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Signaling Pathways in Mouse Embryo Stem Cell Self-Renewal

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1. Introduction

At the pre-implantation blastocyst stage of development, the mammalian embryo is composed of a unique collection of cells of which three major populations predominate. The outermost layer the trophectoderm (TE) gives rise to the placenta, which acts to sustain the developing fetus connecting it to the mother host. The next is a cluster of cells known as the inner cell mass (ICM) these cells are said to be pluripotent (Fig. 1). A third group of cells known as the primitive endoderm, surrounds the ICM cells at the epiblast stage. As development proceeds the ICM cells rapidly divide and eventually begin to differentiate forming the three embryonic germ layers (ectoderm, mesoderm and endoderm). Effectively these pluripotent ICM cells are the precursors of all adult tissues. As these pluripotent cells commit to a specific cellular lineage, they lose their pluripotency. Embryonic stem (ES) cells are euploid pluripotent cell lines isolated directly from cultured preimplantation embryos. The first stable ES cell lines were isolated by immunosurgery from the ICM of implantation-delayed, mouse blastocysts (Martin, 1981; Evans and Kaufman, 1981). Mouse ES cells are very closely related to early ICM cells in terms of their developmental potential (Beddington and Robertson, 1989). This chapter will focus on mouse ES cells (mES) unless otherwise stated. Three features characterize mES cells;

1. They are isolated directly from the embryo (Robertson, 1987).
2. They can colonize the germ line when introduced to the embryo.
3. They possess unrestricted proliferative potential (Suda et al., 1987).

These features effectively mean that under appropriate conditions, a karyotype stable self-renewing, pluripotent population of cells can be propagated indefinitely *in vitro*. mES cells have other characteristics, which prove useful when comparing embryo derived stem cells to their differentiated progenies. mES cells have a euploid (2n) chromosome complement, a feature that allows their participation in germ cell development and the formation of chimeras (Bradley et al., 1984; Evans, 1994). The functional demonstration of mES cell developmental potential through chimera formation is the definitive proof of the pluripotent nature of the cell population in question. Biomarkers are often used as indicators of the stem cell state due to the time consuming and technically more difficult nature of getting functional proof of stemness. Many of the common markers are transcription factors expressed in the ICM and mES cells and have been shown to have functional roles in self-renewal and in the maintenance of pluripotency, in both isolated stem cells or the ICM. The

surface markers expressed depend on species of origin, but common markers include members of the stage specific embryonic antigen (SSEA) family, alkaline phosphatase and Oct4 (Andrews, 2002; Pera et al., 2000; Shamblott et al., 1998). SSEA-1 is expressed in mouse preimplantation embryos from the eight-cell stage until the embryo differentiates into germ layers when it remains only in the ectodermal lineage (Solter and Knowles, 1979; Resnick et al., 1992; Pelton et al., 2002). Some of the best-characterized examples include the POU domain transcription factor Oct4, the homeodomain protein Nanog and the high-mobility group transcription factor Sox2. Of the three factors mentioned our understanding of Oct4 is best developed. Oct4 deficient embryos fail to initiate fetal development, indicating that Oct4 is essential for embryo development (Nichols et al., 1998). In mES cells there is an altered level of expression upon differentiation, a profile for down-regulation into TE and up-regulation in endoderm correlates with the Oct4 profile of expression *in vivo* embryos (Palmieri et al., 1994). mES cells can be maintained in an undifferentiated state *in vitro* with relative ease. They represent pluripotent embryonic cells, which are present only transiently *in vivo*. This enables their use as an *in vitro* model to elucidate the mechanisms of differentiation that these pluripotent cells undergo *in vivo*. The regulatory signaling and transcription networks that play a role in pluripotency and self-renewal it would seem have been conserved between mouse and human ES (hES) cells, however many differences also are found. As distinct from mES cells, hES cells do not appear to express high levels of SSEA1, but do have high levels of SSEA-3, −4, TRA-1–60 and TRA-1–81 (Brimble et al., 2007; Reubinoff et al., 2000). Other difference also exist, both cell types have a high ratio of nuclear to cytoplasmic volume, mES cells grow in nests and form three dimensional embryoid bodies while hES cells often grow in colonies as thin layers. Furthermore hES cells unlike mES can be maintained in a self-renewal cycle in the absence of the cytokine LIF or a feeder layer. Recently a population of cells with pluripotent capability was isolated from a post-implantation mouse epiblast at the later stages of development (Brons et al., 2007; Tesar et al., 2007). These epiblast stem cells (EpiS cells) do not require LIF support as ICM derived mES cells do (Rossant, J. 2007; Nagy et al., 2003). Morphologically EpiS cells are more like hES cells than mES cells as they tend to grow as thin flat layers. Considering these difference and others, the idea that mES and hES cells while both pluripotent, may in fact represent different and distinct stages in development.

Early studies with mES cells showed that the use of mitotically inactivated STO cells (Ware and Axelrad, 1972; Hooper, 1997) was essential in the maintenance of self-renewal and the pluripotent state. Later it was found that the requirement for the feeder layer could be circumvented by the addition of the cytokine LIF in the presence of serum. In the absence of feeder layers or LIF, mES cells differentiate into a variety of cell types (Doetschman et al., 1985) depending on the developmental cue or signaling pathway activated. The process of differentiation can be seen as a loss of pluripotency and mES like their in vivo counterparts are capable of multi-lineage differentiation. mES cells undergo a controlled pattern of differentiation when injected and reintegrated into a pre-implantation blastocyst. Under these circumstance mES cells respond as ICM cells to *in vivo* differentiation cues and fully participate in normal development. Furthermore they are capable of forming a wide range of normal cells including germ cells (Bradley *et al.*, 1984; Robertson *et al.*, 1986). *In vitro* mES cells can be induced to differentiate, by culturing cells in suspension or in a monolayer system under the effect of chemical inducers such as retinoic acid (Robertson, 1987). When mES cells are propagated under conditions that discourage their attachment to the substratum they form small aggregates, termed embryoid bodies (EBs). These three

dimensional colonies organize in such a way that an endodermal layer develops on the outer surface and are now known as "simple embryoid bodies". The inner stock of cells remain undifferentiated not unlike the early events of embryogenesis, where the trophectoderm differentiates from the peripheral cells of the morula, while the inner cells remain pluripotent.

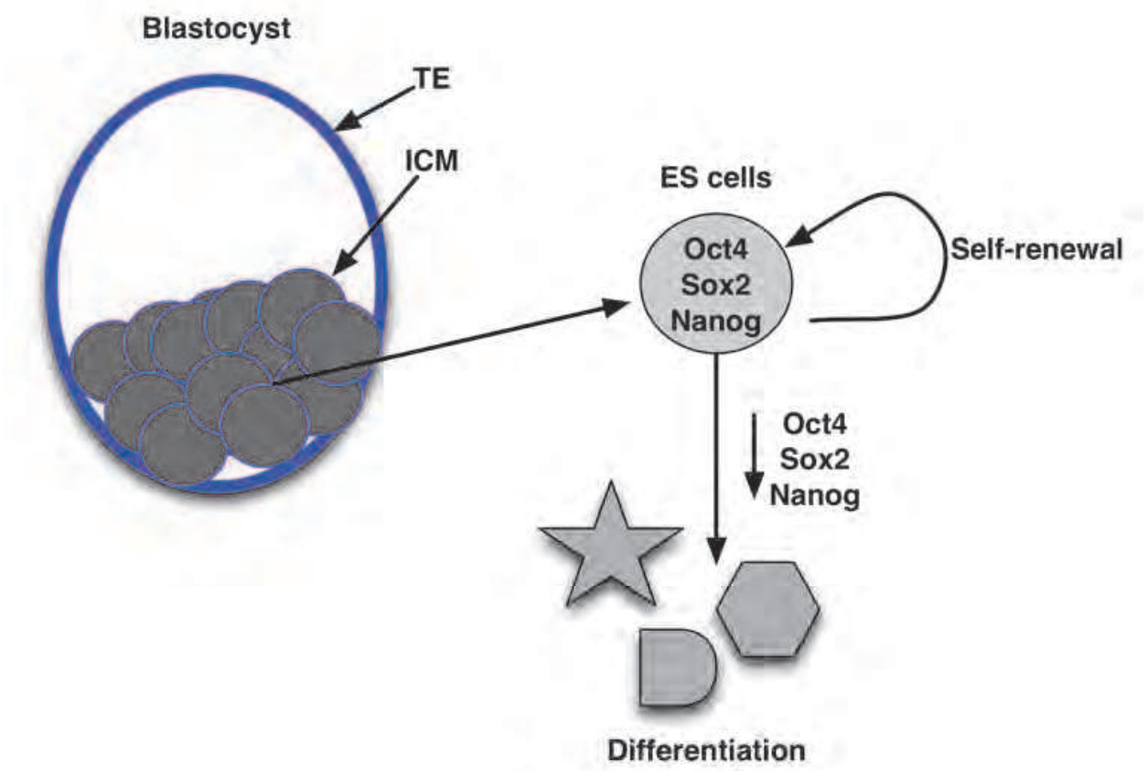

Fig. 1. Key transcription factors involved in self-renewal of ES cells.

Differentiated mES cells from EBs can give rise to a wide variety of cell types including neuronal (Bain *et al.,* 1995), hematopoietic (Suwabe *et al.,* 1998), endothelial (Yamashita *et al.,* 2000), cardiac (Maltsev *et al.,* 1993), smooth muscle (Yamashita *et al.,* 2000), chondrogenic (Kramer *et al.,* 2000) and osteoblastic cells (Buttery *et al.,* 2001). For stem cells there is a constant balancing act that must be maintained between self-renewal and the pluripotent phenotype versus cell lineage commitment and differentiation.

An understanding of the pathways and controlling factors involved in these fundamental cellular events is essential if we are to exploit the full potential of embryo derived stem cells for therapeutic uses in disease treatments and regenerative medicine in the future. This potential is real as it is clear that embryo derived stem cells are capable of unlimited self-renewal capacity and can differentiate into potentially any of over 200 cell types. The potency of these cells is maintained by a number of key regulatory factors, signaling pathways and extracellular signaling agents. The combination and interplay of these elements establishes a patterns of gene expression that sustains the pluripotent phenotype of ES cell. Some of the key regulators are transcription factors such as Oct4, Sox2 and Nanog, the signaling cascades involving phosphatidylinositol 3-kinase (PI3K) and the signaling

molecules like LIF and Wnt proteins. Embryonic stem cells have great therapeutic potential however to fully realize the potential of these cells the signaling pathways that participate in controlling ES cell behavior must be better understood.

2. Pluripotency and Self-renewal

In the developing embryo, pluripotent cells first appear in the ICM, in the mouse blastocyst this is approximately at day 3.5 of embryonic development (E3.5). These cells persist as late as the pre-gastrulation stage. Thus *in vitro* mES cells represent a very transient population of cells existing for a very short period of time *in vivo*. Experimental extraction of these cells facilitates the expansion and maintenance of the pluripotent state *in vitro*. mES cells are pluripotent, which is defined as the ability to differentiate into all cell lineages that make up the adult organism (Buehr et al., 2003). Functional assessment of the pluripotency of mES cells can be determined by the capability of the cells to reintegrate, into the ICM of E3.5 blastocysts contributing to all cell lineages. Strict pluripotency has been shown only in the mouse, where mES cells completely integrate producing a high rate of chimerism in all tissues. In the presence of fetal serum, activation of STAT3 by LIF is sufficient to maintain mES cells in an undifferentiated state (Williams et al., 1988; Matsuda et al., 1999) however, this is not the case for hES cells (Dahéron et al., 2004). Potentially parallel pathways are at play in mouse and human ES cells through which ES cells achieve similar end points and sustain pluripotency. This characteristic is sustained by the ability of ES cells to self-renew. ES cells are capable of differentiation but also symmetrical division generating two identical undifferentiated pluripotent daughter cells. In real terms mES cells can be expanded indefinitely (years) in a self-renewal cycle, once culture conditions prevent differentiation. It appears that maintaining a pluripotent state during mES cell self-renewal is through active suppression of differentiation and the promotion of proliferation. The differentiation of mES cells can be induced by the ectopic expression of certain transcription factors. Forced expression of Gata6 in mES cells drives differentiation toward primitive endoderm lineages (Fujikura et al., 2002), while increasing Cdx2 results in trophectoderm formation (Niwa et al., 2005). Thus, the expression of genes promoting self-renewal, cell proliferation and suppressing cellular differentiation pathways must be stably maintained and passed on to each daughter cell. The regulation of self-renewal is of great interest and importance in developing our basic understanding but also for the development of regimes for cellular therapy. The ability to control and maintain the expansion of pluripotent cells is a cornerstone, if the true clinical potential of ES cell-derived therapies is to be realized for regenerative medicine. mES (Martin, 1981) and hES (Thomson et al., 1998) cells are similar in this regard although not absolutely identical, the molecular machinery and pathways involved is equally complex but involves a number of distinct players in each case (Sato et al., 2003). A good example of this is the vitamin A metabolite, all-trans-retinoic acid which has the effect of silencing self-renewal and driving a differentiation agenda for both mES and hES cells lines (Chen and Gudas, 1996; Mongan and Gudas, 2007). The self-renewal pathways in ES cells comprise complex networks of strategic actions of extracellular agents (including the presence or absence in culture of serum), intracellular signaling pathways and the control of key transcription factors. *In vitro*, LIF supports self-renewal and pluripotency of mES cells through activation of STAT3 (Smith et al., 1998), removal of LIF or suppression of STAT3 results in differentiation (Niwa et al., 1998). LIF receptor knockout mouse embryos are capable of passing the developmental stage required for mES cell

derivation. Thus *in vivo* it is obvious that there is no magic bullet but other factors are also involved in this process (Nichols et al., 2001). The cytokine LIF was among one of the earliest molecules found to be associated with the maintenance of stem cell self-renewal *in vitro* and *in vivo*. More recently other factors have become known including the bone morphogenetic proteins (BMPs) 2 and 4 and glycogen synthase kinase-3. Under serum free culture conditions, mES cells require the presence of both LIF and BMPs to facilitate continued self-renewal (Ying et al., 2003). It appears that linked pathways are at play, LIF supporting self-renewal and proliferation, while BMP4 up-regulates members of the *Id* gene (inhibition of differentiation) family (Ying et al., 2003). Signaling pathway crosstalk with PI3K signaling has also been shown to play a part in self-renewal. The transcription factors Oct4, Sox2, and Nanog are now widely accepted as having a central role in promoting self-renewal and sustaining the undifferentiated phenotype (Ying et al., 2003). As if to emphasize the reliance of self-renewal on networked interactions, recent work has shown that a key set of promoter sequences bind Oct4, Sox2, and Nanog in ES cells (Chambers and Tomlinson, 2009; Avilion et al., 2003; Masui et al., 2007; Niwa et al., 2005). This however is not the total picture as further extensions of the co-dependence of the self-renewal network is coming to light all the time, interestingly some of these newer interactions are independent of the established tri-umbret of Oct4, Sox2, and Nanog (Ivanova et al., 2006). Other work is providing evidence to suggest that two independent pathways may be as play in ES cells. The established Oct4, Sox2, and Nanog networks may be acting to suppress differentiation and thus sustain pluripotency. While other transcription networks play a role in repression of specific cell lineage differentiation. Further extension of these interconnecting pathways includes the addition of the role of miRNA-encoding genes (Marson et al., 2008). In the following sections the role of specific transcription factors and signaling pathways will be expanded upon in the context of their role in the self-renewal of ICM derived mES cells.

2.1 Transcriptional networks

2.1.1 Oct4

Oct4 is a member of the POU (Pit-Oct-Unc) transcription factor family that regulates the expression of target genes by binding to a octameric sequence (Scholer et al., 1990). The key features of this family are the POU domain consisting of two sub-domains each of which bind to DNA. However the C-terminal is cell specific and may be essential for the expression of target gene in an orderly fashion as embryonic development proceeds. It is well established that the Oct4 gene (encoded by Pou5f1) is constitutively expressed in undifferentiated mES cells, in all pluripotent cells during mouse embryo development and is also an essential factor required in the generation of iPS cells (Niwa et al., 2000). Oct4 is also known as Oct3, Oct3/4, Otf3, and Otf4. In the mouse, Oct4 expression is up regulated beginning at the 4-cell stage and becomes localized to the pluripotent cell population (Yeom et al., 1996). The expression of Oct4 is common to human and mouse ES cells, and furthermore expression diminishes in both as cells differentiate. *In vivo* Oct4 knockout mouse embryos crash and do not develop beyond the blastocyst stage, they lack a pluripotent ICM cell population (Nichols et al., 1998), strongly suggesting a central role for Oct4 in maintaining pluripotency. In cells were Oct4 is repressed or in Oct4 knockouts, mES cells differentiate towards a trophoectodermal lineage. It has been reported that Oct4 inhibits trophectoderm lineage formation via an interaction with Cdx2 forming of an inhibitory complex (Niwa et al., 2005). Conversely up regulation or over-expression of Oct4

results in mES cell moving towards primitive endoderm (Niwa et al., 2000). These divergent effects of Oct4 suggest that it regulates the transcription of genes involved in coordination of multiple cellular functions and early cell fate decisions. Thus the actual level of Oct4 expression is important and a key level of expression is required to sustain pluripotency and self-renewal. In mES cells the expression of Oct4 is supported by the action of LIF and down regulated by the chemical inducer all-trans retinoic acid (Faherty et al., 2005; 2007). Suppression of STAT3 and accelerated expression of Oct4 also causes mES cells to differentiate (Niwa et al., 1998; Niwa et al., 2000). The role is Oct4 as a so called master regulator in sustaining pluripotency and self-renewal of mES cells is well known, however it is not a solo run (Nichols et al., 1998; Boyer et al., 2005). Oct4 alone without LIF, is not sufficient to sustain self-renewal and prevent mES cell differentiation, suggesting that additional factors also play a part. More recently it has been shown that Oct4 expression prevents stem cell differentiation by sustaining the expression of other pluripotency factors and inhibiting gene expression of lineage specific factors. Known targets for Oct4 include Fgf4, Rex1/Zfp42, and Sox2 (Zeng et al., 2004; Tomioka et al., 2002). Oct4 has been shown to act in concert with other factors and its DNA binding often occurs in conjunction with the HMG-family protein Sox2, an additional factor required for maintaining mES cell stemness (Chambers and Smith, 2004; Pesce and Schöler, 2001). Oct4 is a key regulator of ES cell fate, particularly in maintaining a pluripotent state. The requirement appears to be that Oct4 protein levels are constrained within the narrow band. It is clear that Oct4 has a critical role in sustaining pluripotency, however its control is unclear. The control of the level of expression appears somewhat auto-regulatory (Chew et al., 2005), but also depends on other factors including the transcription factor Nanog providing a feedback loop to sustain self-renewal (Pan et al., 2006)

2.1.2 Nanog

Nanog is an homeobox containing transcription factor of approximately 280 amino acids. In the developing mouse embryo Nanog plays a key role in determining the fate of the ICM cells, acting to sustain pluripotency and preventing differentiation (Chambers et al., 2003). Nanog was identified as a factor, which when over expressed, supported pluripotency even in the absence of a LIF based signal. In the embryo Nanog expression is first seen at the compacted morulae stage before becoming restricted to the ICM, post-implantation stage Nanog expression is drastically reduced. *In vitro*, Nanog expression is abundant in pluripotent cell types but absent from adult tissues (Chambers et al., 2003). As with their *in vivo* counterparts upon differentiation of mES cells the expression of Nanog is downregulated. Nanog-null embryos fail soon after implantation, stem cells derived from such embryos are pluripotent but are found to quickly differentiate (Chambers et al., 2003; Mitsui et al., 2003). Over-expression of Nanog without any other intervention is sufficient to sustain self-renewal even without LIF albeit the self-renewal capacity under these conditions is reduced. Under the same conditions the level of active STAT3 is not appreciably altered, furthermore increased STAT3 signaling does not appear to alter Nanog expression. These data would suggest that Nanog is neither a target for STAT3, or does it regulate STAT3 activity. However at one least report suggests Nanog is a direct downstream target for STAT3 in the maintenance of pluripotency (Suzuki et al., 2006). BMP signaling normally acts during embryonic development to induce mesoderm formation, but effects in mES cells can be quite different (Winnier et al., 1995). In mES cells low levels of BMPs in the absence of LIF promote mesoderm, while in the presence of LIF, mES cell pluripotency is sustained (Ying et

al., 2003). BMP signaling is facilitated by downstream effectors including SMAD1, an effector which Nanog has been shown to interact with and leads to inhibition of BMP signaling (Suzuki et al., 2006). What is being proposed is that BMPs are at least initially pro-mesoderm lineage formation as evidenced by up-regulation of Brachyury a mesoderm marker. When activated STAT3 interacts with Brachyury and increases Nanog expression, the elevated levels of Nanog inhibit BMPs via SMAD1 interaction and thus maintain the undifferentiated pluripotent state. Nanog is now accepted as an important component in regulating the pluripotent phenotype however the mechanism of its own control and how it effects other genes is not entirely elucidated. It has been shown that Oct4 and Sox2 can bind to the Nanog promoter *in vitro* and *in vivo* (Rodda et al., 2005) suggesting that Oct4/Sox can act to up regulate Nanog expression. However other studies have shown that Nanog expression can be maintained in the absence of Oct4, thus other factors must contribute to the regulation. One such factor is a member of the forkhead family FoxD3, which is found in mES cells and the early embryo. FoxD3 knockout embryos have a similar fate as Nanog knockouts (Hanna et al., 2002). The exact mechanism by which Nanog is regulated and how it effects control of mES cell pluripotency is unknown, however it appears independent of STAT3 activation or the requirement for BMP4 at least when in serum free culture conditions. Nanog is capable of activating the Oct4 promoter forming a negative feedback loop upregulating Oct4 at times and suppressing Oct4 when levels are above normal (Pan et al., 2006). Furthermore it has been shown that Nanog is capable of activating Rex1 a target for Oct4 and Sox2 (Shi et al., 2006). Furthermore it has also been suggested that Nanog may interact with Wnt and BMP4 signaling independent of LIF which may help explain why the forced expression of Nanog in the absence of LIF sustains a level of self-renewal (Chambers et al., 2003; Mitsui et al., 2003). The regulation and comprehensive elucidation of the role of Nanog requires more experimental work before we can paint a true picture of its overall role in pluirpotency.

2.1.3 Sox2

Sox2 is a DNA-binding protein of the HMG family. In the mouse Sox2 is expressed predominatley at the blastocyst stage (Avilion et al., 2003). However unlike Oct4, Sox2 has a major role to play also later in development and in adult stem cells (Wood and Episkopou, 1999; Zappone et al., 2000). At early stages of development and in mES cells, Sox2 activates target genes through interaction with Oct4. Sox2 knockouts are lethal to mouse embryos and they fail to fully develop, furthermore ES cells derived from these embryos are unable to proliferate or self-renew (Avilion et al., 2003). As outlined for Oct4, a precise level of Sox2 appears to be key for pluripotency and to sustain self-renewal. Many studies have highlighted how Oct4 and Sox2 can in a direct way drive the expression of genes required for pluripotency including positive feedback on their own expression and that of Nanog (Chew et al., 2005; Tomioka et al., 2002). Together with the transcription factor Klf4, they activate the expression of Lefty1 (Nakatake et al., 2006). In mES cells a wide range of studies have focused on and delineated the functional role of Oct4, less is known about Sox2. Recent studies are beginning clear up the role of Sox2. As might have been anticipated mES cells deficient in Sox2 lose pluripotency and quickly differentiate supporting the perceived role of Sox2 in maintaining self-renewal. What is interesting is that in the Sox2 protein deficient system Oct-Sox enhancers are still active and up-regulation of Oct4 alone is sufficient to rescue these cells from differentiation. Thus it has been suggested that potentially other

members of the Sox family may substitute for Sox2 in the co-activation process mediated in partnership with Oct4. What has become clear is that Sox2 plays a role in regulating many transcription factors that can affect Oct4 levels including Nanog. Furthermore in cellular reprogramming studies up-regulation of Oct4 in combination with Sox2 is sufficient to generate pluripotent cells (Takahashi and Yamanaka, 2006; Okita et al., 2007). Some fascinating studies looking at global protein phophorylation patterns in hES cells have revealed some interesting dynamics in the Oct4 and Sox2 pattern of activation (Burdon et al., 2002). Thus exploring phophorylation pathways from extracellular signals to gene transcription effects will be key to furthering our understanding of self-renewal, in this context, pathways like those involving LIF and PI3K will be key to disentangling the signaling and transcription circuits involved.

3. Signal transduction pathways

3.1 Leukemia inhibitory factor (LIF)

LIF is expressed in mouse preimplantation embryos from fertilization to the blastocyst stage but not in TE cells (Nichols *et al.,* 1996). LIF transcripts are also found in mES cells (Rathjen *et al.,* 1990) and endometrial glands of the mouse uterus which stops once implantation has occurred. Mouse LIF gene knockouts result in growth retardation and fertilized blastocysts fail to implant (Stewart *et al.,* 1992). Historically mES cells were derived and maintained on a feeder layer of embryonic fibroblast. Subsequently it was found that the use of conditioned media from these fibroblast cultures was sufficient to maintain mES cell self-renewal. It was then shown that a the active agent produced by the feeder layer capable of blocking mES cell differentiation was in fact a cytokine later identified as leukemia inhibitory factor (LIF) (Smith et al., 1988). LIF is the best-characterized effector of self-renewal in mES cells. It is a multifunctional cytokine, which has a wide variety of effects on various cell types (Hilton and Gough, 1991). The name LIF is based on initial observations that *in vitro* it is capable of inducing irreversible differentiation of the murine leukemia cell line M1 to macrophages. LIF is a highly glycosylated single chain polypeptide and a member of IL6 cytokine family (Taga and Kishimoto, 1997). LIF is known to be secreted by a number of cell types including fibroblasts, lymphocytes spleen and liver cells (Gough and Williams, 1989). LIF is a very potent agent, *in vitro* $10^{-9}g/L^{-1}$ (defined as $50U/mL^{-1}$) induces approximately 50% of a population of M1 cells (murine leukemia cell line) to differentiate. LIF induces a wide variety of effects on different cell types e.g. LIF has been shown to sustain the survival of murine primordial germ cells (DeFelici and Dolci, 1991) and stimulate the proliferation of myoblasts in culture. It has also been shown to affect bone growth and remodeling *in vitro* (Lorenzo et al., 1990; Wilson et al., 1992; Gearing et al., 1992; Ip et al., 1992; Taga and Kishimoto, 1997).

The cellular actions of LIF are effected via a specific cell membrane receptor. The LIF receptor is a heterodimeric complex composed of a glycoprotein subunit gp130 and the receptor subunit LIFR (also called LIFRβ) (Ernst and Jenkins, 2004). Studies in mES have shown that the gp130 subunit is the essential component in transmitting self-renewal signals (Nakamura *et al.,* 1998). Binding of LIF to the LIFR subunit induces dimerization with gp130, resulting in the formation of a high affinity receptor complex. The activated receptor switches on the constitutively bound tyrosine kinase Janus kinase (JAK). Activated JAK, phosphorylates both receptor subunits forming SH2 domain bind sites, which are capable of recruiting other signal transduction partners.

The SH2 domains facilitate the binding of signal transducers and activators of transcription (STAT) 1 and STAT3, which are phosphorylated by JAKs (Stahl et al., 1995). The activated STAT proteins form homodimers or heterodimers which then move to the nucleus, where they act as transcription factors (Auernhammer and Melmed, 2000). STAT3 is the principal STAT protein activated in mES cells stimulated with LIF (Niwa *et al.,* 1998). Activation of STAT3, has been shown to be critical for LIF/gp130 dependent self-renewal in mES cells (Niwa et al., 1998). Using a tamoxifen inducible form of STAT3 (fusion of STAT3 to estrogen receptor) it has been shown that activation of STAT3 is capable of sustaining self-renewal of mES in the presence of serum (Mastuda *et al*. 1999).

In the absence of fetal calf serum, in the presence of activated STAT3, BMP4 signaling maintains pluripotency. However, for hES cells LIF-STAT3 signaling cannot maintain pluripotency (Reubinoff et al., 2000) additional factors independent of LIF-STAT3 are required including basic fibroblast growth factor (bFGF) in the presence of Noggin which acts as a BMP pathway inhibitor. The exact mechanism of LIF-STAT3-dependent mES cell self-renewal is still not fully elucidated although models are arising (Fig. 2). A notable target for STAT3 is the transcription factor Myc (Cartwright et al., 2005) which along with others (Klf4, Oct4 and Sox2) has a role in cellular reprograming of somatic cells to a pluripotent state (Takahashi and Yamanaka, 2006). The forced up regulation of Myc supports self-renewal in the absence of LIF. Whereas cessation of LIF signaling results in a decrease in Myc expression presumably through a down-regulation of STAT3. Apart from the above-mentioned STATs a wide range of other downstream effector molecules can be activated through LIF receptor activation including extracellular regulated kinases (ERK), mitogen-activated protein kinases (MAPK) and phosphatydilinositol-3 kinase (PI3K). The network of interactions between intracellular pathways and extracellular ligands continues to develop a pace, with numerous overlaps being identified. In this context another kinase, glycogen synthase kinase 3 (GSK3) a key enzyme in the Wnt pathways is quickly activated resulting in Myc phosphorylation and its degradation. The activity of GSK3 may be controlled by PI3K either directly or indirectly due to LIF signaling. Another possible network connection is that between LIF, PI3K and the Wnt pathway in self-renewal comes from the data that shows improved results in the derivation of mES cells in the presence of the GSK3 inhibitor BIO. Thus from a signaling perspective multiple pathways may be involved in the maintenance of low levels of GSK3 activity to promote pluripotency and mES cell self-renewal. The array of signaling pathways and the level of crosstalk that exist between them and the LIF-STAT3 pathway in mES is slowly being deciphered giving us a clearer picture of the connections between LIF signaling and the transcriptional machinery controlling self-renewal.

3.2 PI3K Pathway

Phosphatidylinositol 3 kinases (PI3Ks) are recognized to modulate a wide range of cellular functions from growth, proliferation and self-renewal to simple metabolic control. They are a family of enzymes, which phosphorylate the 3′-OH position of the inositol ring of phosphoinositides. In 1987 (Whitman et al., 1987) identified two distinct phosphatidylinositol kinases (PIKs) isolated from fibroblasts. They further demonstrated that one of these enzymes associated with activated tyrosine kinase receptors. They called this kinase type I PIK. Subsequently the same group showed that the most abundant form of the previously identified enzymes, type II PIK, phosphorylates the D-4 position on the inositol ring and that type I PIK phosphorylated the inositol ring at the D-3 position.

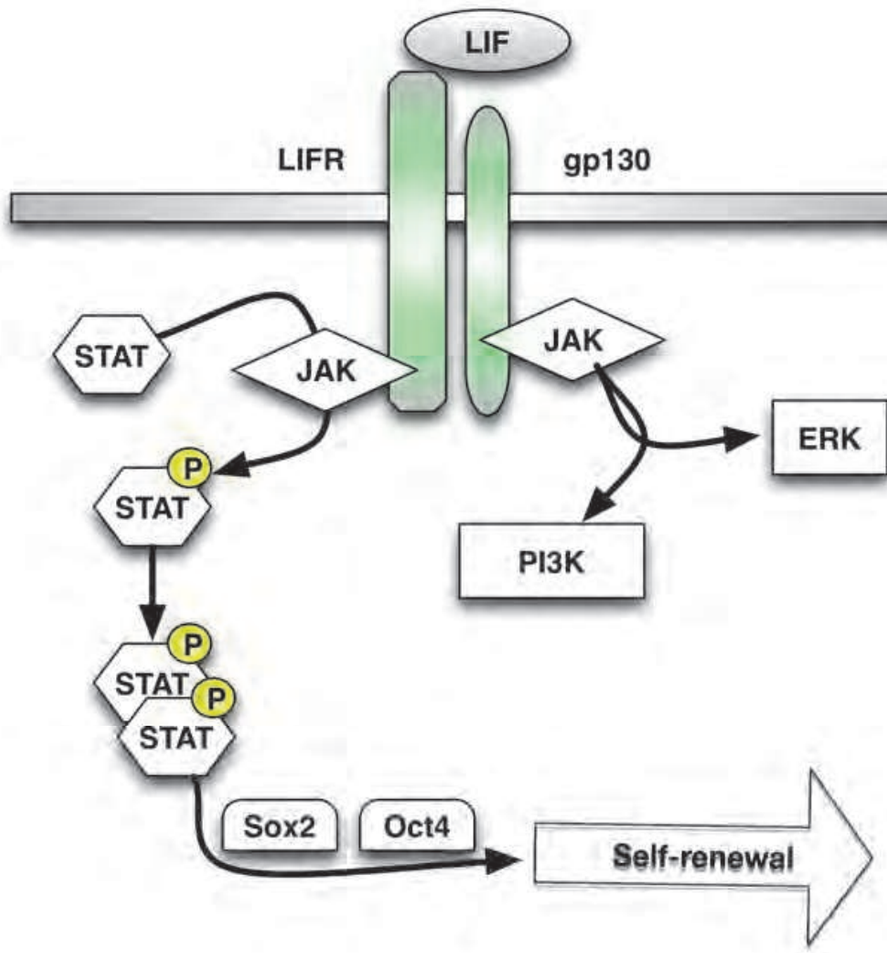

Fig. 2. LIF signal pathways and it integration into transcription machinery of self-renewal. Adapted from Niwa et al., 2009

Currently the family is divided into 3 classes based on structure and substrate preference (Wymann and Pirola, 1998; Vanhaesebroeck et al., 2001). Class I PI3Ks form heterodimers, consisting of a ~110 kDa catalytic subunit, and a regulatory subunit. The regulatory subunit comes in 4 main flavours (p85a, p55a, p50a, p85b, p55g) and a catalytic subunit in 3 major types (p110a, p110b, p110d) (Engelman et al., 2006). In vivo the primary substrate is phosphatidylinositol-4,5, bisphosphate (PtdIns(4,5)P2 or PIP2), which is converted to phosphatidylinositol-3,4,5, triphosphate (PtdIns(3,4,5)P3 or PIP3) (Cantley, 2002). This class of PI3Ks are activated by an array of plasma membrane receptors (for a review see Wymann et al., 2003). Class II PI3Ks produce PI(3)P and PI(3,4)P2 in vitro, but in vivo targets are less clear but the enzyme itself has been localized to the Golgi network. Class III PI3Ks produce only PI(3)P. Much of what we know about the functions of PI3K is because a potent and quite specific inhibitor is available. Wortmannin and LY294002 act as competitive ATP binders targeting the ATP-binding site of catalytic p110 subunit. The most interesting early finding was that wortmannin in the low nanomolar range blocked the respiratory burst of neutrophils (Baggiolini et al., 1987). Studies on purified enzymes have shown that the

mammalian PI3K is the most sensitive to wortmannin (Yano et al., 1993). The use of these inhibitors has proved invaluable in the study of PI3K and its cellular effects (reviewed by Nakanishi et al., 1995). The best known product of PI3K action is PIP3 which has been shown to be an important second messenger capable of recruiting AKT and involved in numerous cellular pathways associated with growth, proliferation and survival (Cantley, 2002). The production of PIP3 facilitates the recruitment of pleckstrin homology (PH) domain containing proteins an important example of which is the protein kinase Akt which itself has multiple intracellular targets (Toker, 2002). Commonly in transformed cells the PI3K/Akt pathway is directly activated by the loss of PTEN, a negative regulator of PIP3 formation and an identified tumor suppressor. Maybe unsurprisingly in mES cells the role of PI3Ks was highlighted by the fact that in PTEN null mES cells, accelerated cell cycle progression was observed (Sun et al., 1999) which can be blocked by the PI3K inhibitor LY294002. However a role for PI3K signaling events has also been identified in the maintenance of pluripotency in mES cell derived for a number of species (Fig. 3) (Armstrong et al., 2006). Blocking PI3K signaling events results in elevated ERK/MAPK signaling (Paling et al., 2004) and there is evidence to suggest that ERK (Hamazaki et al., 2006) and Wnt (Sato et al., 2003) signaling are required to sustain pluripotency in both mouse and human ES cell lines. In the

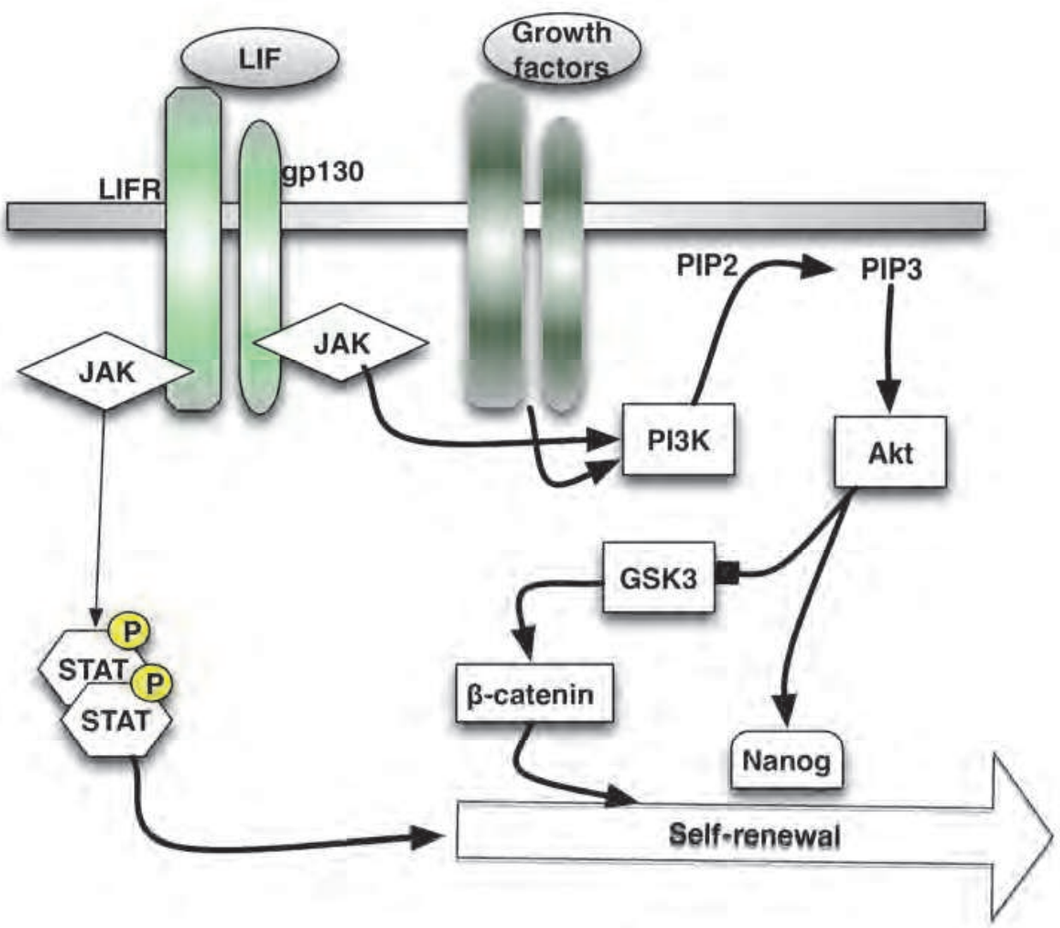

Fig. 3. Potential role for PI3K in self-renewal and LIF signaling in ES cells.

case of mES cells inhibition of PI3K pathways can induce differentiation in the presence or absence of LIF (Paling et al., 2004; Armstrong et al., 2006). However interestingly up regulation of Akt signaling is sufficient to maintain pluripotency of m ES cells (Watanabe et al., 2006). Another linkage for PI3K signaling and self-renewal comes from evidence that Nanog expression as well a number of Nanog target genes are modulated by PI3K signaling. Results have shown that the loss of pluripotent phenotype associated with PI3K blockage by LY294002 can be rescued by exogenous Nanog expression. Also regulation of GSK3 activity acting downstream of PI3Ks plays a role in Nanog expression. The evidence is clearly points out that PI3Ks play an important role in the signaling and maintenance of Nanog expression. PI3K effects are not limited directly to Nanog alone, inhibition of PI3K pathways results in the repression of rfx4, an identified Nanog target (Storm et al., 2007). However, interestingly, of the triad of master factors Oct4, Sox2, and Nanog, it appears that Nanog alone is sensitive to PI3K signaling pathways. However recently it has been shown that suppression of PI3K leads to a reduction in other self-renewal transcription factors including Klf4 (Storm et al., 2009), one of the targets in iPS generation. The role of PI3K in ES cells is complicated by the fact that self-renewal and cell proliferation are linked, and PI3Ks have been cast in major roles for both cellular processes.

3.3 Wnt pathway

The name "Wnt" comes from the fusion of the two names, int (based on the proto-oncogene integration-1 (Tanaka et al., 2002) and wg (based on wingless the segment polarity gene in Drosophila). The Wnt proteins are defined by amino acid sequence rather than by noted functional activities, but all Wnts share a number of common properties like numerous glycosylation sites and target sequences for secretion (Nusse and Varmus, 1992). Upon Wnt binding to its specific receptor, a signaling cascade is activated ultimately upregulating Wnt target genes. The Wnt signaling system is a highly conserved network controlling numerous other signaling transduction pathways from embryonic development to adult tissue homeostasis. Approximately 19 different WNT proteins have been identified acting on at least three different signaling pathways (Nusse and Varmus, 1992). The three pathways are the canonical Wnt pathway, acting via β-catenin and Tcf/Lef factors; the planar cell polarity (PCP) pathway; and the Wnt-Ca2þ pathway (Staal et al., 2008). This section will focus only on the canonical pathway. β-catenin is a well-known cytoplasmic protein and has a role in cell-cell adhesion acting to link membrane bound cadherins to the actin elements in the cytoskeleton. However it is now known to also act as a signaling molecule inside cells as part of the canonical Wnt signaling pathway (Reya and Clevers, 2005). In the absence of Wnt, β-catenin exists in a phosphorylated state in a complex marked for degradation by the ubiquitin-associated proteases. The β-catenin degradation complex includes the tumor suppressor proteins adenomatous polyposis coli gene (APC), Axin, and GSK3. Wnt signaling involves the Wnt ligand binding to the membrane receptor named Frizzled (Fz). Frizzled is a seven transmembrane receptor and the first receptor identified to bind the Wnt ligand (Bhanot et al., 1996). Activation of signal transduction by Wnt binding the Fz receptor requires a co-receptor attachment with a member of the low-density lipoprotein (LDL) family called Lrp5 and −6, this interaction is required for activation of the canonical Wnt signaling pathway (Li and Bu, 2005). Activation of Fz by Wnt results in the protection of β-catenin from proteosomal degradation. Thus the action of Wnt is to maintain the intracellular levels of β-catenin which then translocates to the nucleus where it forms a transcription complex with one of a number of transcription factors including Tcf1, Tcf3,

Tcf4, or Lef1 (Okamura et al., 1998). Tcf1 is found mainly in T lymphocytes, Tcf4 is widely expressed and found in stem cells of gut while Tcf3 is expressed in mES cells. In mES cells there is growing but often-conflicting evidence that Wnt signaling pathways are important components of mES cell self-renewal. Wnt pathways have been shown to sustain pluripotency but also are important for of adult progenitor cell proliferation. The focus on Wnt signaling and its role in pluripotency comes from studies using the GSK3 inhibitor 5-bromoindirubin-3-oxime (BIO) (Fig. 4) (Meijer et al., 2003; Sato et al., 2003).

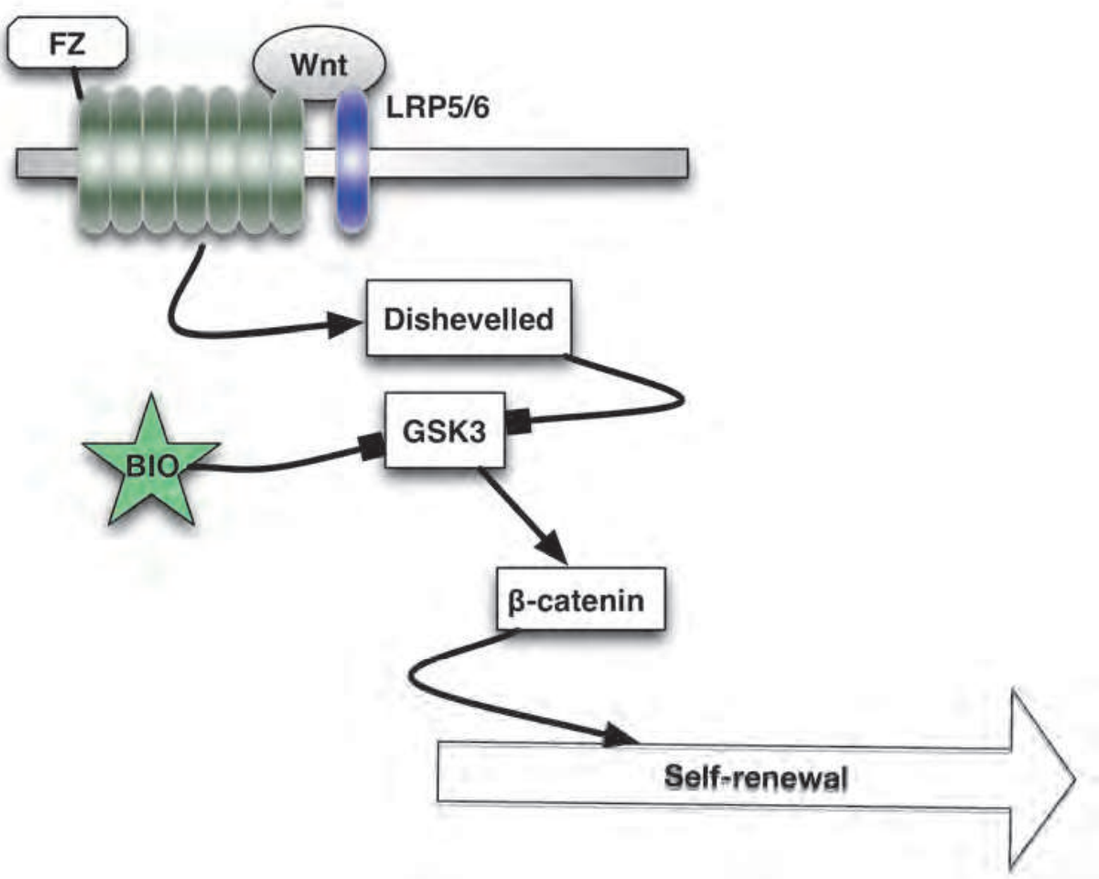

Fig. 4. Wnt signal pathway role in self-renewal.

Inhibition of GSK3 prolongs the existence of β-catenin, causing it to accumulate, increasing the pool, which can translocate to the nucleus and activate gene expression. BIO has been shown to be able to maintain pluripotency of mouse and human ES cells in the absence of LIF (Sato et al., 2003). In a similar vain activation of Wnt signaling indirectly by removing the inhibitory effect of APC sustains pluripotency, suggesting the Wnt signaling is required for self-renewal (Kielman et al., 2002). In addition, treatment with Wnt3a was found to stimulate hES cell proliferation (Singla et al., 2006). Oct4 over-expression increased β-catenin transcriptional activity in progenitor cells. The Wnt controlled transcription factor Tcf3 has been shown to repress Nanog and thus promote differentiation. More recent studies have shown that Lef1 acting along with β-catenin is able to up-regulate Oct4 expression and interact with Nanog and thus promote self-renewal. All these data suggest that Wnt/β-

catenin signaling has some role in the mES cell self-renewal (Takao et al., 2007). In obvious contrast to LIF and BMP signaling in mouse and human ES cells there is no difference between the cell types with regard to Wnt/β-catenin signaling self-renewal (Hao et al., 2006). However, contrary to the above-portrayed role of Wnt in self-renewal, Wnt action has been shown to facilitate differentiation of mES cells into neural precursors and increases the expression of Brachyury a mesoderm marker (Yamaguchi et al., 1999). More work is required to elucidate the role of Wnt signaling in mES cell self-renewal and pluripotency and potential other effect for the non-canonical Wnt pathways.

4. Summary and conclusion

Even after prolonged periods and numerous expansions in culture ES cells retain the ability to respond to normal developmental signals and display no apparent bias for any one cell lineage when reintegrated to a developing embryo. Constructing a stable and coherent map of how ES cells achieve such a feat is a major challenge that must be met if the true potential of these cells is to be realized in a clinical setting. A fundamental breakthrough in this area came with the generation of a tetracycline-suppressible *Oct4* transgene in late 2000. This study clearly defined the effect of Oct4 loss on ES cell self-renewal (Niwa et al., 2000). Silencing *Oct4* resulted in ES cells differentiation into trophectoderm but most surprisingly an increase in Oct4 levels resulted in differentiation into mesoderm and endoderm cell linages (Niwa et al., 2000). *Sox2* null ES cells differentiate into trophoectodermal type lineage (Masui et al., 2007). In the absence of Sox2 expression Oct/Sox targets were sustained, leading to the suggestion that other Sox proteins may replace the role of Sox2. Somewhat like Oct4 but less clear cut it would seem from over-expression studies in mES cells that up-regulation of Sox2 results in formation of neural lineages (Kopp et al., 2008; Zhao et al., 2004). Nanog was initially identified as a molecule capable of supporting the pluripotent phenotype of ES cells in the absence of LIF (Chambers et al., 2003). Suppression of Nanog results in increase differentiation (Chambers et al., 2007; Ivanova et al., 2006). Sufficient evidence indicates that the levels of Oct4, Sox2, and Nanog, govern to a large extent the pluripotency of ES cells (Chambers and Smith, 2004). Interestingly the accumulated work on these factors also points to the fact that Oct4 and Sox2 may perhaps drive a process of differentiation countering the self-renewal process. Oct4 and Sox2 increase the production of fibroblast growth factor 4 (FGF4), a growth factor that pushes ES cells toward differentiation by making them more susceptible to leverage by specific lineage commitment factors. All the while Nanog works to resist ES cells differentiation. Artificial high levels of Nanog through constitutive expression systems prevent ES cell differentiation even when FGF signaling is active (Chambers et al., 2003; Ying et al., 2003). However the true role of Nanog and its functional relationship to Oct4 and Sox2 is increasingly more complex. The level of Nanog found in individual normal ES cells shows a high degree of heterogeneity and Nanog knockouts can sustain self-renewal and are pluripotent (Chambers et al., 2007). In general though it appears that cells that are deficient in Nanog have a high propensity to differentiate (Chambers et al., 2007; Mitsui et al., 2003). Our understanding of the extracellular signaling interactions with these transcriptional networks is to date very unclear. The closest we are coming to tying these elements together is looking at work done on LIF signaling in ES cells. ES cells traditionally maintained on feeder cell layers in the presence of serum or a defined serum replacement with the addition of LIF for mouse, and bFGF for human ES cells. These less than wholly defined conditions are entirely problematic

for many reasons. A fundamental understanding of the pathways involved for both mouse and human stem cells is beginning to emerge. Niwa et al (2009) have shown that the LIF signaling is tightly linked into the transcriptional machinery of ES cell self-renewal. The Stat3 pathway activates Sox2 but not Nanog, while the PI3K–Akt pathway, effects predominately Nanog. Maintaining a pluripotent phenotype can be viewed as a getting the right balance between continued appropriate proliferation and inhibition of differentiation and/or cell death. Thus a key mechanism to sustaining ES cells in a pluripotent state may be to push and pull all at once i.e. push self-renewal factors and simultaneously block differentiation pathways. An example of this strategy is the effect of GSK3 inhibition in conjunction with inhibition of mitogen-activated protein kinase (MEK which facilitates long-term self-renewal of mouse ES cells with no requirement for cytokines). As we get closer to understanding the pathways in mouse ES cells in particular the issue of difference in comparison to human lines become all the more apparent. Human ES cells are significantly different from mouse ES cells in phenotype and signaling pathway profiles. There is the suggestion that the human cell lines may represent a later stage (epiblast stage, Epi Stage Cells (EpiSCs)) of development compared to the mouse lines. LIF for example does not support either human ES cells or EpiSCs, but does support mES cell self-renewal (Xu et al., 2005). Thus as we elucidate the pathways of pluripotency and self-renewal we will have to be mindful of the cells under study and the stage of development they represent. However these are not insurmountable tasks and growing volumes of data are beginning to delineate the signaling pathways and transcriptional networks controlling cell growth, proliferation and self-renewal. Only when we can clearly map the interactions of the many elements involved some competing and often conflicting signal pathways and key transcription regulators can we understand self-renewal and fully realise the potential of the pluripotent phenotype.

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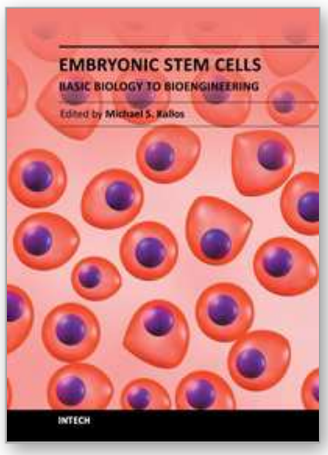

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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