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Mechanisms Controlling Self-renewal and Pluripotency in Human Embryonic Stem Cells

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Introduction

Human embryonic stem cells (hESCs) are an immensely powerful tool for studying the early differentiation of human cell lineages *in vitro*. But more importantly, their proliferative and pluripotent capacity offer the prospective that cell banks of hESCs can generate specific cell phenotypes to a consistent quality for use in clinical treatments for a range of degenerative diseases (Shufaro and Reubinoff, 2004; Keller, 2005). Mammalian embryonic stem (ES) cells were first derived from mouse embryos independently by two laboratories (Evans and Kaufman, 1981; Martin, 1981). These cells displayed extensive proliferation but retained an undifferentiated state when co-cultured with mouse embryonic fibroblasts (mEFs). When ES cells were injected into pre-implantation mouse blastocysts that were returned to

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Abbreviations: APC, axin/adenomatus polyposis coli; bFGF, basic fibroblastic growth factor; BMP, bone morphogenic protein; COUP TF, chicken ovalbumin upstream promoter transcription factor; EB, embryoid body; EC, embryonal carcinoma; EDG, endothelial differentiation gene; ERK, extracellular signal-related kinase; ES cell, embryonic stem cell; GCNR, germ cell nuclear receptor; GDF, growth differentiation factor; GSK3β, glycogen synthase kinase 3β; hCG, human chorionic gonadotrophin; hESCs, human embryonic stem cells; H-IL-6, hyper-interleukin-6; ICM, inner cell mass; IL, interleukin; JAK, janus activated kinase; KGF, keratinocyte growth factor; LIF, leukaemia inhibitory factor; LRH-1, liver receptor homologue 1; MAPK, mitogen activated protein kinase; mEFs, mouse embryonic fibroblasts; mESCs, murine embryonic stem cells; NIC, nicotinamide; PDGF, platelet-derived growth factor; PDK-1, phosphoinositide-dependent kinase 1; PH, plekstrin homology; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PSPB, pluripotent cell specific Sox element-binding protein; PtdIns, phosphatidylinositol; PTEN, phosphatase and tensin homologue deleted on chromosome ten; RNAi, ribonucleic acid interference; RT-PCR, reverse transcriptase-polymerase chain reaction; SF1, steriodogenic factor; SH2, Src homology 2; SHP-2, Src homology protein tyrosine phosphatase 2; SOCS, suppressor of cytokine signalling; S1P, sphingosine-1-phosphate; STAT, signal transducers and activators of transcription; TCF/LEF, T cell factor/lymphoid enhancer; TDGF1, teratocarcinoma-derived growth factor 1; TGF, transforming growth factor; UTF1, undifferentiated embryonic cell transcription factor 1.

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pseudopregnant females, the resulting offspring displayed extensive chimerism, demonstrating the pluripotent capacity of these cell lines. Subsequently, murine ES cells (mESCs) became pivotal in the development of transgenic technology (Gertsenstein *et al.*, 2002) and have been studied and utilized extensively as a vehicle to introduce genes into the early embryo.

Early attempts to derive ES cell lines in other rodents (e.g. rat) or in domestic species failed, and it became dogma that the mouse was perhaps the exception rather than the rule in the generation of ES cell lines. This opinion changed with the derivation of monkey (rhesus monkey, marmoset) ES cells by Thomson and colleagues (Thomson *et al.*, 1995, 1996) and thereafter human ES cells (Thomson *et al.*, 1998). Since 1998, many hESC lines have been produced and characterized to different degrees. While all these cell lines exhibit the fundamental attributes of hESCs in self-renewal and pluripotency, they appear to vary in how easily they can be maintained and propagated under equivalent culture conditions. This is not surprising as they originate from the outbred human population (compared with an inbred mouse strain) and have been derived from the early and late blastocyst and the morula stage of pre-implantation human embryos. Recently, considerable progress has been made in developing more defined culture conditions in which to culture hESCs, but a fundamental understanding of the mechanisms controlling cell self-renewal and pluripotency will be necessary to provide a solid foundation to this burgeoning field.

Derivation of human embryonic stem cells

ES cells result from the transformation of cells from the inner cell mass (ICM) into a cell line that self-renews while maintaining pluripotency. A high proportion of single cells from the mouse ICM have the capability to form ES cells (Brook and Gardner, 1997). Whether this is the case for the human ICM is not known as, to date, the intact ICM or embryo has been used for derivation rather than single cell preparations. hESCs have been derived recently from the morula stage embryo (Strelchenko *et al.*, 2004), although it remains to be confirmed whether such cell lines resulted from transformation of the morula blastomeres or from further development of morula to ICM during the derivation procedure. In either case, ES cells are an artefact of their *in vitro* environment as, *in situ*, the ICM rapidly differentiates to the epiblast and the major cell lineages (endoderm, mesoderm, and ectoderm) during embryo development.

Mitotically inactivated mEFs ('feeder' cells) were used initially with medium supplemented with serum to provide a supportive culture milieu for the murine ICM to generate ES cells, and essentially similar methods were used subsequently to derive the first hESC lines. It is somewhat fortuitous that mouse feeder cells support both murine and human ES cell derivation as it is now clear that the main factor responsible for supporting mESCs, the cytokine, leukaemia-inhibiting factor (LIF) (Smith, 2001), is not responsible for supporting nonhuman primate and human ES cells.

Exactly how mouse feeders support the transformation of the human ICM remains unclear and has to be inferred from the ability of various culture conditions to maintain extant hESC lines. However, the maintenance of established cell lines may not represent the conditions required for initial derivation. Culture protocols are

likely to select cells that propagate the most effectively (a balance between proliferation, apoptosis, and differentiation for ES cells) and, therefore, cell lines may eventually have properties that differ significantly from initial derivation. Such an adaptation process is probably inevitable when cells are passaged over many months, or even years, in culture and can lead to karyotypic abnormalities (Draper *et al.*, 2004b; Enver *et al.*, 2005). More recently, hESC lines have been derived using human feeder cells of various origins, using serum-free medium (but with serum-replacement supplements) and without feeder cells but with extracellular matrix preparations (Hovatta and Skottman, 2005; Klimanskaya *et al.*, 2005). While these various procedures indicate that the combination of growth factors and extracellular matrix components influence hESC derivation, it is difficult to ascertain the relative importance of individual factors in this process.

Factors controlling self-renewal of hESCs

Self-renewal may be defined as the ability to undergo symmetric cell division and produce daughter cells with the same pluripotent capacity and replicative potential of the parent cell. The mechanisms controlling the process of self-renewal can be classified as extrinsic or intrinsic factors. It is our restricted knowledge of the essential factors required for hESC self-renewal that limits the potential applications of these cells at this time. While incomplete, our understanding has evolved steadily in recent years, with many of the discovered pathways previously known to be crucial in developmental biology and fundamental in mESC self-renewal. Although parallels have been drawn between murine and human systems, methods required to culture these species-specific ESCs exhibit key differences. As most studies to date have considered each self-renewal pathway in isolation, the potential for crosstalk between pathways has not been fully explored (*Figure 14.1*).

EXTRINSIC FACTORS/SIGNALLING REQUIREMENTS OF hESCs

LIF/STAT3

LIF signalling plays a major role in maintaining the self-renewal of mESCs, and as such, is routinely used in mESC culture. Supplementation of serum containing media with LIF negated the requirement for co-culture with feeder cells (Smith *et al.*, 1988; Williams *et al.*, 1988) and highlighted the significance of this pathway.

LIF is a member of the IL-6 family of cytokines and signals via a heterodimeric complex composed of a specific low affinity LIFRβ receptor chain and the gp130 chain, which is common to all receptors of the IL-6 family. Cytokine-induced dimerization of the receptor complex results in activation of janus activated kinases (JAKs), which serve to phosphorylate tyrosine residues in the intracellular domain of gp130, creating docking sites for SH2 domain containing proteins. The primary proteins involved are STATs (signal transducers and activators of transcription) 1 and 3 and SHP-2, which mediates mitogen activated protein kinase (MAPK) signalling. Whilst it appears that ERK signalling promotes differentiation (Burdon *et al.*, 1999), the ability of LIF to promote self-renewal results from the activation of the STAT3 pathway (Niwa *et al.*, 1998); indeed, STAT3 activation was deemed sufficient

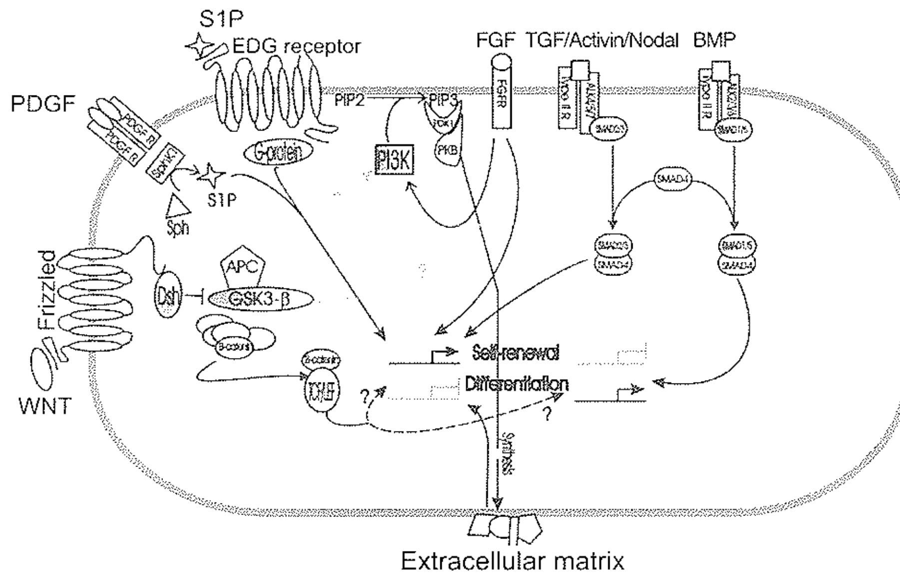

Figure 14.1. Several pathways are implicated in hESC self-renewal, although the mechanisms by which this is controlled are relatively unknown. TGF/Activin/Nodal activate SMAD2/3, which associates with co-SMAD4 and translocates to the nucleus, where they are active as transcription factors; conversely, BMP activation of SMAD 1/5/8 results in differentiation. The role of WNT signalling is unconfirmed, whether it is promoting self-renewal or differentiation (dashed line) and whether the effect is mediated by β-catenin/TCF/LEF or by other downstream effects of GSK3-β inhibition. bFGF signalling supports TGF in self-renewal; one proposed effect is promoting PI3K/PKB activation, which in turn supports stable expression of extracellular matrix proteins, required to maintain self-renewal. S1P signals both extracellularly, through EDG receptors coupled to G proteins, and intracellularly by undefined mechanisms. PDGF promotes intracellular S1P signalling by activating sphingosine kinase, which in turn converts sphingosine to S1P. The combination of extracellular PDGF and S1P supports hESC self-renewal. Crosstalk between pathways is highly probable (two examples are shown (dotted lines)).

to maintain the undifferentiated state (Matsuda *et al.*, 1999). This last point has been questioned more recently by Ying and colleagues, whose serum-free system requires a balance between STAT3 activation by LIF and Id activation by BMP stimulation to maintain self-renewal (Ying *et al.*, 2003). However, the basal culture medium in this system was supplemented with N2B27, developed for the culture of neurons, and so it is possible that the addition of BMP is essential in offsetting the supplements, promoting neural lineage fate.

IL-6 also signals via gp130, although since ES cells lack IL-6 receptor (IL-6R), IL-6 is unable to substitute LIF. However, IL-6 is able to sustain the undifferentiated state of mESCs when in combination with soluble IL-6 receptor (sIL-6R) (Yoshida *et al.*, 1994) or as an IL-6/IL-6R fusion protein (Viswanathan *et al.*, 2002) termed Hyper-IL-6 (H-IL-6) (Fischer *et al.*, 1997), which is active at 100- to1000-fold lower concentrations than unlinked molecules.

LIF supplementation does not appear to increase mESC growth rate, but favours the probability that a cell will undergo self-renewal as opposed to a differentiation division (Zandstra *et al.*, 2000). There is also a threshold of gp130-mediated signalling that influences self-renewal and possible survival outcomes (Viswanathan *et al.*, 2002).

Although it is possible to culture mESCs in the absence of a feeder cell layer for many passages by supplementing the media with LIF, this is not the case for hESCs, which spontaneously differentiate in the absence of feeder co-culture, despite the presence of human LIF (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Using an extracellular matrix preparation such as Matrigel (Invitrogen), it is possible to culture self-renewing hESCs, but this currently requires the use of media conditioned from mitotically inactivated mEFs or the equivalent human cells (Xu *et al.*, 2001; Draper *et al.*, 2004a). Feeder cells are therefore most probably providing soluble factors required to support the self-renewing hESCs. These factors, or combinations of factors, are as yet undefined; however, mEF-provided LIF is unlikely to contribute to this self-renewal since the human LIF receptor complex is irresponsive to murine LIF. These findings, however, do not rule out a role for LIF signalling in an autocrine fashion.

A number of more recent reports further address the relevance of the STAT3 pathway in human and nonhuman primate ES cells (Daheron *et al.*, 2004; Humphrey *et al.*, 2004; Sato *et al.*, 2004; Sumi *et al.*, 2004). The majority of these reports demonstrate the ability to activate STAT3; however, this does not appear to favour self-renewal. The activation state of STAT3 is measured by the phosphorylation of tyrosine residue 705, which promotes dimerization of STAT3 and its translocation to the nucleus, where it acts as a transcription factor. Components of LIFR and gp130 are detected in hESCs by reverse transcriptase-polymerase chain reaction (RT-PCR), and upregulation of activated STAT3 is observed following stimulation of the cells with LIF or H-IL-6 (Daheron *et al.*, 2004; Humphrey *et al.*, 2004). In contrast, Sato and colleagues were able to detect activated STAT3 in non-stimulated hESCs and only a marginal increase following stimulation (Sato *et al.*, 2004). Despite activation of STAT3, cells were unable to self-renew. It has been suggested that activation of STAT3 may even favour differentiation or apoptosis in hESCs (Daheron *et al.*, 2004). This suggestion was based in part upon an inability to generate stable clones expressing STAT3C (a mutant STAT3 that spontaneously dimerizes, translocates to the nucleus, and activates transcription), or a conditionally activated fusion protein of STAT3 and the oestrogen receptor.

Y705 phosphorylation of STAT3 also is observed following stimulation of cynomologus monkey ES lines with LIF of H-IL-6 (Sumi *et al.*, 2004). This is accompanied by detection of transcript for the STAT3 target gene suppressor of cytokines signalling (SOCS) 3. However, not only do LIF and H-IL-6 fail to maintain self-renewal of these nonhuman primate ES cells, but stable transfectants containing a dominant-interfering form of STAT3 (STAT3F) continue to proliferate in an undifferentiated state, retaining their pluripotency (Sumi *et al.*, 2004).

Since a threshold of STAT3 activation is required to maintain mESCs in the undifferentiated state (Matsuda *et al.*, 1999), it is possible this threshold is not reached in these studies with hESCs. Although the receptors are present, expression is at a low level (Brandenberger *et al.*, 2004) and perhaps insufficient for signalling at threshold levels. However, since STAT3 is not an essential component for the self-renewing capabilities of primate ES cells (Sumi *et al.*, 2004) and may adversely affect the capacity of hESCs to renew (Daheron *et al.*, 2004), this may be of little consequence.

TGFβ/BMP and bFGF

Unlike LIF signalling, transforming growth factor (TGF) β family signalling appears to play a major role in maintaining the self-renewing hESC. Signalling can be subdivided into TGFβ/Activin/Nodal and the bone morphogenic protein (BMP)/ growth differentiation factor (GDF) pathways. TGFβ/Activin/Nodal signal via type I receptors, ALK 4, 5, and 7, to activate SMAD2/3, whilst BMP/GDF signalling activates SMAD 1/5/8 via ALK 1, 2, 3, and 6 (reviewed in Shi and Massague, 2003).

Differentiation of hESCs via an embryoid body (EB) stage with TGFβ1 and Activin A favours mesoderm lineage differentiation, as assessed by the expression of a series of cell-specific genes (Schuldiner *et al.*, 2000). However, it is suggested that this may result primarily through inhibiting differentiation into specific cell types, as opposed to their induction. For example, more prolonged culture of hESCs can occur in the absence of feeders by growing the cells on fibronectin in culture medium supplemented with TGFβ1, bFGF, and LIF (Amit *et al.*, 2004). Interestingly, LIF was required for optimal culture on bovine fibronectin, but had little effect when the cells were cultured on human fibronectin. Similarly, efficient feeder-free culture upon laminin is possible with Activin A in combination with nicotinamide (NIC) and keratinocyte growth factor (KGF) (Beattie *et al.*, 2005). In this culture system, Activin A specifically maintains the undifferentiated state, whilst KGF and NIC provide proliferation and survival signals. That there is high expression of Activin A in mEFs and Activin A precursor in media conditioned from mEFs (Beattie *et al.*, 2005) indicate that Activin A may be one of the critical factors provided by mEFs in standard hESC culture protocol.

Teratocarcinoma-derived growth factor 1 (TDGF1), cloned from the human embryonal carcinoma cell line NTERA2 (Ciccodicola *et al.*, 1989), is highly enriched in pluripotent hESCs (Bhattacharya *et al.*, 2004; Brandenberger *et al.*, 2004; Enver *et al.*, 2005) and acts as a necessary accessory receptor for Nodal (Yeo and Whitman, 2001). Significantly, transgenic hESCs that over-express Nodal maintain markers of pluripotency when cultured in feeder-free conditions, which would normally cause wild-type cells to differentiate (Vallier *et al.*, 2004). It has been proposed that there is a positive feed-back loop in which activation of the SMAD2/3 pathway is required for Nodal transcription in hESCs (Besser, 2004). Conversely, LEFTYA and LEFTYB, which inhibit Nodal signalling, are also positively regulated via SMAD2/3 (Besser, 2004), and LEFTYB represents a signature molecule of pluripotent hESCs (Bhattacharya *et al.*, 2004; Brandenberger *et al.*, 2004; Enver *et al.*, 2005).

In contrast to TGFβ/Activin/Nodal signalling, which promotes the maintenance of pluripotent hESCs, BMP signalling is unable to support self-renewal (Beattie *et al.*, 2005) and is associated with differentiation to trophoblast (Xu *et al.*, 2002) or extra-embryonic endoderm cells (Pera *et al.*, 2004). As hESCs differentiate, there appears to be a molecular switch from SMAD2/3 signalling to that of SMAD1/5 (James *et al.*, 2005). SMAD2/3 activation is necessary in maintaining the undifferentiated state, since ablation using a pharmacological inhibitor to the ALK 4, 5, 7 causes cells to differentiate (James *et al.*, 2005; Vallier *et al.*, 2005). Supporting this notion, hESCs can be maintained in the absence of feeders in culture medium containing the BMP inhibitor noggin (Wang *et al.*, 2005; Xu *et al.*, 2005b). Media

conditioned from mEFs, which is sufficient to maintain hESCs on Matrigel, contains BMP inhibitors, noggin and gremlin (Xu *et al.*, 2005b), which will suppress SMAD1/5 signalling. However, unlike TGFβ and Activin A, which supports hESC culture in low concentrations of basic fibroblast growth factor (bFGF) (Amit *et al.*, 2004; Beattie *et al.*, 2005), suppression of BMP signalling with noggin alone is insufficient for prolonged hESC feeder-free culture (Wang *et al.*, 2005) and requires a high dose of bFGF (Wang *et al.*, 2005; Xu *et al.*, 2005b), which in itself suppresses BMP signalling (Xu *et al.*, 2005b).

It appears, therefore, that TGFβ family signalling plays a very important role in the maintenance of pluripotent hESCs, which requires active SMAD2/3 signalling and suppression of SMAD1/5 activity. Correct function of all these receptor-regulated SMADs requires dimerization with the co-SMAD, SMAD4, which may itself be a limiting factor, such that signalling via one branch is at the expense of the other. TGFβ signalling alone is insufficient to maintain the pluripotent state and there is a requirement for bFGF. Thus, there may need to be a balance between the contribution of TGFβ/Activin/Nodal and bFGF signalling, such that supplementation with the former reduces the requirement of the latter. Although high bFGF levels are able to support hESCs in the absence of TGFβ supplementation (Wang *et al.*, 2005; Xu *et al.*, 2005a,b), cells were cultured upon Matrigel-coated plates, which are known to contain, amongst other growth factors, TGFβ. It has been reasoned that the effects of bFGF on hESC pluripotency actually may be dependent upon SMAD2/3 signalling; where the positive effects (regarding the maintenance of pluripotency) of medium dose bFGF in a chemically defined medium is offset by the inhibition of SMAD2/3 signalling (Vallier *et al.*, 2005).

WNT

WNT signalling has an extensive role in controlling animal development, including embryonic induction, the generation of cell polarity and cell fate processes (Cadigan and Nusse, 1997). Canonical WNT signalling involves the binding of WNT ligands to the frizzled receptors. This in turn activates dishevelled which displaces glycogen synthase kinase 3β (GSK3β) from the Axin/adenomatus polyposis coli (APC) complex, preventing ubiquitin-mediated degradation of β-catenin. Subsequently, β-catenin accumulates and translocates into the nucleus, where it associates with T cell factor/lymphoid enhancer (TCF/LEF) proteins to activate transcription of WNT target genes.

WNT signalling is known to be involved in regulating the proliferation of stem cells, including intestinal (van de Wetering *et al.*, 2002; Pinto *et al.*, 2003; Pinto and Clevers, 2005) and haematopoietic cells (Austin *et al.*, 1997), and is involved additionally in the self-renewal of haematopoietic stem cells (Reya *et al.*, 2003). However, inactivation of the β-catenin gene does not appear to adversely affect haematopoiesis or lymphopoiesis (Cobas *et al.*, 2004). WNT also appears to have a role in regulating the differentiation of mESCs, although the reports have been contrary. Treatment of mESC-derived EBs with retinoic acid promotes neural differentiation. This neural differentiation is inhibited through stable expression of WNT1, or the requirement for retinoic acid replaced by forced expression of the WNT antagonist, Sfrp2 (Aubert *et al.*, 2002). Consistent with this finding is the ability to reduce the differentiation capacity of mESC into the three germ layers by

modulating the dose of β-catenin signalling (Kielman *et al.*, 2002). On the other hand, treatment of mESCs and mEC cells with a pharmacological inhibitor of GSK3β actually promotes neuronal differentiation (Ding *et al.*, 2003), which is observed also in mEC cells over expressing WNT1 (Tang *et al.*, 2002).

Components of the WNT signalling pathway are present in hESCs (Walsh and Andrews, 2003; Brandenberger *et al.*, 2004; Sato *et al.*, 2004). In contrast to differentiated cells, the undifferentiated population do not express the transcript for WNT ligands, and levels of antagonist and inhibitors vary between different populations (Brandenberger *et al.*, 2004).

WNT signalling can mediate the self-renewal of hESCs and it has been reported that hESCs cultured under feeder-free conditions with a pharmacological inhibitor of GSK3β or exogenous WNT3a maintain markers of the undifferentiated state (Sato *et al.*, 2004). But some discrepancy remains, at least in our hands, as inhibition of GSK3β with lithium chloride results in differentiation of hEC cells (Giesberts *et al.*, 1999) and hESCs (unpublished data). Also, it is yet to be addressed as to whether the inhibitor (BIO) is able to sustain prolonged culture of the differentiated state. Apart from WNT signalling, GSK3β functions in a number of other signalling pathways (Frame and Cohen, 2001), and therefore, the observed results may reflect modifications elsewhere. A possible candidate here is in the stability of Myc, which promotes self-renewal in mESCs and is targeted for degradation by GSK3β (Cartwright *et al.*, 2005).

Recently, the question of WNT signalling in hESCs has been addressed. WNT was found not only to stimulate hESC proliferation, but also differentiation and β-catenin mediated transcriptional activity was minimal in the undifferentiated state (Dravid *et al.*, 2005). From these findings, the authors concluded that factors secreted by feeders that support hESC were unlikely to be WNT ligands. Hence, the relationship between WNT signalling and GSK3β activity in the regulation of hESCs remains inconclusive.

S1P/PDGF

Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid derived from the breakdown of sphingomyelin, a structural component of eukaryotic cell membranes. It is stored and released from platelets upon their activation or in response to external stimuli, such as growth factors and cytokines (Pyne and Pyne, 2000). S1P has been implicated in a diverse range of biological processes, including cell growth, differentiation, migration, and apoptosis, in many different cell types (Takuwa, 2002). As the prevention of apoptosis is a common self-renewal mechanism, S1P potentially could aid the self-renewal process in hESCs.

Platelet-derived growth factor (PDGF) also has been implicated in the prevention of apoptosis (Heldin and Westermark, 1999). PDGF can activate sphingosine kinase, an enzyme responsible for the conversion of sphingosine to S1P by phosphorylation. This increase in intracellular S1P is thought to be responsible for cell proliferation and survival induced by PDGF. When cultured in the presence of S1P and PDGF in combination, hESCs have shown the ability to retain their pluripotency and undifferentiated state in the absence of co-culture or serum (Pebay *et al.*, 2005), indicating a role for lysophospholipid signalling in the maintenance of stem cells.

PI3K

The phosphoinositide 3-kinase (PI3K) family are lipid kinases that form three classes (I, II, and III). The products of activated class I members, phosphatidylinositol (3,4)-bisphosphate [PtdIns (3,4) P2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns (3,4,5) P3], serve as intracellular second messengers recruiting plekstrin homology (PH) domain containing proteins. The most recognized is protein kinase B (PKB), also referred to as Act, which translocates to the membrane and is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at Thr308 and additionally at Ser437 by PDK2. Activated PKB promotes cell proliferation, survival, growth, and motility, and is therefore implicated in tumourogenicity (Vivanco *et al.*, 2002).

It is perhaps unsurprising that PI3K signalling has been linked to mESC proliferation. PTEN is a tumour suppressor that has PIP3 phosphatase activity, which negatively regulates PI3K/PKB activity. $Pten^{-/-}$ mESCs demonstrate enhanced proliferation and cell survival (Sun *et al.*, 1999), which is associated with downregulation of $p27^{kip1}$, promoting G_1/S transition, and inactivation of the pro-apoptotic protein, Bad. In addition, $Pten^{-/-}$ mESCs are seen to transit more rapidly through G_2/M to G_1 phase of the cell cycle (Kandel *et al.*, 2002). Conversely, mESCs treated with the PI3K inhibitor LY294002 are found to accumulate in G_1, blocked from G_1/S transition (Jirmanova *et al.*, 2002).

More recently, it has been shown that PI3K plays a positive role in LIF regulated mESC self-renewal (Paling *et al.*, 2004), where interference of PI3K signalling results in a reduction in markers of pluripotency. This is associated with increased LIF-induced activation of ERKs, which are known to impair self-renewal (Burdon *et al.*, 1999). These findings would suggest that PI3K favours the balance of STAT3 to ERK signalling upon LIF stimulation. Although PI3K/PKB activity is associated with inactivation of GSK3β by phosphorylation, inhibition of PI3K signalling did not result in changes in phosphorylation levels of β-catenin (Paling *et al.*, 2004). Therefore, potential positive effects of GSK3β inhibition upon mES self-renewal (as observed by Sato *et al.*, 2004 using a GSK3β inhibitor) via PI3K activity are independent of canonical WNT signalling.

PI3K also has been shown to promote self-renewal of hESCs (Kim *et al.*, 2005[1]) independently of LIF, via bFGF signalling. It is believed that FGF2 dependent PI3K/PKB activation is required for the efficient expression of extracellular matrix proteins. Interference at any stage of this proposed pathway has negative effects upon downstream targets and reflects in a loss of markers associated with the pluripotent state of hESCs.

Src kinase

The Src family member, Hck, has been implicated as a downstream target of LIF signalling in mESCs, which, when expressed in a constitutively active form, reduces the requirement of LIF to maintain the undifferentiated state (Ernst *et al.*, 1994). Another family member, cYes, also has been identified in actively maintaining the undifferentiated state (Anneren *et al.*, 2004). cYes is also a downstream target of LIF in mESCs and is found to be active in hESCs. Inhibition of Src family members with

[1]This paper has now been retracted. The information is, therefore, open to evaluation.

a pharmacological inhibitor reduces the capacity of mESCs and hESCs to self-renew, in part by reducing proliferation (Anneren *et al.*, 2004). The Src family may thus present another component required both by mESCs and hESCs, but are activated by different pathways.

INTRINSIC FACTORS

Several key transcription factors are known to be essential in maintaining hESCs in a pluripotent, undifferentiated state. The first gene to be discovered whose function was crucial in maintaining ES cell pluripotency was Oct3/4, which encodes a class V POU transcription factor initially identified in EC cells (Rosner *et al.*, 1990). Most ES cell lines appear to express high levels of Oct3/4, with the precise level of the protein determining the commitment of ES cells to one of three cell fates: self-renewal, trophectoderm or extra-embryonic endoderm, and mesoderm (Niwa, 2004). Over-expression of Oct3/4 in mESCs results in their differentiation into primitive endoderm and mesoderm, with downregulation leading to cells entering the trophectoderm lineage (Niwa, 2001).

The presence of Oct3/4 was confirmed in ES cells of human origin with differentiation of cells leading to its downregulation (Reubinoff *et al.*, 2000). Knockdown of Oct3/4 in hESCs by RNA interference (RNAi) leads to forced de-differentiation of these cells into trophoblast (Hay *et al.*, 2004; Matin *et al.*, 2004), indicating that Oct3/4 is essential for the maintenance of an undifferentiated state in hESCs. With the exception of Oct3/4, genes required to maintain pluripotency in hESCs are largely unknown. STELLAR, a gene with similar expression to Oct3/4, has been identified recently. Its expression is restricted mainly to pluripotent hESCs, pre-meiotic germ cell tumours, and testicular germ cell tumours; furthermore, expression of this gene was clearly downregulated upon hESC differentiation. Although the functional role of STELLAR is not yet established, results suggest that genes with high expression in germ cells or human germ cell tumours, which largely lack expression in somatic tissues, could provide a novel source of genes to investigate regarding self-renewal and pluripotency of hESCs (Hay *et al.*, 2004).

There is scarce knowledge concerning the upstream factors that regulate the expression of the key transcription factors; however, Oct3/4 has been most investigated. The expression of Oct3/4 is regulated by a proximal enhancer and promoter in the epiblast and by a distal enhancer and promoter at all other stages in the pluripotent cell lineage (Minucci *et al.*, 1996; Yeom *et al.*, 1996). Oct3/4 gene expression is activated in murine ES cells by the orphan nuclear receptor, liver receptor homologue 1 (LRH-1), which is expressed in undifferentiated ES cells (Gu *et al.*, 2005). Activation of Oct3/4 is achieved when LRH-1 binds to the proximal promoter and proximal enhancer of the Oct3/4 gene. LRH-1 plays an essential role in Oct3/4 expression in ES cells; however, this is predominantly at the epiblast stage of embryonic development (Gu *et al.*, 2005). Other members of the nuclear receptor family, including steroidogenic factor 1 (SF1), germ cell nuclear receptor (GCNF), and chicken ovalbumin upstream promoter (COUP TF) I/II (Ben-Shushan *et al.*, 1995; Yeom *et al.*, 1996; Barnea and Bergman, 2000), have been implicated previously in the regulation of Oct3/4.

Limited downstream target genes of Oct3/4 have been identified, with FGF4 being the most analysed. When investigated as a possible target of Oct3/4, the enhancer element of FGF4 was found to be specifically active in pluripotent stem cells. Binding elements for both Sox and POU family members were found to be located in the enhancer, and subsequently Sox2 was identified (Yuan *et al.*, 1995). Sox2 is a Sry-related transcription factor and activates the transcription of target genes such as FGF4, in cooperation with Oct3/4. In the case of FGF4, Sox2 binds to the minimal enhancer, but cannot activate the enhancer alone and requires Oct3/4 to bind alongside it to elicit activation (Kamachi *et al.*, 2000). Sox2 expression is controlled by Oct4 and Sox2, suggesting a positive feedback mechanism that could be related to the maintenance of ES cell self-renewal (Tomioka *et al.*, 2002). As found with Oct3/4, the expression of Sox2 declined markedly with the differentiation of hESCs (Wei *et al.*, 2005). Two regulatory elements, SRR1 and SRR2, have been found in the area of the Sox2 gene in undifferentiated ES cells, and both were found to enhance the expression of a single gene. It is assumed that in the presence of these two regulatory elements, a high level of gene expression is achieved through synergistic activity (Tomioka *et al.*, 2002).

Recently, the partnership between Oct3/4 and Sox2 was found to be involved in the expression of the UTF1 gene, found to encode an ES cell specific co-activator (Nishimoto *et al.*, 1999). The binding sites for both factors are located in the second intron of the UTF1 gene, with no intervening spaces (Okuda *et al.*, 1998). These findings indicate that the Oct3/4 Sox2 partnership is indispensable in the maintenance of pluripotency.

Nanog is a relatively new member of the group of transcription factors whose functions are deemed essential for the process of self-renewal in hESCs. Nanog is named after Tir Na Nog, the land of the ever-young in Celtic mythology, as its forced expression in mESCs allows their self-renewal in the absence of LIF (Niwa, 2004). Nanog is an NK2-family homeobox transcription factor and it is likely that Nanog acts by transcriptional activation, achieved by binding to homeobox domains in downstream target genes (Mitsui *et al.*, 2003). Analogous to Oct3/4 and Sox2, Nanog expression is high in hESCs and is downregulated as cells differentiate (Bhattacharya *et al.*, 2004). Downregulation of Nanog leads to a significant downregulation of Oct3/4 and loss of ES cell-surface antigens, consistent with a loss of pluripotency (Hyslop *et al.*, 2005). Differentiation into endodermal lineages is supported by upregulation of several marker genes, including GATA4, GATA6, LAMININ B1, and AFP (Morrisey *et al.*, 1998; Koutsourakis *et al.*, 1999; Fujikura *et al.*, 2002), and trophectoderm specification, as indicated by upregulation of CDX2, GATA2 hCG-alpha and hCG-beta (Kunath *et al.*, 2004).

It has been established recently that Nanog is transcribed under the control of a regulatory region that lies within 332 bp upstream of the transcriptional start site and contains both Octomer and Sox elements (Kuroda *et al.*, 2005). Transcription of Nanog is regulated by the binding of Oct3/4 and Sox2 to the Nanog promoter, as confirmed through mutagensis and *in vitro* binding assays (Rodda *et al.*, 2005). However, in addition to these findings, it has been suggested that an Oct3/4 complex containing an undefined factor, termed pluripotent cell specific Sox element-binding protein (PSPB), preferentially binds to the Octomer/Sox element, implying

that transcription of Nanog may be regulated through an interaction between Oct3/4 and Sox2, or Oct3/4 and PSPB (Kuroda *et al.*, 2005). These findings indicate a possibility of competition between Sox2 and PSPB in binding to the Nanog promoter, and question the requirement for Sox2 in Nanog transcription. In contrast, knockdown of Sox2 by RNAi was found to compromise significantly Nanog transcription levels, and therefore directly implicated the requirement for Sox2 in Nanog regulation (Rodda *et al.*, 2005).

A model of transcription factor hierarchy in pluripotent cells (Chambers *et al.*, 2003) highlights the importance of Nanog and Oct3/4 in sustaining ES cell characteristics, and suggests STAT3 only has an accessory function. Oct3/4 is seen to serve a specific role of blocking differentiation into trophoblast, and exhibits a tendency to promote differentiation into primitive endoderm and germ layers. Nanog (and activated STAT3) may block this differentiation effect. Suppression of differentiation by Nanog also maintains Oct3/4 transcription, which is not downregulated unless cells are committed to differentiation.

It is thought likely that main regulators of stem cell identity bind and control genes encoding other transcriptional regulators. Recent findings revealed Oct3/4, Sox2, and Nanog co-occupy the promoters of a large range of genes, many of which were found to encode developmentally important transcription factors (Boyer *et al.*, 2005). Oct3/4, Sox2, and Nanog reside in a minimum of 353 hESC genes, as many as half of the promoter regions occupied by Oct3/4 also bind Sox2, and 90% of these sites are also occupied by Nanog. Hence, Oct3/4, Sox2, and Nanog contribute to pluripotency and self-renewal by activating their own genes and, in addition, genes encoding key signalling pathways. It is, however, likely that the activity of these main transcription factors is added to by the presence of other co-factors and post-translational modifications (Boyer *et al.*, 2005). Data suggested Oct3/4, Sox2, and Nanog together bind to the promoters of their own genes to form an auto-regulatory pathway, as previously described for mESCs (Catena *et al.*, 2004).

Conclusion

Ultimately, the use of hESC in therapeutic application will require their efficient culture in a system free of animal products, and the development of such systems requires our understanding of the mechanisms that control self-renewal. Although this knowledge is still rather limited, a number of recent advances highlight the importance of a few key pathways. In other biological systems, these same pathways display a vast network of crosstalk, which is probably in operation to support hESC self-renewal. Such information will provide a basis for understanding the response of a cell to varying levels of given signals, and support the development of culture systems that both support hESC self-renewal or directed differentiation. Whilst the key intrinsic factors that control self-renewal of hESCs and mESCs appear similar, notable differences exist regarding the extrinsic requirements, in particular the inability of LIF/STAT3 signalling to support hESCs.

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