colonies at concentrations of 5 and 10μM. Addition of 0.1nM rapamycin in conjunction with TGX-121 further decreased the percentage of alkaline phosphatase positive colonies compared to TGX-121 treatment alone (Figure 5.14**A**). In particular, addition of rapamycin in the presence of 2 and 5μM TGX-121 induced a significant decrease in the percentage of alkaline phosphatase positive colonies compared to 2 and 5μM TGX-121 alone. Using 10μM TGX-121, addition of rapamycin did not significantly decrease alkaline phosphatase staining compared to 10μM TGX-121 alone. This apparent plateau was reached at 36%, similar to the level of self-renewal observed following incubation with LY294002 (Figure 5.12). These results indicate that a minor reduction in proliferation can potentiate a loss of self-renewal but does not induce a loss of self-renewal alone. TGX-121 treatment did not alter the number of colonies per tissue culture dish (Figure 5.14**B**). Additionally, since a low dose of rapamycin had been used, no alteration in colony number was observed with 0.1nM rapamycin treatment compared to vehicle treatment. Furthermore, treatment with both TGX-121 and rapamycin did not significantly alter the number of colonies per treatment dish when compared to vehicle treatment or to TGX-121 treatment alone (Figure 5.14**B**).

**A**

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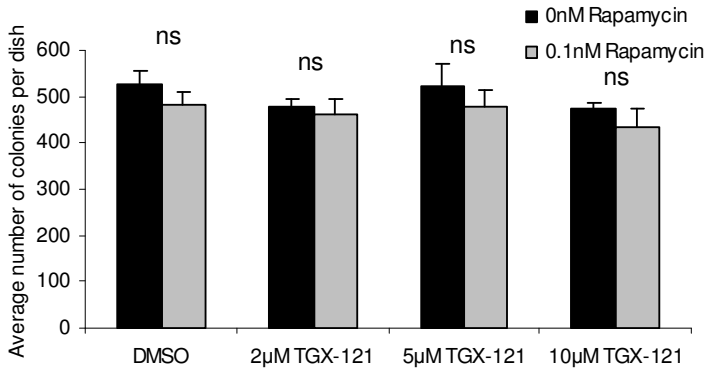


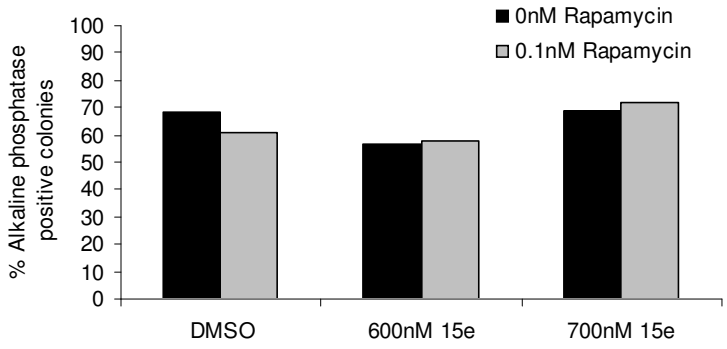

**Figure 5.14 Partial inhibition of mTOR potentiates the loss in self-renewal observed upon inhibition of p110β**

Murine ESCs were incubated in the presence or absence of 0.1nM rapamycin and TGX-121 at the concentrations indicated. After 4 days colonies were fixed and stained for alkaline phosphatase. Data are represented as the mean percentage of alkaline phosphatase colonies (**A**) or as the average number of colonies per tissue culture dish (**B)** with S.E.M (n=3). Statistical analysis was conducted using AVOVA and Fisher's post hoc test to compare each treatment, where * indicates $p<0.05$ and ** indicates $p<0.01$ and ns indicates not significantly different.

At the highest concentration of TGX-121 used, a level of self-renewal similar to that observed with LY294002 is attained. Presumably, the loss of self-renewal induced by LY294002 is the result of combined inhibition of p110α and p110β and possibly mTOR. To confirm that inhibition of p110α and mTOR does not induce a loss of self-renewal, mESCs were incubated in the presence or absence of 0.1nM rapamycin and compound 15e. As previously demonstrated (Figure 4.24 and Figure 5.14), incubation with

compound 15e alone or with rapamycin alone does not alter the level of alkaline phosphatase exhibited by mESCs compared to cells treated with vehicle (Figure 5.15**A**). Furthermore, incubation in the presence of both rapamycin and compound 15e did not induce a reduction in alkaline phosphatase staining, consistent with no change in the level of self-renewal of mESCs (Figure 5.15**A**). Therefore, inhibition of both p110α and mTOR does not perturb self-renewal and, in order to potentiate a loss of self-renewal, inhibition of p110β appears to be required. Furthermore, this data suggests that decreased self-renewal, induced by incubation of mESCs with PI-103, is not attributable to the inhibition of mTOR and p110α but more likely the result of inhibition of mTOR, p110α and p110β.

**A**

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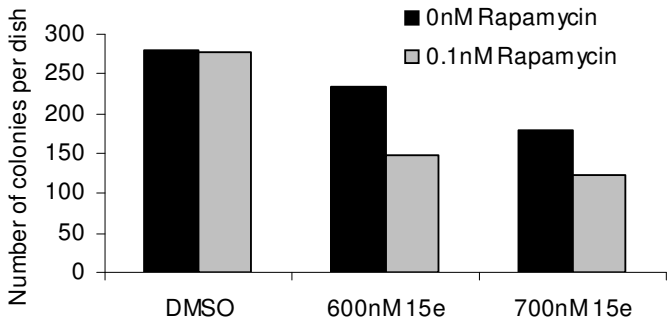



**Figure 5.15 Inhibition of p110α and mTOR does not induce a loss of self-renewal**
Murine ESCs were incubated in the presence or absence of compound 15e and rapamycin at the concentrations indicated. After 4 days, mESC colonies were fixed and stained for alkaline phosphatase. Data are represented as the percentage of alkaline phosphatase positive colonies of all the colonies present (**A**) or as the number of colonies per culture dish (**B**) (n=1).

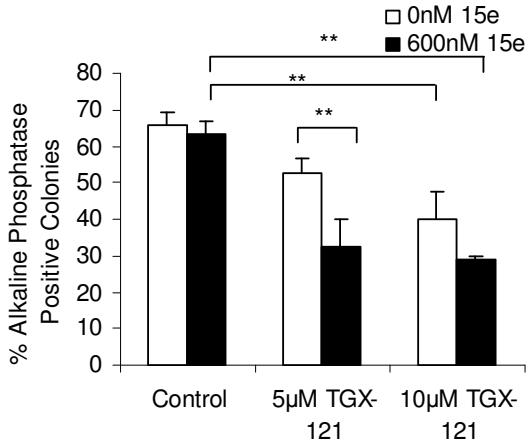
Treatment of mESCs with compound 15e was previously shown to reduce the number of colonies present after 4 days incubation compared to vehicle treatment (Figures 4.25**B** and 4.26**B**). Predictably, treatment of mESCs with both compound 15e and rapamycin potentiated the reduction in colony number observed in the presence of compound 15e alone (Figure 5.15**B**).

### *5.5.3 Simultaneous inhibition of p110α and p110β potentiates a loss of self-renewal*

Given that reduced proliferation leads to a greater decline in mESC self-renewal upon the induction of differentiation by p110β inhibition, the effects observed with LY294002 could well be explained by inhibition of p110β, to induce differentiation, promoted by reduced proliferation by either inhibition of mTOR or p110α. Figure 5.14 demonstrates this potentiation in the loss of self-renewal using rapamycin to induce a partial decrease in proliferation. It has also been demonstrated that inhibition of p110α induces a decrease in mESC proliferation (Figure 5.2). Therefore, reduced proliferation induced by inhibition of p110α could further enhance a loss of self-renewal induced by inhibition of p110β.

Compound 15e was used to reduce mESC proliferation and TGX-121 and TGX-221 used to induce a loss of self-renewal. As demonstrated in the present study, incubation of mESCs with compound 15e does not induce a significant alteration in mESC self-renewal assessed by alkaline phosphatase staining (Figure 5.16**A** and **B**) or expression of Nanog (Figure 5.16**C**). In the presence of TGX-121 or TGX-221 alone, a significant decrease in alkaline phosphatase staining of mESC colonies was observed (Figure 5.16**A** and **B**). Furthermore, this decrease in alkaline phosphatase staining was enhanced in the presence of both TGX-121 and compound 15e (Figure 5.16**A**) or TGX-221 and compound 15e (Figure 5.16**B**). Consistent with this reduction in alkaline phosphatase staining, the presence of inhibitors of p110β and p110α induced a large decrease in the expression of Nanog, indicating a greater loss of self-renewal compared to the presence of p110β or p110α inhibitors alone (Figure 5.16**C**).

**A**

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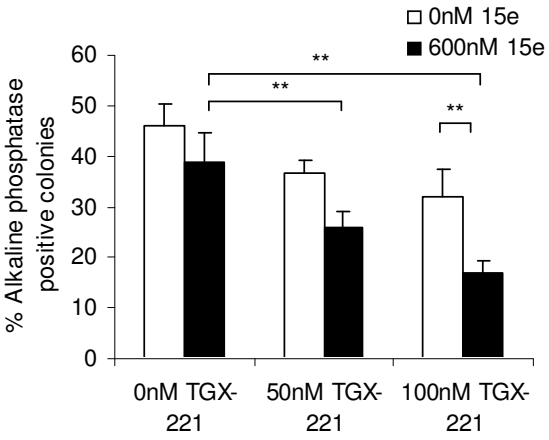


**C**

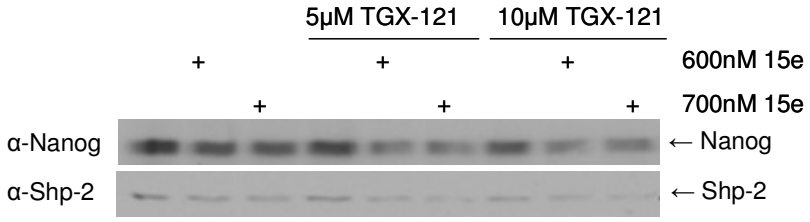


**Figure 5.16 Inhibition of p110α and p110β enhances the loss of self-renewal observed with inhibition of p110β alone**

Murine ESCs were incubated for 4 days in the presence or absence of compound 15e and in the presence or absence of TGX-121 (**A**) or TGX-221 (**B**). Self-renewal was assessed by staining for alkaline phosphatase. Data are represented as the percentage of alkaline phosphatase positive colonies with S.E.M. (TGX-121 n=4 and TGX=221 n=4). **C** After 4 days, mESCs were harvested and protein lysates analysed by immunoblotting with antibodies detecting Nanog and Shp-2.

## 5.6 Summary and discussion: Involvement of p110 isoforms in regulating mESC proliferation

In the previous chapter it was demonstrated that mESCs required neither full expression of p110α nor activity of p110α in order to maintain optimal self-renewal (Figures 4.21, 4.22, 4.24 and 4.25). However, mESCs were shown to display indications of limited growth and proliferation in the presence of selective inhibitors of p110α in that the colonies formed from mESCs incubated in the presence of PIK-75 and compound 15e were smaller and fewer in number compared to untreated or vehicle treated mESC colonies (Figure 4.25). Consistent with this observed reduction in colony size, significantly fewer mESCs were harvested from culture dishes incubated in the presence of inhibitors of p110α (Figure 5.1). This feature could be attributed to a loss of cells through programmed cell death, apoptosis, however this possibility was disproved. Following incubation with inhibitors of p110α, mESCs did not in general display a loss of mitochondrial membrane integrity, indicating that cells were not in the early stages of apoptosis (Figure 5.4). However, the long-term effects of p110α inhibition were not addressed and therefore further investigation would be required to confirm that apoptosis was not induced following 4 days incubation with p110α inhibitors, the same time point used for alkaline phosphatase assay to assess self-renewal. Additionally, only one method of assessing apoptosis was employed, other techniques suitable for mESCs include, detection of p53 translocation to the mitochondria (Han et al., 2008, Mihara et al., 2003), annexin V and propidium iodide staining analysed by flow cytometry and detection of cleaved Caspase 3 by immunoblotting (Duval et al., 2006).

In light of reports indicating a role for p110α in regulating insulin signalling, proliferation and growth of many cell types (Bi et al., 1999, Fan et al., 1999, Foukas et al., 2006, Hooshmand-Rad et al., 2000, Knight et al., 2006, Roche et al., 1994, Vanhaesebroeck and Waterfield, 1999), the present study sought to investigate the role of this isoform in mESCs. Indeed, p110α was found to couple to insulin signal transduction in mESCs (Figure 5.5), but functional redundancy with other p110 isoforms in insulin signalling, reported in other cell types (Chaussade et al., 2007), was not found in mESCs. Neither p110β nor p110δ were found to be involved in mESC insulin signal transduction to any detectable extent (Figure 5.6), which presumably allows p110β to couple effectively to maintenance of self-renewal, as demonstrated in chapter 4.

When the regulation of proliferation and metabolism by p110α were assessed, doses of PI-103 and PIK-75 that affected mESC proliferation, evidenced by reduced cell numbers, did not appear to affect cellular metabolism over a period of 48 hours. Only higher doses of PIK-75, in the range that is likely to inhibit mTOR, induced reduced metabolic activity to a detectable extent. When an alternative p110α inhibitor, compound 15e was used, a reduction in metabolic activity was observed at more typical concentrations of 500nM and above. It is possible that compound 15e also inhibits mTOR, which may explain this reduction in metabolic activity, although this has not yet been reported.

This defined and selective role for p110α in regulating mESC proliferation and insulin signalling is in support of previous studies reporting proliferative effects or glucose metabolism deficiency in mESCs following inhibition of PI3Ks with LY294002 (Jirmanova et al., 2002, Lianguzova et al., 2007, Riley et al., 2005) or disruption of up and downstream signalling molecules of PI3K pathways (Murakami et al., 2004, Stiles et al., 2002, Sun et al., 1999). Furthermore, the present study has identified the PI3K catalytic isoform that mediates these cellular functions and these results are consistent with the over-expression of p110α being able to rescue knockout of ERas mESCs and the subsequent proliferative defects (Takahashi et al., 2003) and consistent with the plasma membrane localisation and interaction with PI3K featured by ERas (Takahashi et al., 2005). Additionally, the embryonic lethality resulting from proliferative defects in mouse embryos deficient for p110α expression is consistent with the regulation of proliferation and insulin signalling of mESCs, isolated from blastocyst stage embryos.

## 5.7 Summary and discussion: Possible synergy of proliferation and self-renewal

The present study has defined roles for p110α in mESC proliferation and insulin signalling and a role for p110β in maintenance of self-renewal (summarised in Figure 5.17). Interestingly, these investigations have found that the rate of proliferation and metabolic activity of mESCs can be reduced without perturbing self-renewal (Figures 5.2, 5.9**B** and **C**, 4.24 and 4.25), while alterations in proliferation without effects on self-renewal has been previously reported (Murakami et al., 2004, Takashi et al., 2003). The contrary is also true; self-renewal can be lost without a change in metabolism in the first 48 hours (Figure 4.5, 5.10**A** and **B**). Additionally, LIF-induced signalling downstream of PI3K does not appear to be regulated by p110β (Figure 4.12) as might be expected given the predominant role of this isoform in regulating self-renewal (Section 4.4.2) and the importance of LIF for maintaining undifferentiated mESCs (Bard and Ross, 1991, Gough et al., 1988, Smith et al., 1988, Williams et al., 1988).

The definition of self-renewal as the proliferation of ESCs whilst suppressing differentiation (Burdon et al., 2002) implies that both of these functions are required in order to satisfy self-renewal. The hypothesis that these functions are linked cooperatively is supported by results generated in the present study. In order to further enhance differentiation induced by inhibition of p110β, inhibition of p110α was also required. Importantly, the possibility that p110α might be functionally redundant in regulating self-renewal, whereby its contribution would only be observed when p110β was also inhibited, was disproved since mTOR inhibition in conjunction with p110β inhibition produced the same effect as inhibition of p110α and p110β. Furthermore, the inhibition of mTOR alone did not alter self-renewal in the present study. In a previous report from our group, 20nM rapamycin was shown to induce a reduction in the expression of Nanog after 48 hours, later than the decrease observed with LY294002 (Storm et al., 2007). In the present study, only partial inhibition of mTOR was required in order to reduce, but not completely suppress, proliferation. Therefore, in this study, only 0.1nM rapamycin was used and at this concentration, no change in Nanog expression was observed after 24 hours (Figure 5.13) and no change in alkaline phosphatase was detected after 4 days incubation with 0.1nM rapamycin alone (Figure 5.14). Additionally, inhibition of mTOR and p110α simultaneously did not perturb self-renewal indicating that inhibition of mTOR and any PI3K isoform is not sufficient to induce a loss of self-renewal, confirming that reduced self-renewal observed with PI-103 must be the result of inhibition of p110β in conjunction with p110α and mTOR as opposed to inhibition of

simply p110α and mTOR. Figure 5.17 details the findings of the present study in terms of the regulation of mESC behaviour by p110α and mTOR.

The present study supports the existence of cross-talk of pathways regulating proliferation and self-renewal, by the coupling to distinct PI3K catalytic isoforms. Evidence for this has been highlighted in other cell types and involving different cellular functions. For example, p110β was demonstrated to set a threshold for p110α in insulin signalling (Chaussade et al., 2007, Knight et al., 2006) and p110γ was reported to be activated in the first phase, prior to second phase p110δ activation, in the neutrophil respiratory bust (Condliffe et al., 2005). It is therefore feasible that p110α activity, possibly regulated by LIF, and proliferation rate regulated by p110α, might set a threshold which sensitises mESCs to changes in the level of activity of p110β. A model demonstrating how PI3K isoforms might regulate mESC behaviour is shown in Figure 5.17.

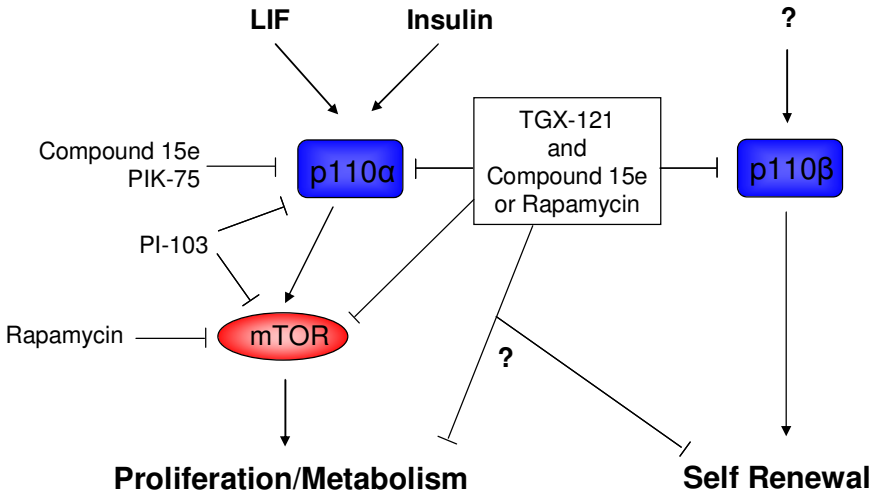


**Figure 5.17 Model of the regulation of mESC fate by p110α and potentiation of loss of self-renewal by reduced proliferation**

PI3K p110α has been implicated in mESC proliferation, metabolism and insulin signalling but does not directly regulate self-renewal. However, a partial reduction in proliferation, achieved by inhibition of either p110α or mTOR, potentiates a loss of self-renewal induced by inhibition of p110β, therefore, linking proliferation rate to the regulation of self-renewal.

# 6 GENERAL DISCUSSION AND CONCLUSIONS

## 6.1 General Discussion and future directions

Self-renewal of ESCs is essential for the maintenance of pluripotency, a feature of ESCs defined as the ability to differentiate into any specialised cell type of the adult organism. The regulation of ESC self-renewal and maintenance of pluripotency involves the coordination of a number of signalling pathways including activation by extrinsic factors, cross-talk of pathways and regulation of core transcriptional components (Boiani and Scholer, 2005, Niwa, 2007). A well defined understanding of the mechanisms which regulate self-renewal and proliferation will enable the therapeutic potential of ESCs to be fully realised. The class $I_A$ PI3K family of lipid kinases have been previously implicated in regulating mESC self-renewal (Paling et al., 2004, Storm et al., 2007) and the activity of PI3Ks and downstream signalling is reported to be important for maintaining undifferentiated mESCs (Bone et al., 2009, Pritsker et al., 2006, Sato et al., 2004, Williams et al., 2000). Furthermore, a role for PI3Ks in regulating hESC self-renewal is also beginning to be defined (Armstrong et al., 2006, Pyle et al., 2006). An alternate role for PI3Ks in regulating mESC proliferation has also been well reported (Jirmanova et al., 2002, Lianguzova et al., 2007, Murakami et al., 2004, Sun et al., 1999).

The hypothesis that maintenance of self-renewal and the regulation of proliferation might be coordinated by PI3Ks via coupling to different PI3K catalytic subunits was tested. Additionally, the present study sought to identify factors, present in routine culture media, which activate PI3Ks. Lineage commitment, often reported to be influenced by a particular pathway, was also investigated under conditions where PI3K activity was inhibited. Finally, cross-talk between the pathways regulating mESC self-renewal and proliferation was investigated.

In realising the aims of this project (defined in Section 1.4), a number of new questions regarding the regulation of mESC fate by PI3Ks have come to light. These future directions will be discussed in the present chapter. Ultimately, it is hoped that a clear understanding of the mechanisms and signalling pathways underlying ESC fate will further the potential for the use of ESCs as a model system of embryonic development, for drug toxicity screens and for regenerative medicine since understanding the regulation of proliferation and self-renewal are important for exploitation in these systems.

### *6.1.1 Inhibition of PI3Ks does not influence lineage commitment of mESCs*

Inhibition of PI3K activity was previously reported to lead to a reduction in mESC self-renewal (Paling et al., 2004, Storm et al., 2007). However, the effect of inhibiting PI3Ks on early lineage commitment of mESCs in 2D culture had not previously been investigated. The present study determined that upon inhibition of PI3Ks with the broad selectivity PI3K inhibitor LY294002, differentiation towards all lineages was neither restricted nor promoted. Although small alterations in the temporal expression of neuroectodermal and ectodermal markers were observed, generally, markers of mesoderm, endoderm, primitive ectoderm and neuroectoderm were detectable in cells incubated in both the presence and absence of LY294002. Most importantly, mESCs incubated in the presence of LY294002 exhibited a differentiation potential comparable to that observed for mESCs undergoing spontaneous differentiation induced by LIF withdrawal.

The concept of the activity of a particular pathway promoting differentiation towards a particular lineage, counteracted by an independent pathway in order to maintain an undifferentiated state is best exemplified by the balance of LIF and BMP4-induced signalling (described in Section 1.3.4.4 and Figure 1.14). Interestingly, the findings presented here demonstrate that PI3Ks regulate and integrate proliferation and self-renewal of mESCs by coupling to independent class $I_A$ PI3K isoforms. Therefore, it is feasible that, independently, PI3K isoforms might regulate lineage commitment and could be studied in more detail using small molecule, isoform selective inhibitors. The present study implicated p110β in self-renewal, a process whereby the undifferentiated state of mESCs is maintained. Selective inhibition or siRNA-mediated knockdown of p110β and subsequent analysis of expression of lineage marker genes may reveal a role for this isoform in maintaining pluripotency by inhibition of differentiation towards a particular lineage or lineages. In a similar manner, using LY294002 to inhibit PI3Ks, the role of PI3Ks in the formation and differentiation of embryoid bodies, a 3D model system of embryonic development, has previously been described (Bone and Welham, 2007), whereas the present study investigated differentiation of mESCs in 2D monolayer cultures. Thus, the requirement for the activity of different PI3K isoforms during different stages of embryoid body formation and differentiation might be of interest developmentally. Microarray data mapping the temporal expression of PI3Ks isoforms in differentiating mESCs, indicate that the expression of p110β decreases upon differentiation while expression of p110δ increases (Storm and Welham, unpublished data). Therefore, different isoforms may regulate different stages of differentiation, lineage commitment and development. Addition of selective isoform inhibitors at

different stages of embryoid body formation and analysis of marker gene expression will allow this possibility to be investigated.

### *6.1.2 Cross-talk between PI3K and MAPK pathways*

Inhibition of MAPK pathway activity has previously been reported to enhance mESC self-renewal (Burdon et al., 1999, Kunath et al., 2007) and inhibition of PI3Ks was reported to enhance Erk phosphorylation (Paling et al., 2004). Hence, the effect of activating the MAPK pathway on self-renewal and the dampening effect exerted by PI3Ks on the MAPK pathway in mESCs warranted investigation.

Using mESC clones that inducibly express a constitutively active form of Mek1 (Mek*) (generated by Prof. M. J. Welham, unpublished data) the MAPK pathway could be artificially and constitutively activated. Colonies formed from mESC clones induced to express Mek* displayed alkaline phosphatase staining that was not significantly different from clones expressing endogenous Mek alone. However, a trend towards fewer alkaline phosphatase colonies was observed following induction of Mek* expression. These results indicated that enhancing MAPK pathway activity causes only a minimal drop in self-renewal suggesting that the balance of self-renewal promoting signals and differentiation promoting signals was not sufficiently tipped towards differentiation. Additionally, the activity of other pathways might be modulated to compensate for an enhancement in MAPK pathway activity, in order to maintain self-renewal. The present study demonstrated that basal and LIF-induced Stat3 phosphorylation was normal in Mek* clones incubated in the presence or absence of tetracycline although the activation of other pathways was not addressed. An assessment of the phosphorylation states of signalling molecules, implicated in the regulation self-renewal of mESCs, in Mek* clones in the presence or absence of tetracycline may reveal alterations in the activity of other pathways.

The hypothesis that activated MAPK pathway activity was compensated by excess LIF concentrations, used for routine culture, was disproved since limiting LIF conditions supported the trend of a non-significant drop in alkaline phosphatase positive colonies when Mek* was expressed. Furthermore, this trend was abolished when mESC clones expressing Mek* were cultured in routine culture media containing serum replacement, indicating that factors present in this serum were capable of overcoming differentiation promoting signals that might be induced by constitutive activation of the MAPK pathway.

Thus, the fate of Mek* mESC clones in chemically defined, serum-free media would be of interest given that serum-free, chemically defined media is thought to contain the minimum requirements for maintaining mESCs in an undifferentiated state. Culture of Mek* clones in this media would therefore provide a more stringent environment to assess the effect of activation of MAPK pathway signalling on mESC fate. Furthermore, self-renewal of these clones was assessed by alkaline phosphatase staining alone and only at the 4 day time point. A greater insight into the survival and fate of these clones might be gained from detecting other markers of self-renewal, Nanog and Rex1, and assessing their expression from induction of Mek* expression to the time point at which alkaline phosphatase staining is conducted. Additionally, the survival and ability of these clones to maintain self-renewal over longer periods of culture is of interest.

It has frequently been observed within our research group that inhibition of PI3Ks with LY294002 in mESCs induces an enhancement in Erk phosphorylation (Paling et al., 2004). Indeed, this phenomenon was also observed in mESCs cultured in chemically defined media in the presence of LY294002. Furthermore, in chemically defined media, LY294002 treatment not only enhanced basal Erk phosphorylation but also enhanced BMP4 and insulin-induced Erk phosphorylation. Although there are reports describing an inhibitory effect of PI3K activity on MAPK pathway activity in other cell types (Kanasaki et al., 2008, Laprise et al., 2004, Romano et al., 2006), the isoforms that might mediate this effect have not previously been described. The present study demonstrated that an enhancement in basal Erk phosphorylation was induced by selective inhibition of class $I_A$ PI3K catalytic subunit p110β, implicating this isoform in cross-talk with the MAPK pathway. Consistent with the present study, an enhancement in Erk2 phosphorylation was reported in MEFs derived from p110β kinase-dead knock-in mice (Ciraolo et al., 2008). In pituitary cells, inhibition of PI3Ks is reported to enhance Raf-1 kinase and Rap-1 activity, both of which are upstream of Mek and Erk (Romano et al., 2006). However, the exact mechanism of how p110β might mediate effects on MAPK signalling in mESCs requires further investigation. In previous studies, inhibition of PKB with the selective inhibitors Akt I and PIA6 lead to enhanced Erk phosphorylation (Romano et al., 2006) while LY294002 was demonstrated to enhance Mek phosphorylation, directly upstream of Erk (Laprise et al., 2004). Similar approaches using inhibitors of PKB and antibodies detecting phosphorylated Mek could be used in mESCs to determine the level of cross talk of these two pathways. Additionally kinase assays detecting Raf-1 kinase activity following treatment with LY294002 or the p110β selective inhibitors TGX-121 or TGX-221 may clarify the involvement of Raf-1 in PI3K/MAPK pathway cross talk in mESCs.

### *6.1.3 The roles of PI3K subunit isoforms in mESC fate*

Prior to the commencement of this project, PI3Ks had been implicated in both proliferation and self-renewal of mESCs (discussed in detail in Section 1.2.6). In other cell types, emerging roles for independent PI3K isoforms in regulating different cellular functions were beginning to arise due to the availability of new, isoform selective small molecule inhibitors (Chaussade et al., 2007, Condliffe et al., 2005, Hennessy et al., 2005, Jackson et al., 2005, Knight et al., 2006) and genetic targeting in mice (Bi et al., 2001, Bi et al., 1999, Ciraolo et al., 2008, Clayton et al., 2002, Foukas et al., 2006, Fruman et al., 1999, Hirsch et al., 1999, Jou et al., 2002, Okkenhaugh et al., 2002, Patrucco et al., 2004, Terauchi et al., 1999). Therefore, the present study sought to test the hypothesis that PI3Ks might regulate both proliferation and self-renewal by coupling to different PI3K class I isoforms. In particular, the class $I_A$ catalytic isoforms of PI3Ks were of interest since they had previously been identified as having an involvement in regulating mESC self-renewal (Paling et al., 2004).

#### *6.1.3.1 Regulation of mESC self-renewal by PI3K p110β*

Pharmacological inhibition and siRNA-mediated knockdown approaches were used to assess the involvement of each class I PI3K isoform in turn, in the regulation of mESC self-renewal.

Evidence for the predominant role of p110β in self-renewal was demonstrated by the requirement of p110β activity and expression in order for mESCs to maintain optimal self-renewal. When the activity of p110β was reduced by selective pharmacological inhibition of this isoform, mESCs exhibited colony morphologies akin to differentiated colony types, reduced alkaline phosphatase staining and decreased Nanog protein expression, all features that indicate a loss of self-renewal (Kingham and Welham, In Press). Consistent with a requirement for p110β activity, reduced expression of this isoform, using three different siRNAs, also led to fewer alkaline phosphatase positive colonies, reduced Nanog protein and RNA expression and led to small decreases in Rex1 and Oct4 expression. Importantly, no significant alterations in p85 or other catalytic subunit isoform expression was found following knockdown of p110β expression, an issue that has plagued the usefulness of knockout mice in such investigations (Bi et al., 1999, Clayton et al., 2002, Jou et al., 2002). This lack of change in the expression of other isoforms indicates that the effects observed are likely to be the direct result of reduced p110β expression as opposed to alterations in the expression of

other isoforms, which might arise as a compensation mechanism (Vanhaesebroeck et al., 2005).

The implication of p110β in maintaining self-renewal marries well with investigations in knockout mice, where mouse embryos homozygous for a partial deletion of *Pik3cb* exhibit early embryonic lethality at E3.5 and cells cannot be derived from these blastocysts (Bi et al., 2001). Isolation of mESCs from blastocysts is typically conducted at E3.5 (Evans and Kaufman, 1981), the same time point at which embryonic lethality is reported to occur in mice deficient in functional p110β (Bi et al., 2001). Therefore, taken together with the present findings, embryonic lethality might be the result of an inability of cells of the ICM to self-renew sufficiently, a process required at this stage to maintain the ICM prior to differentiation and further embryonic development. However, in a previous study, the expression of a catalytically inactive form of p110β led to embryonic lethality with incomplete penetrance (Ciraolo et al., 2008). Surviving mice exhibited some growth retardation but were viable to adulthood, which led the authors to suggest that kinase-independent functions of p110β were sufficient to allow embryonic development and viability to adulthood. In the present study it was demonstrated that pharmacological inhibition of p110β induced a loss of self-renewal indicating that kinase-independent functions are not sufficient to maintain mESC self-renewal although they might be important for other aspects of mESC fate. The functional relevance of kinase-independent functions of p110β in mESC fate could be investigated using mESCs isolated from these mutant mice or transfection of mESCs with the catalytically inactive form of p110β (Ciraolo et al., 2008). An alternate approach could include the use of a site specific recombination system, such as the Cre-*lox*P system (Sauer, 1993). This could be designed to allow inducible expression of kinase-dead mutant form of p110β or provide an inducible p110β knockout mESC line. This method would allow a more detailed analysis and comparison of both kinase-dependent and kinase-independent functions in mESCs. The association of p110β with Rab5, a small GTPase, has previously been reported linking p110β to clathrin-coated vesicle formation and growth factor receptor endocytosis (Christoforidis et al., 1999, Ciraolo et al., 2008). This p110β kinase-independent function could be investigated in mESCs initially by detecting clathrin positive vesicles following knockdown of *Pik3cb* compared to full expression of p110β.

To further the investigation of kinase-dependent functions of p110β in mESCs, it would be interesting to test the hypothesis that over-expression of p110β or inducible expression of a constitutively active form of p110β, previously described (Hu et al., 1995, Logan et al., 1997), would enhance mESC self-renewal. Furthermore, over-

expression of p110β or expression of constitutively active p110β might support self-renewal of mESCs even in the absence of LIF. In contrast, inducible short-hairpin (sh) RNA targeting *Pik3cb* would allow fine regulation over reduced expression of p110β compared to siRNA used in the present study. Following induction of shRNA targeting *Pik3cb*, the expression of self-renewal markers and other gene targets of p110β could be investigated using mircoarray analysis over a number of time-points to determine how p110β regulates self-renewal on a genetic level.

Unexpectedly, LIF-induced signalling downstream of PI3K was not perturbed by inhibition of p110β, indicating that LIF does not engage and activate this isoform to any appreciable extent. Although p110γ is typically activated by G proteins following GPCR stimulation (Brock et al., 2003, Krugmann et al., 1999, Maier et al., 1999, Stephens et al., 1997), a number of reports suggest that in some cell types, p110β can be activated by stimulation of GPCRs particularly in the absence of p110γ or when p110γ is expressed at low levels (Ciraolo et al., 2008, Guillermet-Guibert et al., 2008, Kubo et al., 2005, Maier et al., 1999, Murga et al., 2000, Roche et al., 1998). Since the mESC line used in the present study is known to express GPCRs and very low levels of p110γ (Storm and Welham, unpublished data), the possibility that p110β could be activated by GPCR agonists was explored. Stimulation of mESCs with heat stable lysophospholipids present in serum replacement induced the activation of signalling downstream of PI3Ks, namely PKB and Gsk-3 and this activation was demonstrated to be sensitive to inhibition of p110β. Furthermore, stimulation of mESCs with known GPCR agonists, LPA and S1P, induced the phosphorylation of Erk, which could be ameliorated by pertussis toxin, implicating heterotrimeric G proteins $G_i$, $G_o$, and $G_t$ in this process, and altered by the inhibition of p110β, suggesting coupling to this PI3K isoform. Further investigation into GPCR agonist-induced activation of p110β might explore a time-course of agonist stimulation and assessment of a broader panel of signalling molecules downstream of PI3Ks, in addition to the use of other agonists of GPCRs known to be expressed in mESCs.

Although the present study demonstrated activation of p110β in mESCs by undefined, heat stable lysophospholipids and by the lysophospholipids LPA and S1P, the functional relevance of this in self-renewing mESCs is unknown. Previous reports indicate that in hESCs, S1P can contribute to the maintenance of the undifferentiated state (Pébay et al., 2005). In contrast, sphingosylphosphorylcholine is reported to induce mESCs to differentiate towards cardiac and neural lineages (Kleger et al., 2007) and in embryoid bodies formed from mESCs, S1P promoted cardiac differentiation (Sachinidis et al., 2003). Therefore, it is possible that the coupling of GPCR activation to p110β activity

might promote differentiation towards these lineages but in self-renewing cells, this push towards particular lineages might be counteracted by other signalling pathways. Incubation of mESCs with LPA, S1P or other GPCR agonists in the presence or absence of pertussis toxin, TGX-121 or TGX-221 and subsequent assessment of markers of self-renewal and differentiation would determine if GPCR-induced activation of p110β regulates self-renewal or lineage commitment.

#### *6.1.3.2 Regulation of mESC self-renewal by other p110 isoforms and other PI3Ks*

When other class I catalytic isoforms were independently assessed for a role in mESC self-renewal, no contribution of p110α to maintenance of mESC self-renewal was identified. Pharmacological inhibition, using two structurally distinct selective p110α inhibitors, and siRNA-mediated knockdown of 88%, failed to alter the proportion of alkaline phosphatase positive colonies, *Nanog* and *Rex1* RNA expression and Nanog protein expression, indicating no change in self-renewal. Furthermore, no significant change in the levels of expression of p110β or p110δ were found following knockdown of *Pik3ca*, indicating that a lack of effect of *Pik3ca* knockdown on self-renewal was not the result of up-regulated expression of the other isoforms. Additionally, inhibition of p110α did not restrict the differentiation of mESCs, induced by withdrawal from LIF. As expected, given the low or undetectable expression of p110γ, incubation of mESCs with a selective inhibitor of this isoform did not alter levels of alkaline phosphatase positive colonies. Pharmacological inhibition of p110δ led to a modest decrease in alkaline phosphatase positive colonies whereas siRNA-mediated knockdown did not alter the proportion of alkaline phosphatase positive colonies. The discrepancy in the results obtained from two different approaches was resolved on investigation of the inhibitory profile of the p110δ isoform inhibitor IC87114 (Knight et al., 2006). Only at higher concentrations of IC87114 was a decrease in alkaline phosphatase positive colonies observed, a concentration that could be considered high enough to also inhibit p110β. Given that p110β was already implicated in maintaining mESC self-renewal and that application of both IC87114 and inhibitors of p110β did not induce a further decrease in self-renewal, p110δ is unlikely to contribute to the maintenance of self-renewal and does not appear to exhibit functional redundancy in regulating this cellular function. Interestingly, siRNA-mediated knockdown of p110δ resulted in enhanced Nanog expression which might be explained by the observation that *Pik3cd* expression is enhanced following induction of differentiation (Storm and Welham, unpublished data). Therefore, knockdown of the expression of this isoform in self-renewing mESCs might restrict spontaneous differentiation and prevent a reduction in Nanog expression.

Furthermore, these findings suggest that p110δ might be important for differentiation following an initial induction of loss of self-renewal. Defining a role for p110δ later in the differentiation process requires further investigation using embryoid bodies formed from mESCs and incubated in the presence of IC87114 to inhibit p110δ, followed by detection of lineage marker genes.

The regulation of mESC fate by class II and III PI3Ks was beyond the scope of this project but would make for a complete analysis of PI3Ks in mESCs. Much the same as the class I PI3Ks were investigated in this study, roles for class II and class III PI3Ks could be investigated using selective pharmacological inhibitors, where available, and complimented by gene targeting of specific isoforms, assessing mESC fate in terms of self-renewal, differentiation, pluripotency and proliferation. Additionally, the PH domain containing GTP/GDP exchange factors (GEFs) that are specific for the Rho family of GTPases, namely Rho, Rac and cdc42, interact with $PIP_3$ (Vanhaesebroeck and Waterfield, 1999) and are yet to be investigated in mESCs.

#### *6.1.3.3 Regulation of mESC proliferation by p110α*

Whilst investigating the involvement of p110α in regulating mESC self-renewal, it was observed that inhibition of this isoform gave rise to smaller colonies that were fewer in number. These observations indicated that this isoform might be involved in proliferation and cell growth, cellular functions already reported to be coupled with p110α activity in other cell types (Foukas et al., 2006, Knight et al., 2006). Indeed, selective inhibition of p110α led to the generation of reduced numbers of mESCs but no apparent enhancement in the proportion of apoptotic cells, at least at early time points, indicating reduced proliferation was most likely responsible for the reduction in cell numbers observed (Kingham and Welham, In Press). This defined role for p110α in regulating mESC proliferation is consistent with embryonic lethality resulting from proliferative defects observed in homozygous p110α knockout mouse embryos (Bi et al., 1999) and consistent with a report describing the ability of over-expression of p110α to be sufficient to rescue proliferative defects observed in ERas null ESCs (Takahashi et al., 2003). The involvement of p110α in signalling and mESC fate is summarised in Figure 6.1.

Areas of possible further investigation into the regulation of mESC fate by p110α, predominantly identifying signalling downstream of p110α, are depicted in Figure 6.1. Incubation of mESCs with inhibitors of Gsk-3 produces small, rounded, self-renewing colonies (Bone et al., 2009, Sato et al., 2004) similar to the smaller colonies observed

following inhibition of p110α. Furthermore, Gsk-3 is known to regulate cyclin D1 and GS activity, important for cell cycle regulation and glycogen synthesis respectively (Eisenmann, 2005, Kestler and Kühl, 2008). Thus, determining if Gsk-3 is downstream of p110α in mESCs might help to further define the regulation of proliferation by p110α. This could be addressed by immunoblotting using antibodies detecting Gsk-3 phosphorylation sites following both long and short-term incubation with inhibitors of p110α. Protein synthesis requires the binding of mRNA to the small ribosomal subunit, a process aided by the eukaryotic initiation factor (eIF4) family. The translation repressor proteins, eIF4E-binding proteins (4E-BPs) bind eIF4E and prevent its association with an eIF4 complex, inhibiting translation (Beretta et al., 1996). 4E-BP1 is reported to be phosphorylated and inactivated by mTOR in a wortmannin-sensitive and rapamycin-sensitive manner (Brunn et al., 1997) but can also be phosphorylated and inactivated downstream of PKB in a manner that also relies on mTOR activity (Gingras et al., 1998). This PKB/mTOR/4E-BP pathway might also regulate mESC growth and proliferation by activation downstream of p110α and could be analysed following p110α inhibition by immunoblotting with antibodies to detect phosphorylation of 4E-BP at mTOR and PKB-dependent sites. Furthermore, alterations in proliferation were assessed in the present study following pharmacological inhibition of p110α but proliferation could not be investigated in mESCs transfected with *Pik3ca* targeting siRNA due to the nature of the siRNA transfection protocol and the effect of siRNA transfection on mESCs. However, inducible shRNA targeting *Pik3ca* may make assessment of proliferation following knockdown of this isoform feasible and could be investigated to compliment the findings of this study using pharmacological inhibition of p110α.

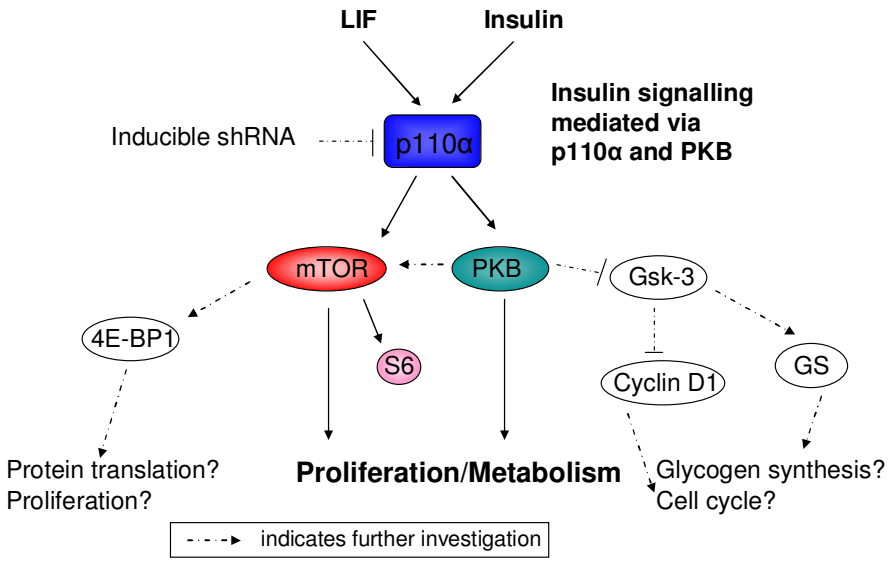


**Figure 6.1 The role of p110α in mESC fate and avenues of further investigation**

The PI3K isoform p110α was demonstrated, in the present study, to be activated by mESC stimulation with LIF and insulin. Additionally, following stimulation with either of these factors, enhancements in PKB and S6 phosphorylation were observed. This isoform was also demonstrated to mediate mESC growth and proliferation. Potential future investigation into the regulation of mESC fate and signalling mediated by p110α are indicated with dashed arrows.

### *6.1.4 Cross-talk between pathways regulating mESC proliferation and self-renewal*

The present study investigated self-renewal and proliferation independently and led to the observation that self-renewal can be lost without an apparent change in proliferation. Also, reduced proliferation did not appear to alter the expression of markers of self-renewal, although a change in self-renewal would not necessarily be expected if the typical definition of self-renewal, as the maintenance of pluripotency with continuous proliferation, is true. Interestingly, when these two features were investigated together, reducing the rate of proliferation of mESCs enhanced a loss of self-renewal. This reduced rate of proliferation was achieved by the inhibition of mTOR or p110α and a loss of self-renewal induced by the inhibition of p110β.

Interestingly, LIF-induced signalling downstream of PI3K was unaltered by p110β inhibition, the isoform highlighted as a regulator of self-renewal, but prevented by the inhibition of p110α, which appeared to have no independent role in regulating self-renewal. Evidence from other systems indicates that the signalling of one PI3K isoform might be regulated by another isoform (Chaussade et al., 2007, Condliffe et al., 2005, Knight et al., 2006). Therefore, a model that might explain the findings of the present study encompasses a threshold being set by p110α for the activity of p110β. This proposed model, taking into account the present findings is depicted in Figure 6.2. In this model, a reduction in the rate of proliferation governed by p110α activity might make mESC self-renewal more sensitive to a reduction in p110β activity. Furthermore, this threshold might be set by LIF or insulin-induced stimulation of p110α, as opposed to p110β.

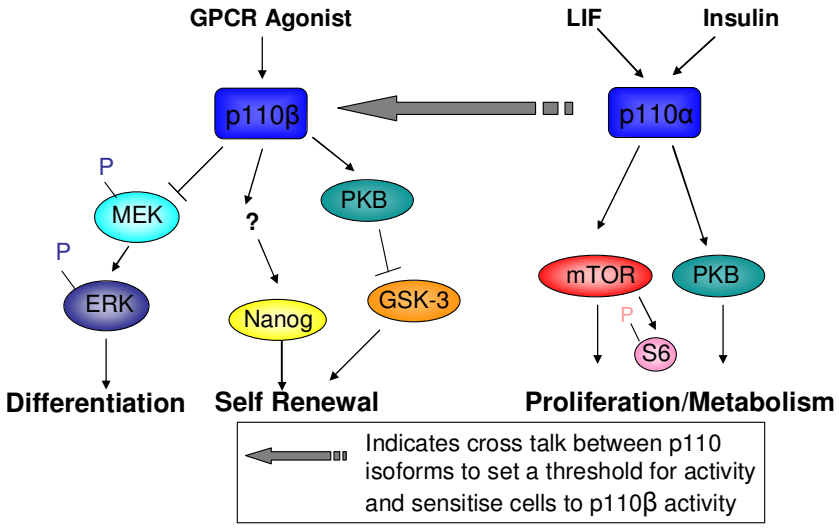


**Figure 6.2 Proposed model of the regulation of mESC proliferation and self-renewal by coupling to different PI3K class $I_A$ isoforms**

The activation of p110α and p110β by extrinsic factors allows the regulation of mESC fate in terms of inhibition of differentiation, promotion of self-renewal and regulation of proliferation and metabolism. However, these pathways appear to work both independently and to display cross-talk. This proposed model explains how the rate of proliferation and signalling via p110α might set a threshold for the activity of p110β to regulate self-renewal where a reduction in proliferation sensitises mESCs to alterations in signalling inducing a loss of self-renewal and further potentiates the induction of differentiation and loss of self-renewal.

#### *6.1.4.1 Rate of proliferation and the implications on differentiation potential*

While the rate of proliferation and the level of self-renewal can be altered independently, a reduction in the rate of proliferation was demonstrated to potentiate an induced loss of self-renewal. The complexities of two cellular events being linked, in addition to the regulation of both by isoforms of PI3Ks makes further investigation somewhat problematic. Although, if the rate of proliferation does set a threshold for sensitivity to p110β activity and loss of self-renewal, then the implications of the rate of proliferation at the time of differentiation induction and also the mode of inducing differentiation and the impact this has on differentiation potential might be worthy of investigation. At the outset, proliferation rates could be crudely controlled using a range of rapamycin concentrations and assessment of self-renewal markers conducted following induction of differentiation. However, analysing loss of self-renewal in a manner employed by the present study provides information about a whole population of mESCs. Recently the technique of laser scanning cytometry has been described that allows investigation of molecular and cellular events on an individual cell basis using imaging and quantitative analysis (Harnett, 2007). Using this technique to detect self-renewal markers and activation of signalling pathways alongside indications of cell cycle or proliferation might reveal a correlation between proliferation rate and differentiation kinetics.

### *6.1.5 Regulation of hESCs by PI3K isoforms*

While a number of differences in the regulation of self-renewal in mESCs and hESCs have been reported, there are some common signalling pathways. As in mESCs, the inhibition of PI3Ks with LY294002 in hESCs is reported to reduce alkaline phosphatase staining, indicative of a loss of self-renewal of hESCs (Pyle et al., 2006). Additionally genes of PI3K-PKB pathway signalling molecules, in particular *PIK3CB,* which encodes p110β in human cells, were highlighted as being important for the maintenance of pluripotency of hESCs (Armstrong et al., 2006). The contribution of individual PI3K isoforms in the regulation of hESC fate remains to be investigated. A similar approach using selective isoform inhibitors complemented by siRNA-mediated knockdown of individual isoforms might reveal if the maintenance of self-renewal by p110β in mESCs is conserved in hESCs.

## 6.2 Conclusions

The present study has further defined the regulation of mESC fate by PI3Ks. PI3Ks do not appear to direct the differentiation of mESCs towards particular lineages but rather allow differentiation towards cell types expressing markers of each of the three embryonic germ layers; endoderm, mesoderm, ectoderm. In mESCs PI3Ks are activated by LIF, insulin, BMP4 and lysophospholipids but differential coupling to class I PI3K isoforms, dependent on extrinsic factor stimulation, is exhibited. It was established that PI3Ks regulate mESC self-renewal and proliferation by differential coupling to the PI3K catalytic subunit isoforms p110β and p110α respectively. Furthermore, cross-talk between p110β signalling and the MAPK pathway, in addition to cross-talk between pathways regulating self-renewal and proliferation was demonstrated. Thus activity of PI3Ks act to coordinate both self-renewal and proliferation of mESCs.

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# 8. Publications

# Phosphoinositide 3-kinases and regulation of embryonic stem cell fate

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## Abstract

**ES (embryonic stem) cell lines are derived from the epiblast of pre-implantation embryos and like the inner cell mass cells from which they are derived exhibit the remarkable property of pluripotency, namely the ability to differentiate into all cell lineages comprising the adult organism. ES cells and their differentiated progeny offer tremendous potential to regenerative medicine, particularly as cellular therapies for the treatment of a wide variety of chronic disorders, such as Type 1 diabetes, Parkinson's disease and retinal degeneration. In order for this potential to be realized, a detailed understanding of the molecular mechanisms regulating the fundamental properties of ES cells, i.e. pluripotency, proliferation and differentiation, is required. In the present paper, we review the evidence that PI3K (phosphoinositide 3-kinase)-dependent signalling plays a role in regulation of both ES cell pluripotency and proliferation.**

## ES (embryonic stem) cells

ES cell lines exhibit the unique property of pluripotency, i.e. the ability to differentiate into all cell lineages comprising the adult organism [1,2]. Pluripotency is underpinned by ES cell self-renewal, the term given to the symmetrical division of ES cells to generate two identical, undifferentiated daughter cells. In essence, self-renewal is proliferation that is accompanied by the suppression of differentiation. Regulation of ES cell self-renewal is of great interest because the ability to maintain and expand pluripotent ES cells is essential if the therapeutic potential offered by ES cell-derived progeny to regenerative medicine is to be realized.

## Regulation of murine ES cell pluripotency

Pluripotency of mES (murine ES) cells is regulated by a complex network of extrinsic factors, signalling pathways and transcription factors. LIF (leukaemia inhibitory factor) was the first cytokine shown to play a role in maintenance of pluripotency [2], via activation of STAT3 (signal transducer and activator of transcription 3) and induction of c-Myc [3–5]. However, like many cytokines, LIF also activates the ERKs (extracellular-signal-regulated kinases) ERK1 and ERK2, which appear to promote differentiation [6], leading to the suggestion that the balance between STAT3 and ERK signals is important in determining ES cell fate [7]. More recently it has been demonstrated that BMPs (bone morphogenetic proteins) BMP2 and BMP4 collaborate with LIF to facilitate mES cell self-renewal, via BMP2/BMP4 activation of Smad4, inducing expression of the Id transcriptional repressors, which actively suppress neuronal differentiation [8]. Wnt signalling has also been implicated, largely as a result of the use of the small molecule BIO [6-bromo-indirubin 3′-oxime; an inhibitor of GSK-3 (glycogen synthase kinase 3)] that facilitates activation of the canonical Wnt signalling pathway [9]. The Src tyrosine kinase also appears to play a role in maintenance of self-renewal of mES cells [10].

Among the transcriptional regulators, Oct-4 (octamer-binding protein-4), Sox-2 and Nanog have all been implicated in the maintenance of mES cell pluripotency [11]. Oct-4 is expressed by undifferentiated mES and hES (human ES) cells, its expression being lost upon differentiation [11]. Nanog (from the Norse *Tir nan Og* meaning 'ever young') was identified by expression cloning [12] and *in silico* analyses [13] as a homeodomain protein that can maintain pluripotency of mES cells independently of LIF. Recent large-scale screening studies have mapped promoters co-bound by Nanog, Oct-4 and Sox-2 in hES cells [14] and Nanog/Oct-4 co-bound promoters in murine ES cells [15], demonstrating that Oct-4 and Nanog target some of the same genes in ES cells. Ivanova et al. [16] have identified a further five genes, *Tcl1*, *Tbx3*, *Esrrb*, *Dppa4* and Unigene Mm.343880, whose down-regulation leads to loss of pluripotency of mES cells. Further evidence suggests that epigenetic mechanisms also play a part in determining ES cell fate [17]. Thus it is increasingly clear that a network of transcription factors and other regulators influence and control the fate of both mES and hES cells [11].

## Regulation of ES cell proliferation

Studies of murine and Rhesus monkey ES cells have demonstrated that the cell cycle of ES cells is not subject to the same regulatory checkpoints as the cell cycle of somatic cells [18–20]. mES cells transit through the cell cycle very rapidly (~8 h), spending as little as 1–2 h in $G_1$-phase and as a

**Key words:** bone morphogenetic protein (BMP), embryonic stem cell (ES cell), phosphoinositide 3-kinase (PI3K), pluripotency, self-renewal.

**Abbreviations used:** BMP, bone morphogenetic protein; ES, embryonic stem; ERas, ES cell-expressed Ras; ERK, extracellular-signal-regulated kinase; GSK, glycogen synthase kinase; hES, human ES; LIF, leukaemia inhibitory factor; mES, murine ES; mTOR, mammalian target of rapamycin; Oct-4, octamer-binding protein-4; PI3K, phosphoinositide 3-kinase; PI(3,4,5)$P_3$, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; STAT3, signal transducer and activator of transcription 3.

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consequence approx. 50% of ES cells in a growing population are in S-phase [18,19]. The unorthodox cell cycle of mES cells, which shows no periodicity in cyclin E expression and no retinoblastoma restriction point [18,19], has made it a challenge to define the molecular mechanisms that regulate ES cell proliferation and the relationship between the signals regulating proliferation and pluripotency is still relatively poorly understood. Recent data have demonstrated that an ES-cell-specific Ras-family member, termed ERas (ES cell-expressed Ras), plays a role in controlling proliferation of mES cells [21] as does mTOR (mammalian target of rapamycin) [22,23]. Both of these pathways involve PI3K (phosphoinositide 3-kinase)-dependent signalling.

## PI3Ks

The PI3Ks are a family of lipid kinases, comprising three subclasses and it is typically the class $I_A$ subgroup that is activated in response to growth factor and cytokine signalling [24]. Class $I_A$ PI3Ks are heterodimers, consisting of a regulatory subunit (for which there are three genes, generating five isoforms, p85$\alpha$, p55$\alpha$, p50$\alpha$, p85$\beta$ and p55$\gamma$) and a catalytic subunit, of which there are three isoforms, p110$\alpha$, p110$\beta$ and p110$\delta$, each encoded by a separate gene. The primary product of class $I_A$ PI3K action is the phosphoinositide PI(3,4,5)$P_3$ (phosphatidylinositol 3,4,5-trisphosphate), which recruits PH domain (pleckstrin homology domain)-containing proteins to the plasma membrane, facilitating activation of a range of downstream signalling cascades [25]. Functionally, PI3Ks have been implicated in a wide array of physiological processes including proliferation, development, growth and migration [25]. Accumulating evidence from mice that have the activity of a selected class $I_A$ catalytic or regulatory isoform ablated have demonstrated that specific isoforms play selective roles both during development and in the function of somatic cells. For example, p110$\delta$ plays a key role in T-, B- and mast-cell biology [24,26], p110$\gamma$ in inflammatory cells [24] and p110$\alpha$ in insulin signalling [27,28]. Interestingly, ablation of the p110$\beta$ isoform leads to lethality at the pre-implantation stage and although the molecular basis of this lethality is unknown, this observation suggests that PI3K signalling plays a critical role during early embryogenesis [29]. One caveat to the gene-targeted mice that completely lack a specific class $I_A$ PI3K isoform is the observation that expression of other subunits can also be affected, complicating interpretation of the data in some studies. For a more detailed discussion see [24].

## PI3K signalling and ES cell proliferation

A role for PI3Ks in control of mES cell proliferation was first suggested when it was discovered that ES cells deficient in PTEN [phosphatase and tensin homologue deleted on chromosome 10; a lipid phosphatase that dephosphorylates the 3′ position of the inositol ring, thereby reducing PI(3,4,5)$P_3$ levels and acting as a negative regulator of PI3K signalling] displayed a 5–10% decrease in the time taken to complete a cell division cycle [30]. Interestingly, PTEN-null ES cells also show decreased dependency on serum for division, raising the possibility that serum-containing factors play an important role in activation of PI3Ks in ES cells. Ablation of the ES cell-specific form of Ras, ERas, also results in decreased ES cell proliferation, while expression of an active form of p110$\alpha$ in ERas-deficient ES cells restores proliferation to levels similar to those of wild-type ES cells [21]. However, ERas cannot be the only activator of class $I_A$ PI3Ks in ES cells, since PKB (protein kinase B) activity (PKB is a critical downstream mediator of PI3K signals) is still observed in ERas-null ES cells [21], although significantly decreased. Hence, the evidence from these essentially gain-of-function approaches suggest that an elevation in PI(3,4,5)$P_3$ levels can enhance proliferation of ES cells. Further data support a role for PI3Ks in regulation of ES cell proliferation since inhibition of PI3Ks with 25 $\mu$M of the broad specificity inhibitor LY294002 decreases ES cell proliferation, cells accumulating in $G_1$-phase of the cell cycle [20]. mTOR signalling plays a key role in regulation of mES cell proliferation, since inducible deletion of mTOR in mES cells or treatment with rapamycin leads to dramatic decreases in ES cell proliferation [22,23]. In contrast however, knockout of the *pik3r1* gene (which produces the p85$\alpha$, p55$\alpha$ and p50$\alpha$ regulatory subunits) leads to only a modest decrease in ES cell proliferation, whereas ES cells deficient in PDK1 (phosphoinositide-dependent kinase 1), one of the key downstream targets of class $I_A$ PI3K signalling, have no reported proliferative phenotype [31]. One caveat to these latter two studies is that compensatory changes could have arisen during selection of the ES cell clones derived and used for these studies such that alternative pathways controlling proliferation are up-regulated in these clones. While this important possibility should be taken into consideration, current evidence collectively suggests that PI3K-dependent signalling does play a role in controlling ES cell proliferation [23], similar to the role of PI3Ks in somatic cells [24].

## PI3K signalling and ES cell self-renewal

In contrast with the reports implicating PI3K signalling in regulation of ES cell proliferation, our group has reported that PI3K-dependent signals are required for optimal maintenance of self-renewal, since inhibition of PI3K signalling (with either 5 $\mu$M LY294002 or upon expression of a dominant-negative class $I_A$ PI3K mutant) in the presence of LIF led to loss of self-renewal [32], with cells under going multi-lineage differentiation (E. Kingham and M. J. Welham, unpublished work). Our findings have recently been supported by the work of other groups. For example, expression of a myristoylated form of PKB maintains pluripotency of murine ES cells in the absence of LIF and also maintains self-renewal of monkey ES cells [33]. In addition, PKB was identified in an RNA interference-based screen for positive regulators of ES cell self-renewal [34]. Importantly, a role for PI3K signalling in maintenance of hES cell pluripotency and survival has been reported [35,36]. To date there has been little commonality demonstrated between the signals regulating murine versus

hES cell pluripotency, so the demonstration that PI3K signalling plays a role in maintenance of pluripotency in both cell types is significant.

Mechanistically, our group has identified a potential role for activation of ERK signalling upon inhibition of PI3Ks in promoting the loss of self-renewal observed [32]. In further studies, we have found that PI3K inhibition leads to down-regulation of the intrinsic regulator, Nanog, at both the RNA and protein levels. We have also revealed a role for GSK-3 in PI3K-dependent regulation of self-renewal and Nanog expression. Significantly, activation of an inducible form of Nanog prevents the loss of self-renewal observed in the presence of PI3K inhibitors, consistent with the relationship between PI3K signalling and maintenance of Nanog expression being a functional one [37].

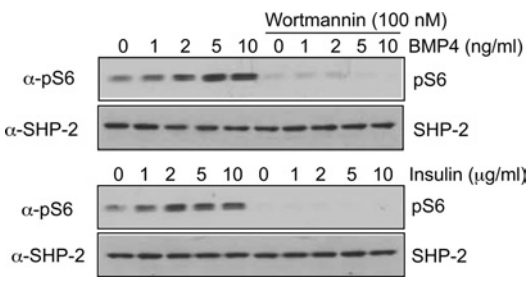
## Recent progress

We have taken a number of approaches to further investigate the molecular mechanisms whereby PI3Ks regulate ES cell fate. Using Affymetrix microarray expression analyses we have defined the PI3K-dependent transcriptome in ES cells generating a dataset of genes whose expression is altered upon inhibition of PI3K signalling in ES cells. We have also investigated the ability of other growth factors and cytokines to activate PI3K signalling in ES cells. Our previous work has shown that LIF can stimulate PI3K signalling in ES cells, while LIF or serum treatment facilitates co-precipitation of the p85 PI3K regulatory subunits with phosphotyrosine proteins, indicative of recruitment/activation of class $I_A$ PI3Ks [32]. To investigate which extracellular factors may further contribute to activation of PI3Ks in ES cells we have used serum-free conditions to cultivate ES cells, as described in [8]. Using this system, we have found that LIF, BMP4 and insulin can all activate PI3K signalling, judged in part by their ability to induce phosphorylation of S6 ribosomal protein at serine residues $Ser^{235}/Ser^{236}$. In each case, S6 phosphorylation can be inhibited by wortmannin (see Figure 1). These results confirm that multiple upstream factors can activate PI3Ks in ES cells and, in view of the observation that PTEN-null ES cells exhibit decreased dependence on serum for their growth, suggest that PI3Ks are normally activated by multiple signals in ES cells.

Interestingly, under the serum-free conditions used, ES cells needed to be maintained at densities of more than $\sim 1.5 \times 10^4$ cells/cm$^2$ in order to retain viability, leading us to consider a contribution from either cell–cell contact or autocrine factors in maintenance of ES cells. To study this, conditioned medium was collected from ES cells for 24 h (either in the presence or absence of LIF) and then used to treat naïve ES cells. Expression of Nanog was monitored by quantitative PCR, as a measure of the ability to maintain expression of pluripotency genes, and the effects were compared with those of fresh medium ± LIF. Our results (Figure 2) suggest that ES cells can produce autocrine factors that co-operate with LIF to ensure maximal expression of Nanog. We have also studied the effects of PDK1 (phosphoinositide-dependent kinase 1) deficiency on ES cell pluripotency. In contrast with the initial report [31], we find that *pdk-1*-null ES cells exhibit enhanced proliferation compared with parental ES cells and while self-renewal of *pdk1*-null ES cells is decreased at low doses of LIF, this is not apparent at optimal doses of LIF (H. K. Bone and M. J. Welham, unpublished work). Most surprisingly, we discovered that *pdk-1* ES cells exhibit elevated levels of Nanog mRNA and protein (H. K. Bone and M. J. Welham, unpublished work). These observations suggest the *pdk1*-null ES cells may have undergone compensatory alterations during either their initial derivation and/or subsequent culture and these findings highlight to us the difficulty in comparing ES lines with targeted homozygous disruption of specific genes.

**Figure 1 | Induction of PI3K signalling by BMP4 and insulin in ES cells cultured under serum-free conditions**

ES cells were cultured under serum-free conditions in the presence of LIF and BMP4, as described by Ying et al. [8]. ES cells were plated at $5 \times 10^5$ cells per 50 mm diameter gelatin-coated Nunc tissue culture plate, cultured for 48 h, washed three times with PBS and then deprived of LIF and BMP4 for 4 h. Samples were pre-treated with either 100 nM wortmannin for 30 min or DMSO, as a control. Stimulations with BMP4 or insulin were carried out for 5 min at the doses indicated. Immunoblotting was performed with an antibody that detects S6 phosphorylation at $Ser^{235}$ and $Ser^{236}$ ($\alpha$-pS6, Cell Signaling Technology). The same immunoblots were stripped and reprobed with anti-SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2) antibodies ($\alpha$-SHP-2; Santa Cruz Biotechnology) to demonstrate equivalent loading.

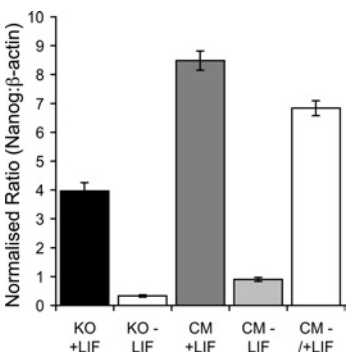



## Summary

How can the evidence supporting a role for PI3Ks in regulation of both mES cell proliferation and regulation of pluripotency be rationalized? We have observed that doses of LY294002 greater than 10 $\mu$M decrease ES cell proliferation [32], while others have shown similar effects with 25 $\mu$M LY294002 [20]. At lower doses, LY294002 perturbs self-renewal [32]. mTOR is a key regulator of ES cell proliferation [22,23] and can also be inhibited by LY294002, but in general this requires higher doses of LY294002 than the dose that inhibits class $I_A$ PI3K isoforms $\beta$ and $\delta$ [28]. Hence, it is possible that the effects of higher doses of LY294002 on proliferation result from additional inhibition of mTOR. Taking these findings into consideration we propose two models that may explain the involvement of PI3Ks in the control of both proliferation and self-renewal of ES cells. In model 1, distinct PI3K isoforms could couple selectively with either ERas or mTOR, to regulate ES cell

**Figure 2 | Regulation of Nanog expression by autocrine factors**

Conditioned medium (CM) was harvested from ES cells cultured for 24 h in knockout (KO) DMEM (Dulbecco's modified Eagle's medium) alone (CM − LIF) or with LIF (CM + LIF). Aliquots of CM were placed on to fresh ES cultures, with or without addition of LIF. As controls, fresh cultures of ES cells were also treated with fresh medium to which LIF was added (KO + LIF) or not (KO − LIF). RNA was harvested following 48 h and Nanog expression, normalized to $\beta$-actin, determined by quantitative PCR. ES cells cultured in CM + LIF exhibited a 2-fold increase in Nanog RNA expression compared with ES cells cultured in fresh medium supplemented with LIF (KO + LIF). Incubation with conditioned medium generated in the absence of LIF, to which fresh LIF was then added (CM−/+LIF), also led to enhanced expression of Nanog compared with KO + LIF. Results are the means ± S.D. for quadruplicate samples, representative of four independent experiments.




proliferation, while other isoforms couple specifically with GSK-3/Nanog pathways, thereby contributing to regulation of self-renewal. In an alternative model, the PI3K-dependent pathways controlling pluripotency and proliferation could be regulated by different threshold levels of PI(3,4,5)$P_3$. A modest reduction in PI(3,4,5)$P_3$ levels could perturb PI3K-dependent signals involved in regulation of self-renewal, while proliferation is not affected. A more significant reduction in PI(3,4,5)$P_3$ levels could lead to a decline in the PI3K-dependent signals regulating proliferation, resulting in decreased ES cell proliferation. Future investigations will help delineate the mechanisms by which PI3Ks contribute to regulation of ES cell fate.

We thank all the members of the Welham laboratory for discussions and encouragement. This work was supported by funding from BBSRC (Biotechnology and Biological Sciences Research Council), EU FP6, MRC and Wellcome Trust.

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Received 22 November 2006

# Distinct roles for isoforms of the catalytic subunit of class-IA PI3K in the regulation of behaviour of murine embryonic stem cells

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*Accepted 3 April 2009*
*Journal of Cell Science 122, 000-000 Published by The Company of Biologists 2009*
*doi:10.1242/jcs.046557*

## Summary

**Self-renewal of embryonic stem cells (ESCs) is essential for maintenance of pluripotency, which is defined as the ability to differentiate into any specialised cell type comprising the adult organism. Understanding the mechanisms that regulate ESC self-renewal and proliferation is required before ESCs can fulfil their potential in regenerative therapies, and murine ESCs (mESCs) have been widely used as a model. Members of the class-IA phosphoinositide 3-kinase (PI3K) family of lipid kinases regulate a variety of physiological responses, including cell migration, proliferation and survival. PI3Ks have been reported to regulate both proliferation and self-renewal of mESCs. Here we investigate the contribution of specific class-IA PI3K isoforms to the regulation of mESC fate using small-molecule inhibitors with selectivity for particular class-IA PI3K catalytic isoforms, and siRNA-mediated knockdown. Pharmacological inhibition or knockdown of p110β promoted mESC differentiation, accompanied by a decrease in expression of Nanog. By comparison, pharmacological inhibition or siRNA-mediated knockdown of p110α had no effect on mESC self-renewal per se, but instead appeared to reduce proliferation, which was accompanied by inhibition of leukaemia inhibitory factor (LIF) and insulin-induced PI3K signalling. Our results suggest that PI3Ks contribute to the regulation of both mESC pluripotency and proliferation by differential coupling to selected p110 catalytic isoforms.**

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/??/????/DC1

Key words: Embryonic stem cells, Pluripotency, Cell proliferation, PI3 kinase, Signal transduction

## Introduction

Murine embryonic stem cells (mESCs) are derived from the inner cell mass (ICM) of the preimplantation blastocyst at embryonic day 3.5 (E3.5) (Evans and Kaufman, 1981). When grown in culture in the presence of serum, leukaemia inhibitory factor (LIF) is required to maintain mESCs in an undifferentiated, pluripotent state (Smith, 2001). Pluripotency is defined as the ability to produce all the differentiated cell types that comprise an adult organism. So that pluripotency is maintained, ESCs in culture need to self-renew, which in essence is proliferation accompanied by the suppression of differentiation. LIF promotes an array of intracellular signalling pathways that are important for promoting self-renewal, including the activation of Stat3 (Boeuf et al., 1997; Cartwright et al., 2005; Niwa et al., 1998) and phosphoinositide 3-kinase (PI3K) signalling (Paling et al., 2004). The MAPK pathway is also activated by LIF and FGF4 but, rather than contribute to self-renewal, activation of this pathway promotes differentiation (Burdon et al., 1999; Kunath et al., 2007; Stavridis et al., 2007).

The PI3K family of lipid kinases catalyse the addition of a phosphate group to the D3 position of the inositol ring of phosphoinositides, generating PtdIns(3)*P*, PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$, which subsequently act as secondary messengers for signal transduction (Vanhaesebroeck and Waterfield, 1999). Mammalian PI3Ks are categorised into three classes, with the class-IA PI3Ks comprising a 110-kDa catalytic subunit (p110α, p110β or p110δ, encoded by the genes *Pik3ca*, *Pik3cb* and *Pik3cd*, respectively) coupled to a regulatory subunit (p85α, p55α or p50α, encoded by *Pik3r1*; p85β, encoded by *Pik3r2*; or p55γ, encoded by *Pik3r3*) (Cantrell, 2001; Vanhaesebroeck and Waterfield, 1999). The phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten) dephosphorylates PtdIns(3,4,5)$P_3$, the key product of class-IA PI3K activity and so acts as a negative regulator of this pathway. In mESCs, PI3Ks were first implicated in the regulation of cell proliferation, initially owing to the fact that *PTEN*-null mESCs proliferate in reduced levels of serum and show a modestly enhanced growth rate (Sun et al., 1999). Direct inhibition of PI3K signalling with LY294002 (at a dose of 25 μM) was reported to lead to a decrease in mESC proliferation (Jirmanova et al., 2002), whereas deletion or pharmacological inhibition of mammalian target of rapamycin (mTOR), a downstream effector of PI3K signalling, also resulted in proliferative defects (Murakami et al., 2004). Furthermore, deletion of the mESC-specific Ras protein, ERas, leads to a reduction in proliferation of ESCs, which was rescued by expression of an activated form of p110α (Takahashi et al., 2003). By contrast, ESCs lacking the key PI3K target phosphoinositide-dependent kinase-1 exhibit no changes in proliferation compared with wild-type cells (Williams et al., 2000).

We, and others, have also implicated PI3Ks in the regulation of ESC self-renewal. We demonstrated that inhibition of PI3Ks with the broad-selectivity PI3K inhibitor LY294002 (at a dose of 5 μM) leads to a loss of self-renewal, without demonstrating appreciable effects on proliferation, and implicated class-IA PI3Ks in this response (Paling et al., 2004). Furthermore, we have shown that

both LIF and BMP4 activate PI3K signalling in mESCs (Welham et al., 2007). Consistent with our observations, expression of a myristoylated, active form of protein kinase B (PKB, also known as Akt) promotes self-renewal of mESCs in the absence of LIF (Watanabe et al., 2006). PKB normally inhibits glycogen-synthase kinase-3 (GSK-3) activity; consequently, it is interesting to note that inhibition of GSK-3 with 6-bromoindirubin-3′-oxime (BIO) (Sato et al., 2004), or a series of bisindolylmaleimides (Bone et al., 2009), enhances mESC self-renewal. In addition, PKB was identified in a screen for activators of self-renewal (Pritsker et al., 2006), whereas a short-interfering RNA (siRNA)-based investigation demonstrated a requirement for the PKB-binding protein Tcl1 in the maintenance of mESC pluripotency (Ivanova et al., 2006). Mechanistically, we have recently reported that PI3K activity appears to be coupled with the regulation of expression of the homeodomain transcription factor Nanog (Storm et al., 2007), which, along with Oct4 and Sox2, forms the core transcriptional circuitry that is involved in the maintenance of pluripotency (Niwa, 2007). A requirement for PI3K signalling in the maintenance of pluripotency of human ESCs has also been reported (Armstrong et al., 2006; Pyle et al., 2006).

Class-IA PI3Ks are expressed from the single-cell stage of mammalian development and their activity is required in order for development to proceed normally (Riley et al., 2005; Riley et al., 2006). Evidence supports the notion that individual isoforms play specific physiological roles in vivo. For example, mouse embryos lacking p110α exhibit proliferative defects and die at E9.5-E10.5 (Bi et al., 1999). Replacement of endogenous p110α, by knock-in of a kinase-dead version of p110α, also results in embryonic lethality of homozygous embryos, whereas heterozygotes are viable and fertile but exhibit disrupted insulin signalling (Foukas et al., 2006). Interestingly, embryos that are deficient in p110β perish at a very early stage of development, with a failure in blastocyst implantation (Bi et al., 2002). By contrast, mice that are deficient in active p110δ are viable and fertile but are subject to impaired immune responses (Okkenhaug et al., 2002). In addition to distinct physiological roles, there is evidence emerging that class-IA PI3K isoforms are also differentially regulated in distinct cellular contexts (Papakonstanti et al., 2008).

To develop a greater understanding of the role of PI3Ks in the regulation of ESC behaviour, we investigated the contribution of individual isoforms to the control of mESC fate. Using inhibitors that are selective for different isoforms, complemented by siRNA-mediated knockdown, our findings suggest that p110β plays the predominant role in the regulation of ESC self-renewal, whereas p110α appears to regulate ESC proliferation.

## Results

PI3Ks have previously been implicated in the regulation of both ESC proliferation and self-renewal (see Introduction), and, in this study, we sought to investigate whether individual isoforms of the class-IA PI3K catalytic subunit are responsible for regulating distinct aspects of mESC behaviour. Recently, a number of cell-permeable, small-molecule inhibitors have been characterised regarding their selectivity for the class-I PI3K family (Condliffe et al., 2005; Hayakawa et al., 2006; Hennessy et al., 2005; Jackson et al., 2005; Knight et al., 2006; Sadhu et al., 2003). We used a range of these small molecules, complemented with siRNA-mediated knockdown of individual isoforms, to investigate the roles of different isoforms of the class-IA PI3K catalytic subunit in the regulation of ESC self-renewal.

### PI3K p110β-mediated signalling regulates self-renewal

Mouse embryos homozygous for a partial deletion of *Pik3cb*, the gene encoding the p110β subunit, exhibit early embryonic lethality (E3.5) and cells cannot be derived from these blastocysts (Bi et al., 2002). mESCs are isolated from blastocysts at E3.5; hence, the role of p110β in mESCs was of particular interest. Treatment of mESCs, cultured in the presence LIF and serum, with the p110β-isoform selective inhibitor TGX-221 (Condliffe et al., 2005; Hennessy et al., 2005; Jackson et al., 2005) generated colonies displaying a more differentiated morphology and reduced staining for alkaline phosphatase, a marker of undifferentiated mESCs, compared with controls (Fig. 1A). Treatment with 25 nM or 50 nM TGX-221 led to significant decreases in the proportion of alkaline-phosphatase-positive colonies, as was observed following incubation with LY294002 (Fig. 1B), whereas higher doses did not lead to further decreases (supplementary material Fig. S1A). No significant change in the total number of colonies generated was observed. A less-potent p110β inhibitor, TGX-121, had similar effects to TGX-221 (supplementary material Fig. S1B). These results, using two different p110β selective inhibitors, suggest that this isoform is involved in the regulation of mESC self-renewal.

To further characterise the involvement of p110β in the regulation of mESC self-renewal, mESCs were pre-treated with TGX-221 prior to acute stimulation with LIF (Fig. 1C). Surprisingly, inhibition of p110β with TGX-221 did not affect basal or LIF-stimulated levels of phosphorylation of Ser473 of PKB (Fig. 1C, pPKB). Similarly, basal levels of both GSK-3α/β phosphorylation and ribosomal protein S6 phosphorylation (sites Ser20/9 and Ser 235/236, respectively) were unchanged by TGX-221 treatment. Whereas LIF-stimulated phosphorylation of the MAPKs Erk1 and Erk2 was unaffected by TGX-221, basal levels of phosphorylated Erk2 were modestly enhanced in unstimulated cells following incubation with TGX-221. Pre-treatment with TGX-121 demonstrated similar effects on basal signalling and negligible consistent changes on LIF-stimulated signalling (supplementary material Fig. S1C). We also examined the longer-term effects of inhibiting p110β on downstream signalling in mESCs cultured in the presence of LIF. As shown in Fig. 1D, treatment for 4 days with TGX-221 led to a decrease in PKB phosphorylation and a small decline in S6 phosphorylation, whereas levels of Erk phosphorylation were unaltered.

### p110β is required to maintain optimal mESC self-renewal

To complement the use of TGX-221, we further investigated the role of p110β in mESC self-renewal using siRNAs targeting *Pik3cb* to knockdown the expression of p110β. Both Smartpool siRNAs (Dharmacon), supplied as a pool of four single siRNAs, and individual Silencer Select siRNAs (Ambion, Applied Biosystems) were used to decrease *Pik3cb* gene expression. As a positive control, we used Smartpool siRNAs targeting Nanog, a homeodomain transcription factor known to play a key role in controlling ESC fate (Chambers et al., 2003; Chambers et al., 2007; Mitsui et al., 2003) that we have shown is downregulated following inhibition of PI3Ks over a 72-hour timecourse (Storm et al., 2007).

Quantitative reverse transcriptase (RT)-PCR revealed significant knockdown of p110β expression using Smartpool *Pik3cb* siRNAs (Fig. 2A), with 50 nM being most effective. No effect on expression of *Pik3ca* or *Pik3cd* RNAs observed (supplementary material Fig. S2A,B). Owing to antibody-sensitivity issues and low expression levels, we were unable to consistently detect catalytic-isoform protein expression, so cannot entirely rule out compensation at the protein level. However, levels of p85 subunit were either unchanged

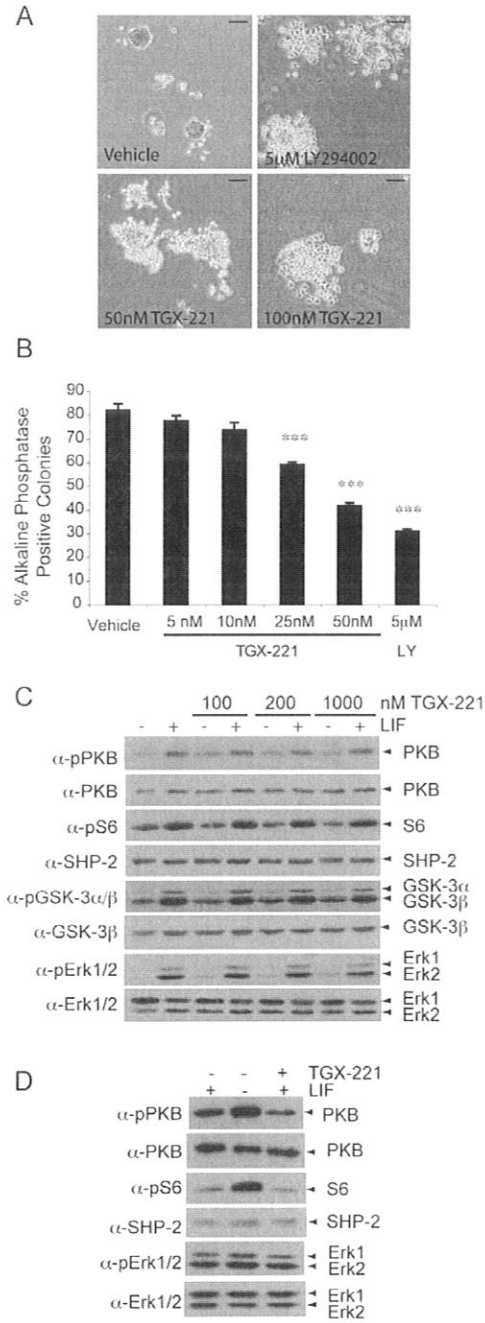


**Fig. 1.** Pharmacological inhibition of p110β leads to a decrease in self-renewal of murine ESCs. (A) Murine ESCs (mESCs), cultured in the presence of LIF and serum, were treated for 4 days with LY294002 or TGX-221 at the doses shown or with vehicle alone (DMSO). Self-renewal was assessed by staining for alkaline-phosphatase activity and representative colonies are shown. Scale bars: 100 μm. (B) The average percentage of alkaline-phosphatase-positive, self-renewing colonies in each condition are shown with s.e.m. ($n$=4). ANOVA and Dunnett's post-hoc test were applied; ***$P$<0.001. (C) Cells were treated with TGX-221 for 30 minutes prior to stimulation with $10^3$ U/ml LIF for 10 minutes. ??WHAT IS SHOWN???. (D) Cells were cultured for 4 days in the presence or absence of LIF or in the presence of LIF and 100 nM TGX-221. Cell lysates were resolved by SDS-PAGE and immunoblotting carried out using the antibodies indicated to detect PI3K downstream signalling.

or slightly decreased following knockdown of p110β (Fig. 2Eii), the latter being consistent with the overall stoichiometry of p85:p110 being preserved. No decrease in the level of mRNA encoding p110β was detected following Nanog knockdown (Fig. 2A), whereas expression of *Nanog* mRNA was decreased following knockdown of p110β (Fig. 2B), consistent with our previous observation that PI3K inhibition leads to the downregulation of Nanog expression (Storm et al., 2007). On assessment of self-renewal, knockdown of p110β led to a reduction of approximately 50% in the proportion of alkaline-phosphatase-positive colonies (Fig. 2C). Furthermore, a significant reduction in the proportion of pure self-renewing alkaline-phosphatase-positive colonies was observed using 50 nM of p110β-targeting siRNA (Fig. 2D). Importantly, the levels of self-renewal following siRNA-mediated knockdown of p110β were comparable to pharmacological inhibition of p110β with TGX-221 (Fig. 1B) and siRNA-mediated knockdown of Nanog (Fig. 2C). Consistent with a decrease in self-renewal, the level of Nanog protein was also reduced following knockdown of p110β expression (Fig. 2Ei); however, the effect of targeting p110β on Oct4 expression was not as clear, despite cells appearing morphologically differentiated. This is reminiscent of the effects we observed upon inhibition of PI3Ks with LY294002, in which Oct4 levels were maintained even though cells appear differentiated, possibly owing to the slower decline in Oct4 protein expression upon loss of pluripotency (Paling et al., 2004). For subsequent analyses, we used Nanog expression as an indicator of ESC pluripotency.

We further verified the role of p110β in the regulation of mESC pluripotency using siRNAs that target different *Pi3kcb* exons to those targeted by the Smartpool siRNAs. As shown in Fig. 3A, the siRNAs *Pik3cb* s93107 and *Pik3cb* s93108 (which reduced p110β RNA expression between 40 and 60%, Fig. 3B, but did not affect levels of *Pik3ca* or *Pik3cd* RNAs) (supplementary material Fig. S2C,D) reduced the ability of mESCs to generate alkaline-phosphatase-positive, self-renewing colonies (Fig. 3A). Consistent with the loss of alkaline-phosphatase staining, Nanog RNA (Fig. 3C) and protein (Fig. 3D) expression were also reduced following p110β knockdown, whereas levels of the p85 regulatory subunit were unchanged (Fig. 3D). Taken together, these results indicate that p110β plays a role in the maintenance of pluripotency of mESCs. When expression of p110β is reduced or when p110β is selectively inhibited, mESCs lose their alkaline-phosphatase expression and exhibit a decrease in Nanog RNA and protein expression, features that are consistent with a loss of self-renewal.

## p110δ and the control of mESC fate

p110δ plays a predominant role in cells of the lymphohaemopoietic system owing largely to its restricted expression in cells of the immune system (Okkenhaug et al., 2002; Vanhaesebroeck et al., 2004; Vanhaesebroeck et al., 1997). Although expressed at low levels by mESCs, given the importance of low levels of p110γ in cardiomyocytes (Patrucco et al., 2004), we investigated whether p110δ contributes to the regulation of ESC fate. Treatment of mESCs with the p110δ selective inhibitor IC87114 (Knight et al., 2006; Sadhu et al., 2003) only led to a significant decrease in self-renewal at a dose of 10 μM (Fig. 4A), whereas siRNA-mediated knockdown of p110δ (Fig. 4B) had little effect on the levels of alkaline-phosphatase-positive colonies (Fig. 4C). Furthermore, expression of *Nanog* RNA was not reduced following p110δ knockdown (Fig. 4D) and levels of p85 regulatory-subunit protein were unaltered (Fig. 4E). These data suggest that, if p110δ is playing a role in ESCs, it is minor in comparison to p110β.

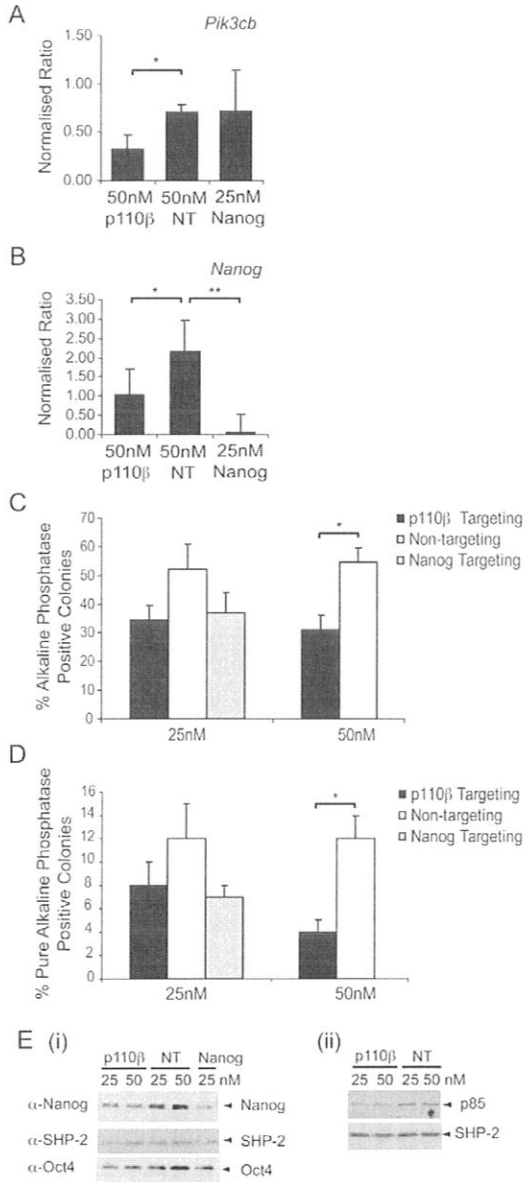


**Fig. 2.** Knockdown of *Pik3cb* reduces self-renewal and decreases Nanog expression. mESCs were transfected with *Pik3cb* (p110β), *Nanog* or non-targeting (NT) Smartpool siRNAs and harvested 72 hours later. (A) Quantitative RT-PCR was conducted on quadruplicate samples to measure knockdown of *Pik3cb* RNA and levels of RNA encoding p110β were normalised relative to levels of β-actin RNA. Representative data from four independent experiments are shown with s.d.; $*P<0.05$ following a Student's *t*-test. (B) Quantitative RT-PCR was conducted to detect knockdown of *Nanog* RNA. Representative data are shown with s.d.; $*P<0.05$ and $**P<0.01$ following a Student's *t*-test. (C) Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline-phosphatase staining. The average percentage of alkaline-phosphatase-positive, self-renewing colonies in each condition are shown with s.e.m. ($n=4$); $*P<0.05$ following a Student's *t*-test. The number of total colonies formed in each condition did not vary significantly. (D) Assessment of self-renewal by alkaline-phosphatase staining is depicted by the percentage of pure alkaline-phosphatase colonies (compact round red-stained colonies) with s.e.m. ($n=4$); $*P<0.05$ following a Student's *t*-test. (E) Immunoblotting was used to detect (i) Nanog and Oct4 protein levels or (ii) p85 protein levels, with SHP-2 reprobing used to confirm equal loading.

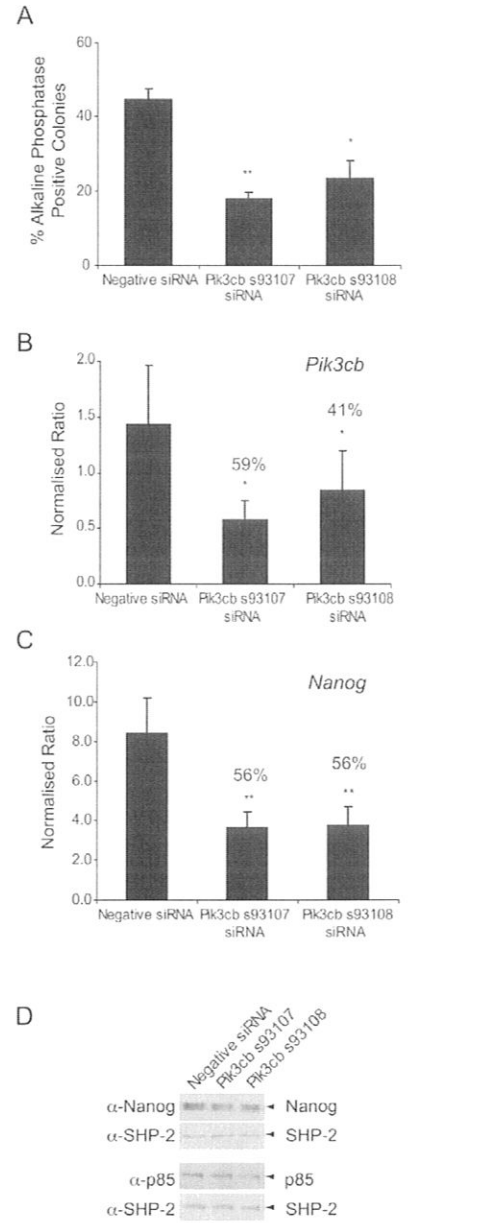


**Fig. 3.** siRNA-mediated knockdown of p110β expression reduces self-renewal. mESCs were transfected with *Pik3cb*-targeting siRNAs or with Negative Control Silencer Select siRNA (Ambion). (A) Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline-phosphatase staining. The average percentage of alkaline-phosphatase-positive colonies are shown, plus s.e.m. ($n=4$); $*P<0.05$ and $**P<0.01$ following a Student's *t*-test. The number of total colonies formed in each condition did not vary significantly. (B,C) Following transfection, cells were re-plated for a further 4 days then harvested for RNA analysis. Quantitative RT-PCR was conducted to detect knockdown of *Pik3cb* RNA (B) or expression of Nanog (C). Representative data, with s.d., are shown; $*P<0.05$ and $**P<0.01$ following a Student's *t*-test. The percentage of knockdowns, compared with the negative control, are indicated. (D) Immunoblots detecting expression of Nanog and p85 proteins in mESCs after transfection and 4 days of culture are shown and the position of proteins indicated. Blots were stripped and reprobed with antibodies detecting SHP-2 to assess loading.

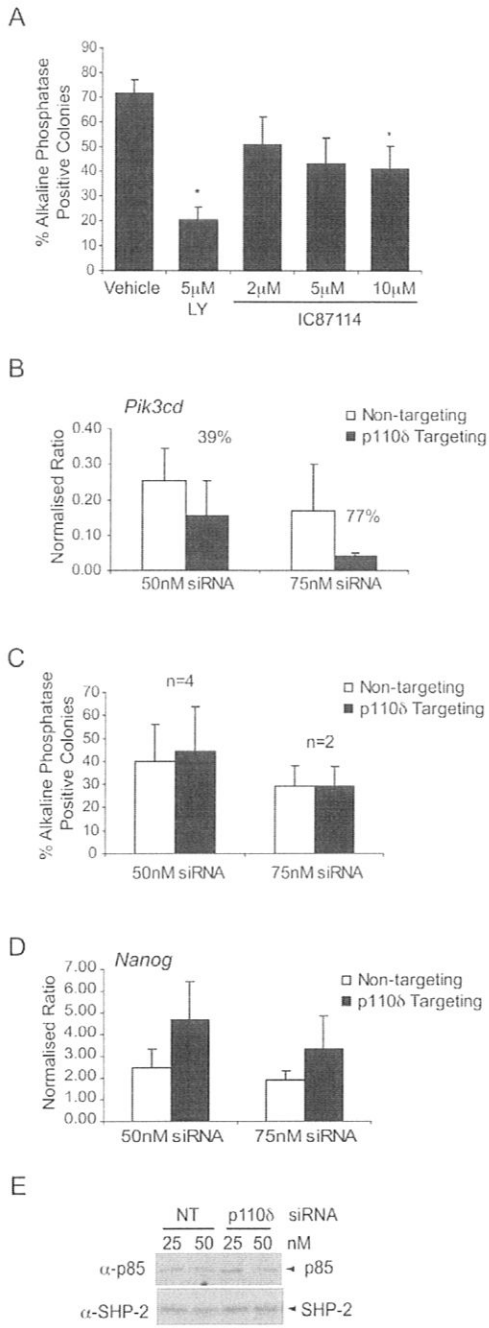


**Fig. 4.** A minor role for p110δ in mESC self-renewal. (A) mESCs were treated with a p110δ inhibitor, IC87114, for 4 days and self-renewal assessed by staining for alkaline-phosphatase activity. The average percentage of alkaline-phosphatase-positive, self-renewing colonies in each condition are shown with s.e.m. ($n$=4); *$P$<0.05 following a Student's $t$-test. The number of total colonies formed in each condition did not vary significantly. (B) Quantitative RT-PCR was conducted to detect knockdown of *Pik3cd* RNA following transfection with *Pik3cd*-targeting siRNA or with non-targeting siRNA. Representative data is shown with s.d. and the percentage of knockdowns, compared with control, are indicated. (C) Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline-phosphatase staining. The average percentage of alkaline-phosphatase-positive, self-renewing colonies in each condition are shown with s.e.m. The number of total colonies formed in each condition did not vary significantly. (D) Expression of Nanog was assessed by quantitative RT-PCR following knockdown of *Pik3cd*. Representative data is shown with s.d.
(E) XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX.

### p110α does not regulate pluripotency in mESCs

p110α has been widely implicated in the regulation of cell proliferation (Bi et al., 1999), particularly in response to insulin signalling (Foukas et al., 2006; Knight et al., 2006). To investigate whether p110α plays a selective role in the control of mESC fate, we first assessed its importance using siRNA-mediated knockdown. Smartpool *Pik3ca*-targeting siRNAs effected an 88% knockdown of mRNA encoding p110α (Fig. 5A), but had no significant effect on the expression of *Pik3cb* or *Pik3cd* RNAs (supplementary material Fig. S2G,H) or protein levels of the p85 regulatory subunit (Fig. 5Cii). Despite achieving a high level of knockdown of p110α, we observed no change in the proportion of alkaline-phosphatase-positive, self-renewing colonies (Fig. 5B). Consistent with this, we observed no alteration in the expression of the pluripotency markers Nanog or Rex1 (Fig. 5C,D). These studies suggest that p110α might not be required for the maintenance of mESCs in an undifferentiated state.

To further probe the role of p110α in mESCs, we used some of the recently described small-molecule inhibitors that exhibit selectivity for p110α. Compound 15e has been characterised as a selective inhibitor of p110α with, as yet, no reported off-target inhibition of mTOR (Hayakawa et al., 2006). PIK-75 has been used more widely and is a selective p110α inhibitor with a half-maximal inhibitory concentration ($IC_{50}$) value in the range of 5-10 nM (Chaussade et al., 2007; Knight et al., 2006). Importantly, unlike the broad-selectivity PI3K inhibitors LY294002 and PI-103, at concentrations at which PIK-75 is selective for p110α (5-10 nM) it does not inhibit mTOR, which is reported to occur at concentrations of 1 μM or higher (Knight et al., 2006). Treatment of mESCs with either 15e (Fig. 5E) or PIK-75 (Fig. 5F) did not lead to a decrease in the proportion of self-renewing colonies, in agreement with our siRNA studies. However, we noted a decrease in the total number of colonies formed in these cultures (Fig. 5E,F) and observed that colonies were smaller than those with vehicle treatment alone (Fig. 5G), suggesting that p110α plays a role in regulating the proliferation of mESCs.

### p110α-mediated signalling appears to regulate mESC proliferation

In light of earlier reports (Jirmanova et al., 2002; Murakami et al., 2004; Sun et al., 1999; Takahashi et al., 2003), and our results demonstrating that inhibition of p110α had no discernable effect on self-renewal but rather led to a reduction in colony size and number, we considered that p110α might couple to regulation of ESC proliferation, distinct from a role in the maintenance of pluripotency. We found that both 15e and PIK-75 reduced mESC growth (Fig. 6A), which appears to predominantly be the result of reduced cell division, as levels of apoptosis were similar in control and treated samples, with the exception of cells treated with 800 nM 15e, in which levels of apoptosis were slightly elevated (supplementary material Fig. S3). p110α has been linked to the regulation of insulin signalling in many cell types (Foukas et al., 2006; Knight et al., 2006), including ESCs (Welham et al., 2007).

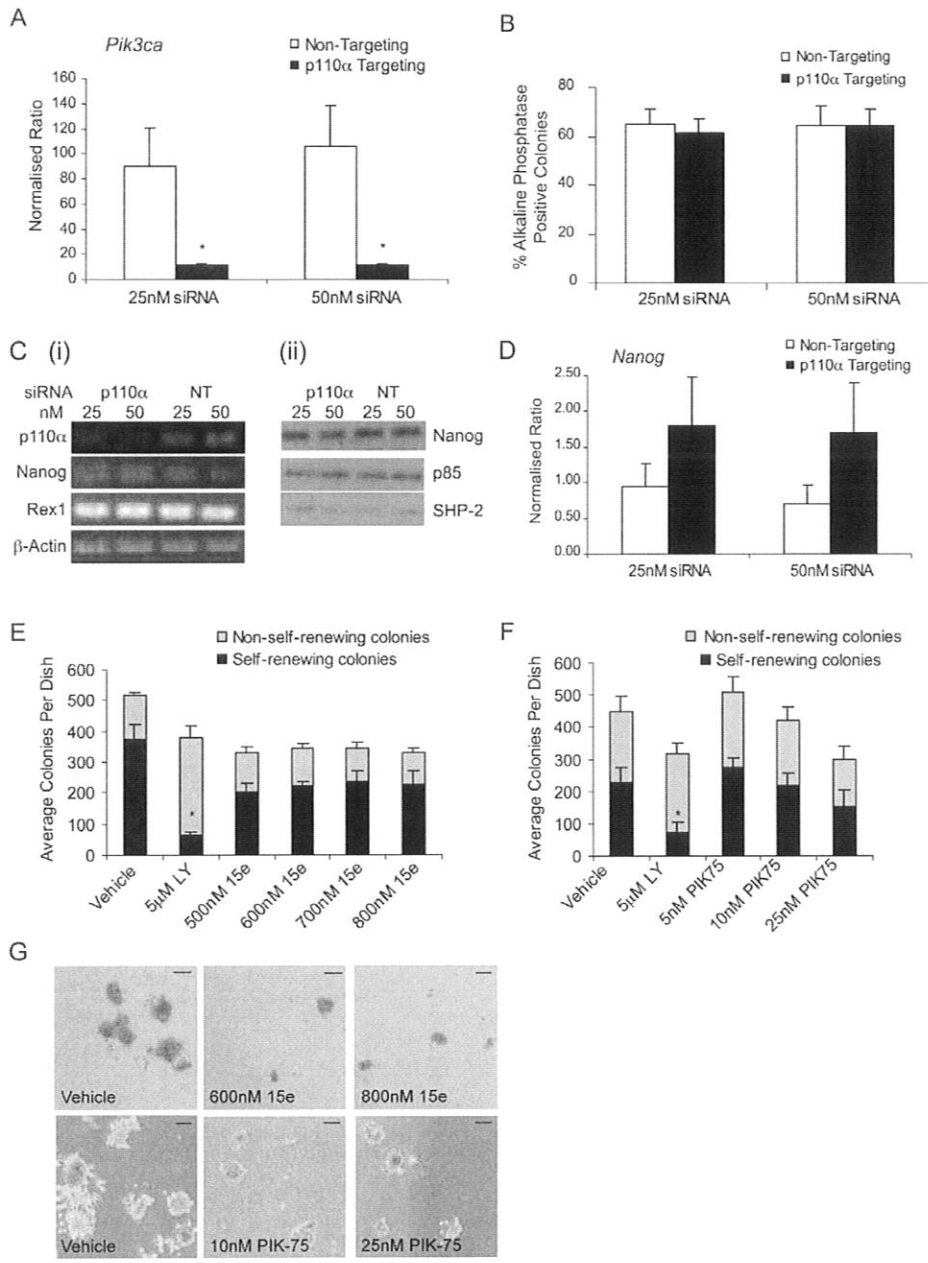


**Fig. 5.** p110α does not regulate mESC self-renewal. mESCs were transfected with *Pik3ca*-targeting (p110α) or with non-targeting (NT) siRNAs. (A) Quantitative RT-PCR of quadruplicate samples was conducted to detect knockdown of *Pik3ca* RNA. The averages, with s.d., are shown and are representative of four independent experiments. $*P<0.05$ following a Student's *t*-test. (B) Following transfection, cells were plated for 4 days and self-renewal assessed by alkaline-phosphatase staining. The average percentage of alkaline-phosphatase-positive, self-renewing colonies in each condition are shown with s.e.m. ($n=4$). The total number of colonies formed in each condition did not vary significantly. (Ci) Semi-quantitative RT-PCR was used to assess knockdown of RNA encoding p110α, Nanog or Rex1 following transfection with the indicated siRNAs. (Cii) Immunoblotting was used to detect Nanog, p85 and SHP-2 protein levels. (D) Nanog expression was also assessed by quantitative RT-PCR as in B. (E,F) mESCs were treated for 4 days with the indicated concentrations of LY294002, compound 15e (E), PIK-75 (F) or with vehicle alone (DMSO). The average number of self-renewing alkaline-phosphatase-positive and non-self-renewing colonies in each condition is shown with s.e.m. (15e, $n=5$; PIK-75, $n=3$); $*P<0.05$ following a Student's *t*-test. (G) Following treatment with 15e or PIK-75, colonies were fixed and stained for alkaline phosphatase. Representative colonies are depicted. Scale bars: 100 μm.

We found that basal and insulin-stimulated phosphorylation of Ser473 on PKB were inhibited in a dose-dependent manner by both 15e (Fig. 6Bi) and PIK-75 (Fig. 6Bii), whereas S6 phosphorylation was also reduced (Fig. 6Bi,ii). In contrast to the p110β selective inhibitor TGX-221, 15e and PIK-75 also reduced LIF-stimulated PKB Ser473 and S6 Ser235/236 phosphorylation (Fig. 6Ci,ii).

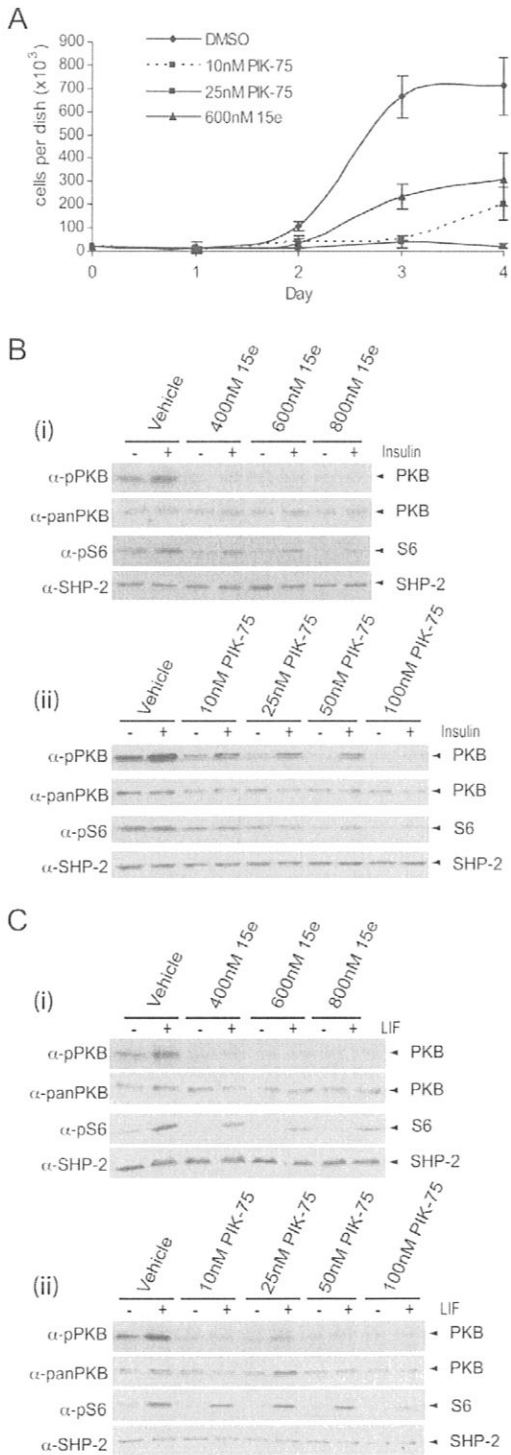


**Fig. 6.** p110α appears to be coupled to proliferation, insulin signalling and LIF signalling in mESCs. (A) To assess cell proliferation, viable cell numbers were determined in triplicate at 24-hour intervals for up to 4 days. The average number of cells per dish, ± s.d., are shown for each treatment. (B,C) Following 30 minutes pre-treatment with either (i) 15e or (ii) PIK-75 at the doses indicated, mESCs were stimulated with 10 μg/ml insulin for 5 minutes (B) or $10^3$ U/ml LIF for 10 minutes (C). Signalling downstream of PI3K was assessed by SDS-PAGE and immunoblotting with the antibodies indicated. Data, representative of three independent experiments, are shown.

### Inhibition of p110α activity does not prevent differentiation

Inhibition of p110α activity or knockdown of p110α do not appear to result in loss of pluripotency, but rather our data with PIK-75 and compound 15e suggest a role in cell division and proliferation. Alternative interpretations of our data are that inhibition or knockdown of p110α could either enhance self-renewal, block ESC differentiation or lead to selective apoptosis of spontaneously differentiating ESCs. To distinguish between these possibilities, we examined the effects of PIK-75 and 15e on mESCs cultured in the absence of LIF but in the presence of serum. The results reveal that treatment with neither PIK-75 nor 15e is sufficient to maintain self-renewal in the absence of LIF, demonstrated by the very low numbers of alkaline-phosphatase-positive colonies that were generated (Fig. 7A). However, colonies did form in the absence of LIF, arguing against selective apoptosis of differentiating cells, and these colonies had a differentiated morphology, suggesting differentiation was not blocked to any significant extent (Fig. 7B). We also investigated what effect inhibition of p110α would have on the loss of self-renewal observed following p110β selective inhibition. Interestingly, a greater loss of self-renewal was observed when mESCs were treated with both TGX-221 and 15e, compared with TGX-221 alone (Fig. 7C). These data suggest that, although effects on mESC self-renewal and proliferation seem to be coupled to different isoforms of the class-IA PI3K catalytic subunit, there is crosstalk between the isoforms.

## Discussion

ESC pluripotency is regulated by the coordinated actions of a network of extrinsic factors, signalling pathways and core transcriptional components (Boiani and Schoeler, 2005; Niwa, 2007). Self-renewal of ESCs, which is widely defined as proliferation accompanied by suppression of differentiation (Burdon et al., 2002), is required in order that pluripotency is retained. Thus, a comprehensive understanding of the regulatory mechanisms that control self-renewal is essential. The PI3K family of lipid kinases has been implicated in the regulation of ESC proliferation (Jirmanova et al., 2002; Murakami et al., 2004; Sun et al., 1999; Takahashi et al., 2003) and also in the control of self-renewal (Armstrong et al., 2006; Paling et al., 2004; Pritsker et al., 2006; Pyle et al., 2006; Watanabe et al., 2006). We rationalised that specific class-IA PI3K catalytic subunits might couple to particular physiological responses in mESCs, providing an explanation for these findings. We tested this hypothesis using isoform-selective pharmacological inhibitors and siRNA-mediated knockdown. Our data clearly demonstrate that p110β is the main isoform that contributes to the regulation of mESC pluripotency. By contrast, our data suggest that p110α plays a role in regulating mESC proliferation.

The p110β-specific inhibitors TGX-221 and TGX-121 caused a loss of mESC self-renewal, which was exemplified by significant decreases in the proportion of alkaline-phosphatase-positive colonies formed and a decline in expression of the master regulator of pluripotency, Nanog. Importantly, we observed very similar results using three different siRNAs, all of which selectively knocked down expression of p110β. In view of the lack of effect of knockdown

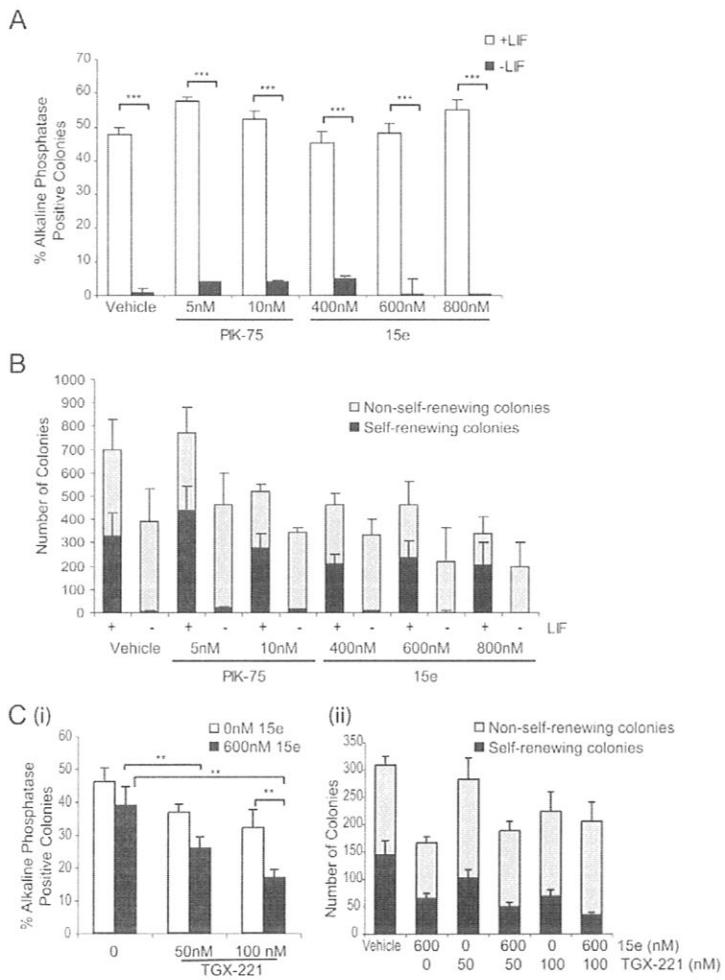


**Fig. 7.** p110α inhibition does not prevent loss of self-renewal or block mESC differentiation. (A) mESCs were treated with the p110α inhibitors 15e or PIK-75 for 4 days, at the doses indicated, in the presence or absence of LIF. The average percentage of alkaline-phosphatase-positive colonies, with s.e.m. ($n$=3), is shown, ***$P$<0.001 following a Student's $t$-test. (B) The average number of self-renewing alkaline-phosphatase-positive and non-self-renewing colonies in each condition is shown with s.e.m. ($n$=3). (C) mESCs cultured in the presence of LIF were treated with TGX-221 for 4 days in the presence or absence of 600 nM 15e. Self-renewal was assessed by staining for alkaline-phosphatase activity and the average percentage of alkaline-phosphatase-positive colonies, with s.e.m., are shown ($n$=4). ANOVA ($P$=0.002) and Dunnett's post-hoc test were applied; **$P$<0.01.

of p110δ, it is likely that the reduction in self-renewal that was observed with the p110δ selective inhibitor IC87114 is also due to inhibition of p110β, which would be predicted at the dose of this inhibitor at which an effect was observed (Knight et al., 2006). It has been known for some time that complete knockout of p110β results in very early embryonic lethality, at the blastocyst stage of development (Bi et al., 2002), which corresponds to the exact stage of development that the ICM, from which ESCs are derived, is present (Evans and Kaufman, 1981). Our results are consistent with a key requirement for PI3K signalling, mediated at least in part via p110β, for the maintenance of cells of the blastocyst. More recently, mice have been generated that contain a knock-in of a catalytically inactive form of p110β, which retain p110β protein expression. These mice demonstrate lethality, but with incomplete penetrance, the reasons for which are not currently clear (Ciraolo et al., 2008; Guillermet-Guibert et al., 2008). One proposal to rationalise the different phenotypes observed in mice that completely lack p110β (Bi et al., 2002) compared with those with knock-in of a kinase-inactive form (Ciraolo et al., 2008; Guillermet-Guibert et al., 2008), is that p110β has non-catalytic functions (Ciraolo et al., 2008). For example, the small GTPase Rab5, located mainly on endosomes, associates with p110β (Cristoforidis et al., 1999) and, when p110β expression is reduced, endocytosis of epidermal growth factor receptor is impaired (Ciraolo et al., 2008). In ESCs, we have demonstrated that inhibition of p110β activity or knockdown of p110β expression both result in decreased self-renewal, suggesting that the catalytic functions of p110β are functionally important. However, at this stage we cannot rule out the possibility that p110β also has non-catalytic functions in mESCs or that compensatory changes in expression of other PI3Ks are involved.

Somewhat surprisingly, we discovered that neither TGX-221 nor TGX-121 affected acute LIF-induced activation of PI3K signalling, although, following a 4-day treatment, TGX-221 did lead to a reduction in PKB phosphorylation in cells cultured in LIF, perhaps reflecting a role for p110β in long-term LIF action, which requires further investigation. The results of our acute LIF-stimulation experiments are very reminiscent of reports that suggest that p110β is not a major effector of tyrosine-kinase-coupled signalling pathways, but rather is linked to G-protein-coupled receptors (GPCRs) (Ciraolo et al., 2008; Guillermet-Guibert et al., 2008; Roche et al., 1998). p110γ is expressed at extremely low levels, if at all, by mESCs (Storm and Welham, unpublished data), and treatment with the p110γ selective inhibitor AS605240 has no measurable effect on mESC self-renewal (our unpublished data). Thus, p110β is likely to be the sole PI3K isoform present in mESCs that is able to couple to GPCRs. There is little information relating

to signalling via GPCRs in mESCs, although, in human ESCs, sphingosine-1-phosphate, in conjunction with platelet-derived growth factor, has been reported to promote pluripotency (Pebay et al., 2005). Components in serum, media supplements or autocrine factors might be capable of activating GPCRs, which could contribute to what might be termed 'basal' levels of signal generation. In relation to this, it is interesting to note that inhibition of p110β with TGX-221 or TGX-121 led to an increase in phosphorylation of Erk2 in unstimulated mESCs, similar to effects we have previously observed following treatment with LY294002 (Paling et al., 2004). Erk1/2 MAP-kinase signalling promotes differentiation of ESCs (Burdon et al., 1999; Kunath et al., 2007; Stavridis et al., 2007); hence, the increase we observe here could favour differentiation. A modest enhancement in basal Erk2 phosphorylation is also apparent in MEFs derived from p110β kinase-dead knock-in mice (Ciraolo et al., 2008). The mechanism underlying this enhancement in Erk2 phosphorylation is unclear, but could be related to the ability of PKB to control Raf1 phosphorylation (Rommel et al., 1999). Further studies will be required to unravel the mechanisms regulating p110β activity in ESCs.

Despite highly effective siRNA-mediated knockdown of p110α, mESCs retained their undifferentiated phenotype. Consistent with these effects, we found that two structurally distinct p110α selective inhibitors, 15e and PIK-75, did not perturb the proportion of self-renewing mESCs. Rather, our results show that treatment with 15e and PIK-75 lead to a reduction in ESC proliferation, which is exemplified by the direct measurement of cell growth and indirectly via a reduction in the size of colonies observed in self-renewal assays, similar to that reported upon knockdown of Pim-1 and Pim-3 (Aksoy et al., 2007). We also observed a decrease in the total number of colonies generated at higher doses of PIK-75, which could be due to the fact that cells are dividing more slowly and that, below a certain size, colonies will not be scored. Overall, these findings suggest that p110α is not playing a major role in regulating ESC pluripotency, but instead might regulate mESC proliferation. We also considered alternative explanations of our results. A common issue relating to PI3K inhibitors has been their tendency to inhibit mTOR, a protein that has previously been shown to regulate ESC proliferation (Murakami et al., 2004). Although much higher concentrations of PIK-75 than those we used here are required to inhibit mTOR (1 μM, compared with 6 nM to inhibit p110α) (Knight et al., 2006), no data have been reported on the possible effect of compound 15e on mTOR activity, so there remains a formal possibility that the reduction in proliferation could be due to inhibition of mTOR directly, rather than directly via p110α, although for PIK-75 we believe this is unlikely. Other alternatives include the possibility that inhibition of p110α potentiates self-renewal, blocks differentiation or causes apoptosis of spontaneously differentiating mESCs, but our data did not support these alternatives. There is extensive evidence for a role for p110α in the control of proliferation in many cell types (Bi et al., 1999; Foukas et al., 2006; Hooshmand-Rad et al., 2000; Kang et al., 2006; Knight et al., 2006; Roche et al., 1994; Vanhaesebroeck et al., 1999). Our data suggest that p110α might play a similar role in mESCs, although definitive proof requires conditional deletion of p110α in mESCs by either siRNA or gene-targeting approaches, as well as confirmation that there are no compensatory changes in expression of other PI3K isoforms. While bearing these caveats in mind, our findings now provide a rationale for the reported role of PI3Ks in the regulation of ESC proliferation (Jirmanova et al., 2002; Murakami et al., 2004; Sun et al., 1999; Takahashi et al., 2003). Most of these previous studies had not distinguished between the isoforms of the p110 catalytic subunit, although, interestingly, Takahashi et al., did show that expression of p110α could rescue the defect in proliferation that arises from a lack of ERas (Takahashi et al., 2003).

The signals that integrate the self-renewal machinery of ESC and their control of proliferation remain enigmatic. Self-renewal is widely defined as proliferation with the suppression of differentiation (Burdon et al., 2002), but it is evident that the rate of ESC proliferation can be altered while ESCs retain their undifferentiated status (Murakami et al., 2004; Takahashi et al., 2003). Although our data implicate the coupling of p110β to self-renewal and p110α to proliferation, we also present evidence that there is crosstalk between these two pathways. We found that inhibition of p110α concomitantly with p110β led to a greater decline in ESC self-renewal compared with inhibition of p110β alone. Evidence from other systems suggests that one PI3K isoform can regulate signalling via another isoform, e.g. p110β sets a threshold for p110α in insulin signalling (Chaussade et al., 2007; Knight et al., 2006) and, in priming the neutrophil respiratory burst, p110γ activation in the first phase is required for activation of p110δ in the second phase (Condliffe et al., 2005). In ESCs, p110α could set a threshold linked to ESC proliferation, acting potentially via LIF or insulin signalling. Inhibition of p110α would reduce the rate of proliferation and at the same time make self-renewal more sensitive to levels of p110β activation. Thus, PI3K signalling could potentially link the machinery controlling pluripotency with that regulating proliferation. Further detailed analyses will be required to test this hypothesis.

## Materials and Methods

### ESC culture

E14tg2a mESCs were routinely cultured as previously described (Paling et al., 2004). E14tg2a mESCs were also cultured in N2B27-defined media containing a 1:1 ratio of Neurobasal media to DMEM F-12 media, supplemented with N2 (Bottenstein and Sato, 1979) and B27 (Brewer and Cotman, 1989; Brewer et al., 1993) supplements (Invitrogen), 2 mM glutamine, 50 mg/ml bovine serum albumin (BSA), 0.0125% (v/v) Monothioglycerol (Sigma), 1000 U/ml LIF (Chemicon) and 10 ng/ml BMP4 (R&D Systems), as described previously (Ying et al., 2003a; Ying et al., 2003b). Stimulation experiments were conducted using cells cultured for at least 2 days in N2B27-defined media. Cells were washed and incubated in 1:1 Neurobasal media to DMEM F-12 media alone for 4 hours prior to stimulation.

### Inhibitors

LY294002 (Calbiochem), compound 15e (Alexis Biochemicals), PI-103 (Patent WO01083456), TGX-121 (Patent WO0153266), IC87114 (Patent WO0181346) (all generous gifts from Tom Crabbe, UCB, Slough, UK) or PIK-75 (Patent WO2001083481), TGX-221 (Patent WO2004016607) [generous gifts from Peter Shepherd, University of Auckland, New Zealand (Chaussade et al., 2007)] were used in this study at the concentrations indicated. To verify their activity and cell permeability, the effects of these small molecules on IL-3-induced signalling in the mouse pro-B cell line BaF/3, which expresses all class-IA catalytic isoforms (Vanhaesebroeck et al., 1997), were examined (supplementary material Fig. S4). All inhibitors used in this study perturbed IL-3-induced signalling in BaF/3, confirming their activity.

### Self-renewal assays

Self-renewal assays were performed as previously described (Paling et al., 2004). mESCs were allowed to adhere for a minimum of 4 hours before the addition of inhibitors. The number of alkaline-phosphatase-positive colonies (total self-renewing), the number of pure, compact, highly self-renewing colonies (pure self-renewing) and the total number of colonies per dish were determined. The mean numbers are shown for each treatment with s.e.m. Statistical significance of the results was assessed using ANOVA or *t*-test where appropriate; $*P<0.05$, $**P<0.01$ and $***P<0.001$.

### Cell lysates and immunoblotting

ESCs were washed with phosphate-buffered saline (PBS) and incubated for 4 hours in media lacking supplements and cytokines. Inhibitors were added 0.5-1.0 hour prior

to a 10-minute stimulation with $10^3$ U/ml LIF or a 5-minute stimulation with 10 μg/ml insulin. Culture plates were placed on ice, washed three times with ice-cold PBS and lysed with solubilisation buffer (Welham et al., 1994). Insoluble cell debris was removed by centrifugation for 2 minutes at 14,000 rpm at 4°C. Protein concentration was determined with a Bradford Assay using the Bio-Rad protein assay kit according to the manufacturer's instructions. A total of 10-20 μg of protein was separated by SDS-PAGE and transferred onto nitrocellulose. Immunoblotting was performed as previously described (Welham et al., 1994) using the following primary antibodies at 1:1000 dilution: rabbit polyclonal antibodies against phosphorylated p44/42 MAPK (anti-pERK-1/2; Santa Cruz Biotechnology, sc93), p44/42 MAPK [anti-panERK-1/2; Cell Signalling Technologies (CST), 9102], phosphorylated Ser473 PKB (anti-pPKB; CST, 4058), PKB (anti-panPKB; CST, 9272), phosphorylated ribosomal protein S6 (anti-pS6; CST, 2211), SH2-domain tyrosine phosphatase (anti-SHP-2; Santa Cruz Biotechnology, sc280), phosphorylated Ser 20/9 GSK-3 (anti-pGSK-3α/β; CST, 9331), GSK-3β (anti-panGSKβ; CST, 9315) and Nanog (anti-Nanog; Abcam, 21603). Horseradish-peroxidase-conjugated anti-rabbit secondary antibodies (Dako, UK) were used at 1:20,000 dilution and blots were developed using ECL or ECL Advance (Amersham Biosciences, UK). Blots were stripped and re-probed as described previously (Welham et al., 1994).

### Analysis of apoptosis using DiOC6 staining

Mitochondrial-membrane-permeability transition, a hallmark of and an irreversible point in programmed cell death (Rottenberg and Wu, 1998), was assessed by DiOC6 (3,3′-dihexyloxacarbocyanide iodide) staining. Cells were pre-treated with compound 15e, PIK-75 or DMSO for 18 hours, then media and cells were collected, washed in PBS and incubated with 1 nM DiOC6 for 30 minutes at room temperature. Samples of unstained cells were retained. An aliquot of DMSO-treated cells were pre-treated with 150 μM carbonyl cyanide *m*-chlorphenyl hydrazone (CCCP) for 20 minutes. After staining, cells were washed and fluorescence measured by flow cytometry.

### Growth-curve analyses

Cells were plated at $2\times10^4$ cells/5-cm plate and incubated with DMSO or p110α inhibitors for 1-5 days. Cells were counted each day, in triplicate, using Trypan Blue vital dye.

### Gene silencing with siRNAs

Cultures of mESCs were transfected with doses of *Pik3ca*-, *Pik3cb*-, *Pik3cd*- or *Nanog*-targeting, or non-targeting, siRNAs (Dharmacon) as indicated, using Lipofectamine 2000 transfection reagent (Invitrogen). After 48 hours, cells were re-transfected and incubated for a further 24 hours. Transfected cells were plated into a self-renewal assay, or harvested for protein or RNA expression analysis. Cultures of mESCs transfected with 10 nM *Pik3cb*-targeting or non-targeting negative siRNA (Ambion, Applied Biosystems) were plated into self-renewal assays after 48 hours, harvested for protein or RNA expression analyses at the same time or re-plated for a further 4 days before protein and RNA were harvested.

### Gene-expression analyses

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. DNase treatment, reverse transcription and quantitative RT-PCR (qRT-PCR) were conducted as described previously (Storm et al., 2007). Briefly, qRT-PCR was conducted using LightCycler FastStart DNA Master SYBR Green (Roche Applied Science, East Sussex, UK) according to the manufacturer's instructions. The primers used in this study were: Nanog Forward 5′-CTCTTCAAGGCAGCCCTGAT-3′; Nanog Reverse 5′-CCATTGCTAGTCTTCAACCAC-3′; Rex1 Forward 5′-CGTGTAACATACACCATCCG-3′; Rex1 Reverse 5′-GAAATCCTCTTCCA-GAATGG-3′; p110α (*Pik3ca*) Forward 5′-AAATGGCGACGACTTACG-3′; p110α (*Pik3ca*) Reverse 5′-TTGTTCTTGTCCTTGAGC-3′; p110β (*Pik3cb*) Forward 5′-TAATGTGTCAAGTCGTGG-3′; p110β (*Pik3cb*) Reverse 5′-CAGCCTACAG-CGTATTCC-3′; p110δ (*Pik3cd*) Forward 5′-CTGGACCTGAGGATGACG-3′; p110δ (*Pik3cd*) Reverse 5′- GGCTCAAGTCCAAGAACC-3′; β-Actin Forward 5′-TAGGCACCAGGGTGTGATGG-3′; β-Actin Reverse 5′-CATGGCTGGGGTG-TTGAAGG-3′.

Representative experiments, showing expression of quadruplicate samples normalised to β-actin, are presented with standard deviations. Expression following targeted siRNA transfection was compared with expression following transfection with non-targeting siRNA controls and statistical significance assessed using a Student's *t*-test, where $*P<0.05$ and $**P<0.01$.

This work was supported by an MRC Capacity-Building studentship and a BBSRC Research Development Fellowship to M.W. The authors are grateful to Tom Crabbe (UCB) and Peter Shepherd (Auckland, New Zealand) for the gifts of isoform-selective PI3K inhibitors. Deposited in PMC for release after 6 months.

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