



Self-renewal of teratocarcinoma and embryonic stem cells

Ian Chambers*¹ and Austin Smith*¹

¹MRC Centre Development in Stem Cell Biology, Institute for Stem Cell Research, University of Edinburgh, King's Buildings, West Mains Rd., Edinburgh EH9 3JQ, Scotland, UK

Pluripotent stem cells derived from preimplantation embryos, primordial germ cells or teratocarcinomas are currently unique in undergoing prolonged symmetrical self-renewal in culture. For mouse embryonic stem (ES) cells, self-renewal is dependent on signals from the cytokine leukaemia inhibitory factor (LIF) and from either serum or bone morphogenetic proteins (BMPs). In addition to the extrinsic regulation of gene expression, intrinsic transcriptional determinants are also required for maintenance of the undifferentiated state. These include Oct4, a member of the POU family of homeodomain proteins and a second recently identified homeodomain protein, Nanog. When overexpressed, Nanog allows ES cells to self-renew in the absence of the otherwise obligatory LIF and BMP signals. Although Nanog can act independent of the LIF signal, a contribution of both pathways provides maximal self-renewal efficiency. Nanog function also requires Oct4. Here, we review recent progress in ES cell self-renewal, relate this to the biology of teratocarcinomas and offer testable hypotheses to expose the mechanics of ES cell self-renewal.

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Introduction

The first differentiative events during mouse embryogenesis partition the embryo into extraembryonic and embryonic components (Gardner, 1983). The embryonic component, located on the interior and referred to as the inner cell mass (ICM), is the source of all the tissues of the developing embryo, fetus and ultimately adult organism. The ICM is also the source of embryonic stem (ES) cells (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). The value of ES cells is derived from their capacity to contribute to all three germ layers, ectoderm, endoderm and mesoderm. This can be demonstrated *in vitro* in response to particular differentiative regimes or more strikingly when ES cells are introduced into a blastocyst. In this case, ES cell

derivatives can colonize all the tissues to produce a chimaera in which cells derived from the host ICM and from the injected ES cells are intimately associated throughout the organism (Beddington and Robertson, 1989). When the ES cells colonize the germline, they can pass their genetic inheritance to the next generation (Bradley *et al.*, 1984). This latter property has been extensively exploited in the analysis of gene function *in vivo* (van der Weyden *et al.*, 2002). The ability of ES cells to differentiate into all germ layers of the organism is one of their defining features and is an attribute that we define as pluripotency. Some authors describe ES cells as totipotent, but this overlooks the fact that they do not ordinarily give rise to trophoblast nor do they produce primitive endoderm *in vivo*. Other cells sometimes referred to as pluripotent such as haemopoietic stem cells can be defined more rigorously as multipotent, since they can differentiate into a restricted but distinct range of cell types. The fact that ES cells can be derived from and reintroduced into the ICM does not necessarily mean that ES cells and cells of the ICM are equivalent. The ICM exists only transiently and does not operate as a stem cell compartment *in vivo*. Thus, it is not evident that cells identical to ES cells ever exist *in vivo*. Rather, these remarkable cells may arise through selection and adaptation to the culture environment (Smith, 2001; Buehr and Smith, 2003). A second defining feature of ES cells is the ability to undergo cell divisions without differentiation so as to produce identical pluripotent progeny. This property is referred to as self-renewal and in ES cells occurs via symmetrical cell division. At present, we do not know if asymmetric division ever occurs in ES cell culture.

In this review, we first summarize pertinent findings from the seminal work conducted on teratocarcinomas and the origin of embryonal carcinoma (EC) cells. We then discuss the molecular basis of self-renewal of ES cells both in terms of what the literature provides evidence for and in terms of hypotheses that may help formulate experimental tests. Finally, we relate recent findings on ES cell biology to the stem cell component of teratocarcinomas.

Teratocarcinomas and the origin of EC cells

The intellectual framework for investigations into the biology of ES cells was laid during the 1970s when the imagination of many investigators had been captured by

*Correspondence: I Chambers; E-mail: ichambers@ed.ac.uk or A Smith; E-mail: austin.smith@ed.ac.uk

observations made on teratocarcinomas and the pluripotent cells that they harbour, EC cells. Here, we simply summarize a few points about these tumours; for fuller discussions, the reader is referred to some excellent reviews (Stevens, 1983; Andrews, 2002). In pioneering work carried out from the mid-1950s, Stevens demonstrated that teratomas arise spontaneously in the testes of ~1% of mice of strain 129. These tumours can also form in other strains of mice when male genital ridges from embryos at gestational days 11–13 are transplanted to the testes of adult recipients. Teratomas contain a mixture of differentiated cell types derived from all three germ layers that are present in the tumour mass in an organizationally deranged manner. These tumours can either be benign or malignant. In the latter case, they are referred to as teratocarcinomas. The cell biological distinction is that teratocarcinomas contain undifferentiated stem cells allowing them to form tumours when transplanted into a secondary recipient. Subsequent studies demonstrated that the origin of experimentally induced teratocarcinomas was not restricted to male germ cells; transplantation of pregastrulation stage embryos as young as the two-cell stage to either testes or kidney also results in teratocarcinoma formation (Stevens, 1968, 1970; Solter *et al.*, 1970). Interestingly, in this case it was possible to establish teratocarcinomas from a strain (C3H/H) that would not produce teratocarcinomas when genital ridges were transplanted (Solter *et al.*, 1970). The cellular origin of teratocarcinoma-forming cells within the pregastrulation embryo was shown to be the epiblast (Diwan and Stevens, 1976). The clonal potency of teratocarcinoma stem cells was established by the formation of secondary teratocarcinomas upon transfer of individual undifferentiated cells (Kleinsmith and Pierce, 1964). These undifferentiated cells can also be cultured *in vitro* to give rise to lines of EC cells (Finch and Ephrussi, 1967). Interestingly, although EC cells form malignant teratocarcinomas when transplanted to ectopic sites, when reintroduced into a blastocyst, they may sometimes become incorporated into the embryo and contribute to tissues of the developing fetus (Brinster, 1974). This indicates that the proliferation of undifferentiated EC cells can be brought under control in response to the appropriate environmental cues. Conversely, proliferation of the epiblast becomes deregulated in an ectopic environment to generate a teratocarcinoma, indicating that the pluripotent embryo founder cells have an intrinsic propensity for extended proliferation.

EC cells are almost always aneuploid and most do not contribute to chimaeras nor differentiate well *in vitro*. Nevertheless, it was this work on teratocarcinoma stem cells that informed experiments leading to the isolation of pluripotent ES cells. The success of these experiments hinged on the use of a supporting layer of fibroblasts upon which to explant the embryonic material; the maintenance of pluripotent EC lines had been found to be favoured by co-culture with fibroblasts (Martin and Evans, 1975). In the early 1980s, the same workers reported the isolation of stem cell lines directly from preimplantation embryos explanted into culture without

an intervening tumour phase (Evans and Kaufman, 1981; Martin, 1981). Initially, these cells were characterized as teratocarcinoma stem cell lines. However, when they were reintroduced into mouse embryos and found to contribute widely to tissues of the resulting chimaera and subsequently to pass through the germline, it became clear that they differed qualitatively from EC cells in that their quasi-tumorigenic status could be entirely controlled (Bradley *et al.*, 1984). These cells are now known as ES cells.

Molecular basis for ES cell self-renewal

Signalling through cytokine receptors ES cells can be propagated under culture conditions similar to those used for EC cells; in other words, in the presence of serum and in co-culture with a layer of fibroblasts. An indication that the fibroblasts act by producing a signal that inhibits ES cell differentiation (Smith and Hooper, 1983) was substantiated by the ability of medium conditioned by the Buffalo rat liver cell line to replace the fibroblast requirement (Smith and Hooper, 1987). Subsequent fractionation of the conditioned medium identified the active component as leukaemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). Although many workers continue to use feeders, it is worth noting that the first targeted germline modifications generated via ES cells used feeder-free conditions (Hooper *et al.*, 1987; Thompson *et al.*, 1989) and increasing numbers of investigators now use cell lines such as E14Tg2a and CGR8, which have little or no history of feeder co-culture.

LIF is a member of the IL6 family of cytokines that signal through receptor complexes including the transmembrane receptor, gp130 (reviewed in Heinrich *et al.*, 2003). LIF binds directly to a receptor (LIFR) that contains a long cytoplasmic tail with homology to gp130. The LIF–LIFR complex then recruits gp130, to form a trimeric complex (Zhang *et al.*, 1997). IL-6, in contrast, binds to an IL6 receptor (IL6R) lacking a significant intracellular domain and dependent on the recruitment and dimerization of gp130 in order to mediate downstream signalling. ES cells do not express IL6R and so cannot respond to IL6. However, if a soluble form of IL6R is supplied to ES cells along with IL6, then the cells can be propagated in the undifferentiated state (Yoshida *et al.*, 1994) and can in fact be isolated *de novo* (Nichols *et al.*, 1994). This indicates that the requirement for LIFR is not absolute and can be substituted for by a second gp130 molecule. The signalling events occurring downstream of gp130 in ES cells have been reviewed previously (Burdon *et al.*, 1999a). In summary, the key positive event following gp130 dimerization is the tyrosine phosphorylation, dimerization and nuclear translocation of the signal transducer and activator of transcription, STAT3 (Niwa *et al.*, 1998). Evidence that activation of STAT3 is sufficient for ES cell self-renewal has been presented (Matsuda *et al.*, 1999). These studies used a chimaeric STAT3-estrogen receptor that could be dimerized by treatment of cells with 4-OH-tamoxifen. However, these

analyses were conducted at relatively high cell density leaving open the possibility of contributions from autocrine factors, including LIF, to ES cell self-renewal. Moreover, we now know that the additional presence of serum contributes self-renewal signals, establishing that STAT3 is not strictly sufficient for self-renewal (Ying *et al.*, 2003).

Other extrinsic regulators of ES cell self-renewal

Bone morphogenetic protein (BMP)/GDF It has recently been demonstrated that BMP4, BMP2 or GDF6 can replace the requirement for serum both during clonal propagation of ES cells and during their *de novo* derivation (Ying *et al.*, 2003). This suppression of differentiation appears to be restricted to BMP receptor agonists as it is not shared by other members of the TGF- β superfamily, specifically activin and TGF- β 1. The BMP/GDF function is dependent on co-stimulation with LIF; in the presence of BMP alone cells are driven into non-neural differentiation. In LIF without serum or BMP, there is limited self-renewal and neural differentiation ensues. BMP treatment suppresses neural determination and in combination with LIF is sufficient to sustain ES cell self-renewal without feeders or serum factors. BMP induces expression of members of the Id family of negative transcriptional modulators in various cell types (Nakashima *et al.*, 2001; Ruzinova and Benezra, 2003). This induction of Ids also occurs in ES cells and is the critical contribution of BMP/GDF signalling to ES cell self-renewal, since constitutive expression of Id1, Id2 or Id3 circumvents the BMP/GDF requirement. It is possible that Id molecules act to prevent the effects of precocious expression of the proneural basic helix-loop-helix (bHLH) transcription factors such as the Mash genes in ES cells. Alternatively, the Ids may exert their effect by interaction with non-bHLH proteins, such as Pax factors (Norton, 2000).

Wnt Wnts also appear to suppress neural determination of ES cells (Aubert *et al.*, 2002). Recently, evidence has been presented that the wnt pathway could be involved in the short-term maintenance of pluripotency of both mouse and human ES cells (Sato *et al.*, 2004). This assertion relies heavily on the use of a recently discovered pharmacological inhibitor of glycogen synthase kinase-3 and is thus subject to the usual specificity provisos with regard to chemical inhibitors (Bain *et al.*, 2003). Moreover, GSK-3 is involved in more than just wnt signalling (Frame and Cohen, 2001). RT-PCR analysis suggests that wnts are expressed during preimplantation development (Lloyd *et al.*, 2003) and may be expressed in undifferentiated ES cells (Lako *et al.*, 2001). However, the acceptance of a role for wnts in ES cell self-renewal will require the demonstration that specific activation of wnt signalling allows maintenance of ES cell pluripotency during clonal propagation of ES cells through multiple passages.

Other unidentified factors The requirement of LIF for maintenance of pluripotency of ES cells represents

something of a paradox. Mutations in genes encoding LIF-like cytokines, components of the LIF receptor or STAT3 do not produce phenotypes in embryos during the period of establishment and maintenance of pluripotency in the mouse embryo. This is despite reciprocal blastocyst expression patterns of LIF and LIFR with LIFR being expressed in the ICM (Nichols *et al.*, 1996) and of clear evidence that these molecules play critical roles in the *in vitro* maintenance of pluripotency. The fact that mutations in gp130 and, to a lesser extent, LIFR, impair the ability of preimplantation embryos to survive a period of implantational delay (Nichols *et al.*, 2001) suggests a resolution of this conundrum. Delayed implantation is a physiological adaptation to the presence of a suckling litter that allows embryos to persist for several weeks without implanting; upon cessation of suckling, the embryos implant and development proceeds normally. gp130 mutants lose the epiblast component after 6 days in delay and can no longer generate a fetus on implantation. This indicates that there is a specific and obligate requirement for gp130-mediated signalling in order to extend the period of pluripotency. Nevertheless, the lack of a phenotype in the unperturbed embryo suggest that other mechanisms are normally responsible for the maintenance of pluripotent identity. The ability of *lif*^{-/-} ES cells to self-renew in the presence of neutralizing gp130 antibody provided evidence of an additional pathway for ES cell maintenance (Dani *et al.*, 1998). This ES cell self-renewal factor (ESRF) was detectable in the conditioned medium produced by a parietal endoderm-like cell line. However, this activity has proven refractory to standard protein purification and COS cell expression cloning strategies. The production of LIF by fibroblast feeder cells suggests that LIF is a major determinant of the ability of feeder cells to support ES cell self-renewal. In fact, fibroblasts carrying deletions in LIF have a reduced capacity to support ES cell self-renewal (Stewart *et al.*, 1992). We reasoned that if the only activity supplied by a feeder layer of primary fibroblasts was LIF, then ES cells unable to respond to LIF due to deletion of both *lif* alleles would completely differentiate when co-cultured with fibroblasts. Indeed, a reduced number of alkaline phosphatase positive colonies was formed in this case (Figure 1), underscoring the role of LIFR in this system. However, undifferentiated colony formation was not completely inhibited, indicating that there are LIFR-independent means by which fibroblasts can support ES cell self-renewal.

Intrinsic determinants of ES cell self-renewal

Oct4 Oct4 is a member of a group of transcription factors that bind the octamer sequence ATGCAAAT. In addition, Oct4 can act on a subset of target genes by binding to alternative A/T-rich sites (Saijoh *et al.*, 1996). Expression of Oct4 occurs in the unfertilized egg and the early embryo prior to segregation of the ICM from the trophoctoderm (Pesce and Scholer, 2001). Oct4 is zygotically expressed during cleavage stages (Palmieri *et al.*, 1994). Subsequent to the allocation of ICM cells,

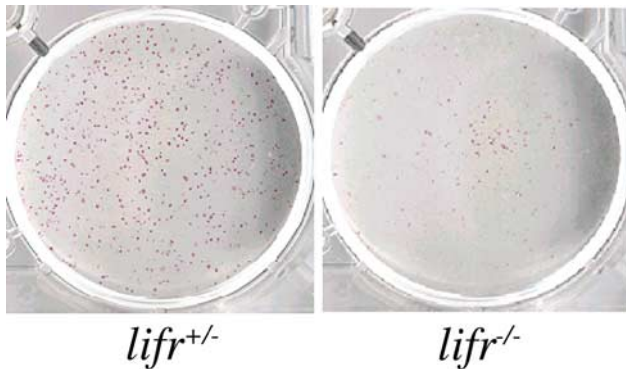


Figure 1 ES cells lacking LIFR self-renew in fibroblast co-culture. 1000 ES cells heterozygous or homozygous for deletion of the *lifr* gene were plated onto a confluent 10 cm² layer of irradiated primary mouse embryonic fibroblasts in standard ES cell medium lacking LIF. After 6 days incubation, undifferentiated ES cell colonies were visualized by staining for alkaline phosphatase. Alkaline phosphatase-positive colonies show a pink colouration

Oct4 is downregulated in the trophectoderm but is readily detected in the ICM. Expression of Oct4 is maintained in the epiblast of pre- and postimplantation embryos before becoming restricted to the migratory primordial germ cells where it persists through the formation of the genital ridges in both sexes.

Mouse embryos in which Oct4 is mutated, form superficially normal but developmentally compromised blastocysts. Ordinarily, after blastocyst formation, a layer of primitive endoderm forms on the blastocoelic surface (Gardner, 1983). When Oct4^{-/-} embryos are allowed to attach *in vitro* and outgrow on plastic, the colonies that form consist entirely of trophectodermal cells (Nichols *et al.*, 1998). The failure of Oct4 mutant outgrowths to differentiate into primitive endoderm or definitive germ layers indicates a severe defect in the potency of the cells that become allocated to the interior in the absence of Oct4. Experiments performed *in vitro* using ES cells carrying an Oct4 cDNA under tetracycline control showed that when Oct4 is deleted from ES cells transdifferentiation into trophectodermal cells occurs (Niwa *et al.*, 2000). This demonstrates that Oct4 is continuously required by ES cells in order to maintain their pluripotent identity. Furthermore, when the level of Oct4 was artificially raised by more than 50% of wild-type levels, a significant proportion of the cells formed differentiated colonies expressing markers of endodermal and mesodermal origin. This finding suggests that a factor that acts co-operatively with Oct4 to direct expression of the ES cell transcriptome is present at limiting amounts. One possibility is that this factor interacts directly with Oct4 such that when Oct4 levels are raised it either becomes titrated away or displaced from specific target genes.

In genetically unmanipulated ES cells, withdrawal of LIF causes differentiation and leads to a coincident and gradual reduction in Oct4 expression. This means that it is not normally possible to examine the effect of Oct4 expression in the absence of LIF. However, in the cells referred to above in which Oct4 expression is regulated

solely by tetracycline, LIF can be withdrawn without reducing Oct4 expression. Under these conditions, the cells differentiate. This establishes that Oct4 cannot act alone to maintain pluripotency and that other factor(s) whose activity is influenced by the gp130 pathway act in combination with Oct4.

Sox2/FoxD3 Two transcription factors that may interact with Oct4 and for which roles in pluripotency have been proposed are FoxD3 and Sox2. Expression of the forkhead transcription factor FoxD3 is detectable in the blastocyst and later in the postimplantation egg cylinder epiblast. Although interaction with Oct4 has been claimed (Guo *et al.*, 2002) evidence that FoxD3 is involved in pluripotency remains indirect. Mutant FoxD3 embryos survive until about E6.5 by which time there is an obvious defect in the embryonic compartment (Hanna *et al.*, 2002). Thus, FoxD3 is required at a stage beyond establishment and maintenance of a pluripotent ICM.

Sox2 is a transcription factor containing a HMG DNA-binding domain that plays an essential role in the transcription of several Oct4 target genes (Niwa, 2001). In the case of the FGF4 enhancer, which is the best studied example, Sox2 and Oct4 bind to adjacent sites within the enhancer and act synergistically to stimulate transcription (Ambrosetti *et al.*, 2000). Sox2 mutant embryos are present at E3.5, but by E6 Sox2^{-/-} implantation sites lack Oct4-expressing cells (Avilion *et al.*, 2003). Outgrowths of Sox2^{-/-} ICMs do not sustain an epiblast component but, in contrast to Oct4 mutants, generate both trophectodermal and primitive endodermal cell types. However, the situation here is complicated by the persistence of maternal Sox2 protein. It would be interesting to know whether Sox2^{-/-} embryos lacking maternal Sox2 protein would reproduce the Oct4-null phenotype. It would also be informative to remove Sox2 from ES cells by conditional deletion or siRNA ablation. Such studies may reveal whether Sox2 is required to enable Oct4 to block trophectodermal differentiation.

Nanog Recently, using either functional cDNA expression cloning or an *in silico* differential expression analysis, two groups independently identified a novel intrinsic determinant of pluripotency (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). This is Nanog. Like Oct4, it is a homeodomain containing protein. However, the identity within the Nanog homeodomain to the homeodomains of other mouse proteins is at best 50%. This is too low to place Nanog in a previously identified family (Kappen *et al.*, 1993) and indicates that Nanog is a divergent homeodomain protein. Nanog is expressed in a quite restricted range of cell types and apparently only in a subset of cells that express Oct4, including ES cells. Nanog is not present in the unfertilized egg and expression is not initiated at the onset of zygotic transcription. Rather, Nanog expression is first detected in morulae being most obvious in cells in the interior of the embryo. High levels of Nanog mRNA persist in the

early blastocyst, but interestingly Nanog expression declines prior to implantation. This dynamic expression pattern could indicate that downregulation of Nanog may be important to avoid an uncontrolled expansion of pluripotent cells. Following implantation, Nanog mRNA is expressed in a subset of epiblast cells with highest levels in the proximal posterior region; upon entry into the primitive streak, Nanog mRNA is rapidly downregulated (Hart *et al.*, 2004 and our unpublished data). Nanog is detectable in PGCs during migration to and residence in genital ridges between days 9 and 13 of gestation (Chambers *et al.*, 2003 and unpublished)

To confirm that Nanog is capable of conferring cytokine independent self-renewal, a Nanog transgene flanked by loxP sites was integrated into otherwise unmodified ES cells. When Nanog expressing cells were taken through two passages at clonal density in the presence of an LIF receptor antagonist (Vernallis *et al.*, 1997), self-renewing ES cells persisted, whereas similarly treated parental cells completely differentiated. The subsequent excision of the floxed Nanog cassette restored LIF dependence. Blastocyst injection of the Cre derivative cells showed contribution of donor cells to the tissues of chimaeric mice, indicating that pluripotency was maintained during the period of LIF-independent ES cell self-renewal (Chambers *et al.*, 2003).

These experiments also allowed a phenotypic comparison of parental cells, Nanog-expressing cells and their Cre derivatives. By all criteria, Cre derivative cells and parental cells were indistinguishable establishing that the phenotypic alterations in Nanog-expressing cells were a consequence of Nanog expression and did not result from some other mutation in the cells. In addition to confirming that Nanog overexpression could direct ES cell self-renewal under conditions in which they would otherwise differentiate, biochemical analysis indicated that Nanog overexpression does not effect STAT3 activation nor does STAT3 activate Nanog expression. Signals from gp130 include a negative feedback mechanism whereby STAT3 induces expression of SOCS3, which then binds to gp130 and inactivates signaling (Heinrich *et al.*, 2003). The ability of Nanog to function separately from the STAT3 pathway is further supported by the ability of Nanog-overexpressing cells to self-renew in the presence of constitutive SOCS3 expression (unpublished). Despite being able to act separately, Nanog and STAT3 do act

synergistically to provide maximal self-renewal efficiency. This is evident from the fact that Nanog-overexpressing cells form pure stem cell colonies at clonal density with significantly increased efficiency when the cultures are supplemented with LIF (Chambers *et al.*, 2003, Figure 5).

Nanog function requires the continued presence of Oct4. When ES cells that express Oct4 from the tetracycline-regulated locus have their Oct4 transgene repressed the cells differentiate along the trophectodermal lineage. This effect can be prevented by transfecting an Oct4 cDNA into the cells (Niwa *et al.*, 2002) but not by transfecting Nanog cDNA (Chambers *et al.*, 2003). This eliminates the possibility that Oct4 functions simply to activate Nanog expression and establishes that Nanog function requires Oct4. The fact that overexpression of Nanog, in otherwise wild-type cells, confers self-renewal implies that Nanog can determine Oct4 expression. However, this effect is most probably indirect. The requirement of Nanog for the expression of the Oct4 gene was tested directly by examination of blastocysts from a Nanog^{+/-} × Nanog^{+/-} intercross. The presence of Oct4 mRNA in all the embryos indicates that Nanog is not essential for Oct4 expression (I Chambers, A Smith, J Nichols and S Yamanaka, unpublished data). The reciprocal experiment suggests that Oct4 is not essential for the initiation of Nanog expression. However, the relative kinetics of the expression of Oct4 and Nanog in the embryo and the presence of a potential Oct4-binding site immediately adjacent to a potential Sox2-binding sites in the Nanog promoter suggest that Oct4 may contribute to the dynamic and/or quantitative regulation of Nanog expression (Figure 2).

Molecules inhibitory to ES cell self-renewal

The activation of the STAT3 target gene SOCS3 represents a negative regulatory signal that is delivered from gp130. SOCS3 is a member of the family of suppressors of cytokine signaling, which act as classical negative regulators to attenuate the signal leading to their production. Members of the SOCS gene family were isolated through an elegant functional strategy in which M1 myeloid cells, which differentiate and stop proliferation when treated with LIF, were transfected with a cDNA library and screened for continued proliferation in the presence of LIF (Starr *et al.*, 1997).

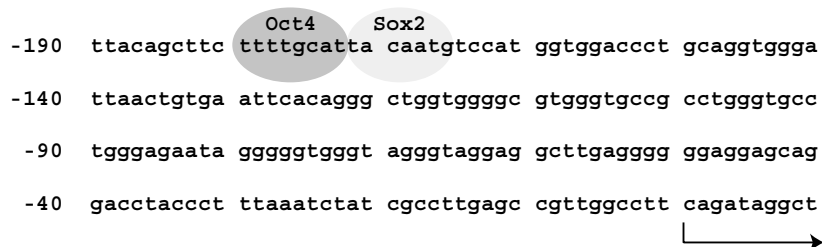


Figure 2 Sequence of the Nanog gene upstream from the transcription initiation site (indicated by the arrow). Matches to the consensus binding site for Oct 4 (ATGCAAAT; 7/8) and Sox 2 (CTTGT/AT/A; 6/7) are shown shaded in dark and light grey, respectively. These sequences are juxtaposed relative to one another in the same manner as they are on known Oct 4/Sox 2 target genes such as Utl1

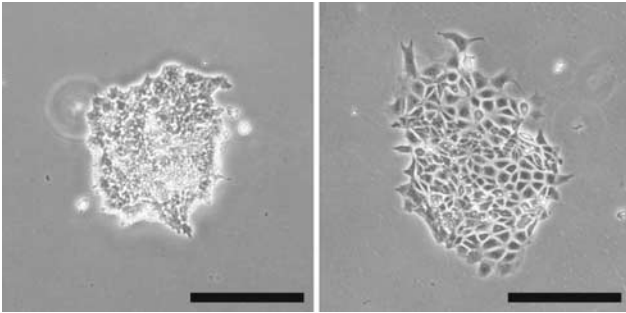


Figure 3 Enforced expression of SOCS3 blocks self-renewal of ES cells directed by LIF. E14/T cells were supertransfected with an episomal expression plasmid (pPyHPGK-) containing no insert (left) or SOCS3 ORF (right) and plated in the presence of LIF and Hygromycin B. Colonies were photographed after 5 days of selection

In ES cells, the only SOCS gene to be significantly induced following gp130 stimulation is SOCS3 (unpublished data). Evidence has been presented that SOCS3 overexpression in ES cells has an apoptotic effect (Duval *et al.*, 2000). This conclusion was based largely on the observation of an absence of colonies following transfection of a SOCS3 expression plasmid into ES cells. We have also noted that when ES cells are transfected with an expression plasmid driving SOCS3 at high levels, there is a decrease in colony formation. However, this may simply be due to non-specific effects of the expression of a gene product at levels far in excess of the physiological norm. Indeed, when SOCS3 is expressed at a more modest level either from a weaker episomal expression plasmid or from a tetracycline-inducible locus, colonies do form in the presence of LIF, but are morphologically differentiated (Figure 3) and do not express alkaline phosphatase. A negative interfering form of STAT3 (STAT3F) similarly directs ES cell differentiation in the presence of LIF when overexpressed (Niwa *et al.*, 1998). In both cases, morphological differentiation is similar to that seen upon LIF withdrawal or addition of LIF antagonist. This indicates that the primary role of LIF is as a differentiation inhibitor rather than a stem cell survival factor.

Induction of differentiation by GATA factors

Transcription factors that have been demonstrated to induce ES cell differentiation into primitive endoderm are GATA6 and GATA4 (Fujikura *et al.*, 2002). These molecules are unique to date in their ability to cause ES cells to differentiate in an apparently uniform manner. An interesting feature of the differentiation induced by GATA4 and GATA6 is the positive feedback on the endogenous gene expression seen when either transcription factor is exogenously expressed in ES cells (Fujikura *et al.*, 2002). Both GATA4 and GATA6 are also expressed, albeit at a lower level, following supertransfection of ES cells with either Oct4 or STAT3F. This could reflect heterogeneous differentiation. Alternatively, GATA expression may occur broadly in the population but a threshold level may need to be

exceeded to initiate positive feedback and dictate uniform endodermal differentiation. A further point of note from this work is the rapidity of loss of ES cell-specific transcripts following induced GATA6 expression from a tetracycline-responsive GATA6 transgene. The rate of decline of Oct4 and Sox2 mRNAs was relatively shallow, whereas the rate of disappearance of mRNAs corresponding to the Oct4 target genes Rex1 and Utf1 (also a Sox2 target) was much more rapid. Of course, it is possible that differences in the half-life of the mRNAs underlie this effect. On the other hand, it could reflect the rapid loss or functional inactivation of a positively acting transcriptional coregulator of Rex1 and Utf1 expression.

Is there a relationship between GATA4/6 function and Nanog?

As mentioned above, Yamanaka and co-workers used an *in silico* differential display algorithm to identify expressed sequence tags specifically expressed in undifferentiated mouse ES cells. Importantly, several of the identified genes were previously known to be specifically expressed in ES cells, including Oct4 and Rex1. More interesting, however, were the novel genes (including Nanog) that Yamanaka's group have subsequently gone on to analyse by targeted gene deletions. Nanog^{-/-} embryos fail to develop following implantation (Mitsui *et al.*, 2003). When ICMs from Nanog^{-/-} embryos were cultured *in vitro*, epiblast cells were not evident. Instead, the outgrowths were composed entirely of primitive endoderm-like cells. Furthermore, when Nanog was deleted from ES cells, the resulting Nanog^{-/-} cell lines lost their ES cell character and displayed a rounded morphology typical of parietal endoderm. This assignment is underpinned by the expression of endodermal rather than ES cell mRNAs in the Nanog^{-/-} cells. Taken together, these observations indicate that Nanog functions to prevent primitive endodermal differentiation.

Given the similar phenotypic consequences of overexpression of GATA factors and elimination of Nanog, this raises the question of whether Nanog acts either partially or wholly by preventing the expression or function of GATA6. As GATA6 can act as a positive mediator of its own transcription, a small increase in GATA6 expression may rapidly become amplified (Fujikura *et al.*, 2002). A DNA sequence to which Nanog binds has been determined by SELEX. Unsurprisingly, this sequence contains the core ATTA sequence recognized by all homeodomain proteins. Outwith this sequence, there is limited specificity. A homeodomain-binding site exists in the GATA6 promoter, which has been hypothesized to bind Nanog and mediate repression of GATA6 expression (Mitsui *et al.*, 2003). Whether this sequence is indeed involved in the regulation of GATA6 expression will require experiments in which the GATA6 promoter is used to drive expression of a reporter gene in ES cells. An alternative method of demonstrating an antagonistic biological effect of Nanog on GATA6 function would be to

examine the effect of expression of GATA6 from a constitutive, heterologous promoter in Nanog-over-expressing ES cells. If Nanog is found to block the expression or activity of GATA6, an important next question will be whether this is the only important function of Nanog. This can be investigated by creating Nanog^{-/-}, GATA6^{-/-} cells and determining whether they retain ES cell identity or differentiate.

Human ES cells

Human ES cells express Oct4 and Nanog. Orthologues of Nanog have been identified in humans and rats and cDNAs have been tested functionally in mouse ES cells; both are capable of sustaining alkaline phosphatase-positive colonies in the absence of LIF (Chambers *et al.*, 2003 and unpublished). The efficiency with which human Nanog directs mouse ES cell self-renewal is low compared with mouse Nanog, which probably reflects divergence in the protein sequence between the two species. A requirement for Nanog in direction of self-renewal of human ES cells seems probable but remains to be established. However, Oct4 appears to be required to suppress extraembryonic differentiation in human ES cells as it does in mouse ES cells (Hay *et al.*, 2004; Matin *et al.*, 2004). Extrinsic signals that support human ES cell self-renewal have yet to be definitively identified, but may be different from those effective on mouse ES cells. Several reports (Xu *et al.*, 2001; Pera *et al.*, 2003; Amit *et al.*, 2004) suggest that bFGF promotes human ES cell self-renewal, an effect that has not been observed on mouse ES cells. Human ES cells can self-renew in the absence of exogenously added LIF (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). However, this does not necessarily mean that STAT3 activation is not important for self-renewal of human ES cells. It is possible that autocrine signalling through gp130 occurs in these cultures and that the extent of this is sufficient for the self-renewal of human but not mouse ES cells. There is also a potentially trivial explanation for the reported lack of effect of LIF on human ES cells in some studies that stems from the widespread use of ESGRO, a commercially available form of recombinant mouse LIF. Mouse LIF does not bind to the human LIFR (Layton *et al.*, 1994). An alternative explanation is that human ES cells express a higher effective level of Nanog than mouse ES cells. Thus, human ES cells may not require a cooperative interaction between STAT3 and endogenous Nanog in order to self-renew efficiently.

Teratocarcinomas and ES cells

Teratocarcinomas are the classical stem cell tumour (Pierce, 1967). In other words, they contain stem cells as well as differentiated cells, whereas the related benign tumours, teratomas, lack an undifferentiated compartment. Of the two tumour types only the stem cell containing teratocarcinomas can form secondary tumours when serially transplanted. This concept of cancers being composed of a heterogeneous mixture of cell types including cancer stem cells has recently found

favour within the cancer research community (Pardal *et al.*, 2003) (see also the article by JE Dick in this issue). It can explain the fact that chemotherapy often removes the bulk of a tumour mass without preventing tumour recurrence, suggesting the survival of a subset of cancer stem cells. Therefore, it may be informative to contrast the cryptic tumorigenic potential of epiblast and ES cells with the overt malignancy of EC cells. An unanswered question here is whether the epiblast cell is directly tumorigenic, or are genetic or epigenetic changes obligatory to confer continuous tumour growth and transplantability? In this regard, it is interesting that the majority of EC lines can self-renew in the absence of LIF or of a feeder layer. In the past, there has been strong selective pressure applied to cells during the course of formation and serial transplantation of teratocarcinomas and subsequent derivation of EC cells, which historically was performed without LIF and in many cases without feeders. It would be surprising therefore if some genetic alterations did not occur. In fact, EC cells exhibit a range of chromosomal abnormalities, common among which is trisomy for chromosome 6. This is of interest because of the location of Nanog on chromosome 6. In humans, testicular germ cell tumours account for the majority of cancers in young postpubertal men. These invariably exhibit trisomy for chromosome 12 often present as a small isochromosome, which contains the region syntenic to mouse chromosome 6 on which Nanog is located (Skotheim *et al.*, 2002). Interestingly, during routine culture hES cells repeatedly gain sequences from human chromosome 17, whereas following subcloning or attempts to culture hES cells in the absence of feeders, duplication of chromosome 12 sequences occurs (Draper *et al.*, 2004). These observations have generated some excitement given the fact that not only Nanog but also Stella and GDF3 are localized to the same region of synteny in rats as well as humans and mice (Payer *et al.*, 2003; Clark *et al.*, 2004). It is possible that like the related molecules GDF6 and BMP4, GDF3 could enhance ES cell self-renewal, although at present there is no direct evidence to support this notion. In contrast, the case for an involvement of Stella is less clear (Payer *et al.*, 2003). However, the genetics of testicular germ cell tumour susceptibility are controversial, with some studies mapping the region encompassing Nanog, Stella and GDF3 outwith the region of minimal duplication in isochromosome 12 (Mostert *et al.*, 1998; Rodriguez *et al.*, 2003; Skotheim and Lothe, 2003).

Recent studies have found comparatively high levels of Oct4 during early stages of human testicular germ cell tumorigenesis (Looijenga *et al.*, 2003; Gidekel *et al.*, 2003). To investigate the significance of this expression, further use was made of the cells carrying the tetracycline regulatable Oct4 transgene referred to above (Gidekel *et al.*, 2003). In ES cell culture ZHTc6 cells express ~75% of wild type Oct4 protein levels when cultured without tetracycline and ~150% wild type levels when cultured with tetracycline (Niwa *et al.*, 2000). ZHBTc4, cells only express Oct4 in the presence of tetracycline and do so to about 60% wild-type levels

(Niwa *et al.*, 2000). Teratocarcinomas were formed when these cells were subcutaneously injected into mice. ZHTc6 cells produced tumours containing a greater proportion of relatively undifferentiated tissue when the recipients were allowed fresh drinking water as opposed to water containing tetracycline. The tumour incidence was lower when ZHTc4 cells were injected and was negligible when similarly injected mice were given tetracycline to shut off transgene expression. More significantly, when mice carrying ZHTc4-derived tumours were given tetracycline the tumours regressed. These experiments not only show that the proportion of relatively undifferentiated cells in a tumour can be affected by the Oct4 level but also that Oct4 is required continuously to maintain the malignant stem cell component of teratocarcinomas.

Insight into the teratogenic nature of ES cells has come from analysis of another of the ES cell-specific transcripts identified by Yamanaka's group. The corresponding gene is X-linked and encodes a constitutively active ras molecule termed Eras (Takahashi *et al.*, 2003). Mutations at codons 12, 59 and 63 of Hras render the molecule constitutively active. When compared to Hras both human and mouse Eras have amino-acid substitutions at each of these codons. Importantly, Eras does not engage the Raf-Mek-erk pathway, but does stimulate PI3 kinase (see below). Eras⁻ ES cells expand more slowly than unmutated ES cells despite continuing to express Oct4 and retaining a normal morphology. Moreover, when injected subcutaneously into nude mice Eras⁻ ES cells produce significantly smaller tumours than wild-type cells. This could indicate that Eras⁻ tumours do not sustain a pluripotent compartment and are teratomas rather than teratocarcinomas. This would manifest in a loss in the ability of tumour cells to produce secondary teratomas upon serial transfer, although this remains untested. Intriguingly, Eras⁻ mice show no abnormalities, indicating that Eras is dispensable for the epiblast. However, it would be interesting to know whether loss or mutation of Eras affects the ability to derive ES cells *de novo* and whether Eras may be involved in the nonpermissiveness for ES cell derivation from certain mouse strains (Kawase *et al.*, 1994). A related issue is the timing and localization of Eras expression *in vivo* and how this relates to the ease with which ES cells can be isolated from embryos of particular developmental stages (Brook and Gardner, 1997). A further point of interest is whether there is a deficit in the ability of Eras⁻ embryos to survive a period of implantational delay as has been demonstrated for receptor components involved in LIF signalling (Nichols *et al.*, 2001). This would implicate Eras in the STAT3 pathway. It is not currently known if Eras is a downstream target of either STAT3 or Nanog.

ES cells have a rapid cell cycle with an unusually short G1, possibly due to the fact that hypophosphorylated retinoblastoma (Rb) is essentially undetectable (Burdon *et al.*, 2002). Transfection of an oncogenic form of Hras caused growth retardation and differentiation of ES cells (Cheng *et al.*, 1998), an effect not seen with overexpression of Eras (Takahashi *et al.*, 2003). This is

consistent with a prodifferentiative rather than proliferative function for the SHP2-ras-MAPK pathway in ES cells. Inhibitors of the MAPK pathway allow more efficient ES cell self-renewal than mock-treated controls (Burdon *et al.*, 1999b). Furthermore, the efficiency of ES cell derivation is enhanced by MAPK pathway inhibitors (Buehr and Smith, 2003). The MAPK pathway is linked to gp130 via binding of SHP2 to the same residue that binds SOCS3. Consistent with a prodifferentiative effect of the MAPK pathway, homozygous mutant SHP2 ES cells have enhanced sensitivity to LIF (Qu and Feng, 1998). Unlike oncogenic Hras, Eras does not associate with Raf and does not activate the MAPK pathway. However, Eras does interact with PI3K and phosphorylation of the downstream target Akt was decreased in Eras⁻ cells. The slow growth and reduced size of teratomas produced by Eras⁻ cells could be rescued by the expression of active PI3K. This fits with the increased proliferation of ES cells lacking PTEN (the phosphatase that hydrolyses PIP3) (Sun *et al.*, 1999) and with the incidence of testicular teratocarcinomas in PTEN^{-/-} mice (Kimura *et al.*, 2003). However, while the PI3K inhibitor LY294002 increases the fraction of ES cells that are in G0/G1 phase of the cell cycle (Jirmanova *et al.*, 2002), the cell cycle status of Eras⁻ cells appears unchanged. This suggests that PI3K may have a cell cycle-independent effect on cell growth and could indicate that LY294002 has an additional effect upon ES cells that alters the cell cycle. The phenotype of PTEN^{-/-} ES cells suggests that these cells may be primed for unrelenting proliferation. The cells lack a significant G1 phase and have constitutively high PIP3 levels. In fact, PTEN^{-/-} ES cells give rise to tumours upon transplantation into mice that appear to consist almost entirely of undifferentiated cells (Di Cristofano *et al.*, 1998). It may be that all that is required to achieve tumorigenicity is a suppression of differentiation. However, the stem cell nature of PTEN^{-/-} tumour cells was not tested by serial transplantation.

It is notable that Eras activates a subset of the pathways activated by more conventional ras molecules. How general such restricted pathway activation is for stem cells is unclear. However, it is interesting that variant SHIP and Gab1 signalling molecules lacking subcellular localization motifs have been identified in haematopoietic and ES cells (Tu *et al.*, 2001; Burdon *et al.*, 2002).

ES cell self-renewal circuit

The level of overexpression of Nanog is correlated to the efficiency of the formation of cytokine-independent undifferentiated ES cell colonies. A dosage effect is also observed for STAT3 and Oct4. These observations can be synthesized to provide a model of the contributions of these molecules to ES cell self-renewal (Figure 4). Mouse ES cell self-renewal requires the expression of both Nanog and Oct4. Elimination of either of these molecules results in a change in cell identity from epiblast-like to the extraembryonic cell type formed after the first (Oct4^{-/-}) or second (Nanog^{-/-})

differentiative decision to occur during embryogenesis. Propagation of the pluripotent state normally requires STAT3. When STAT3 activation is reduced either by withdrawal of LIF or by interference with STAT3 function, cells cultured in serum differentiate into a mixed cell population expressing markers of endoderm and mesoderm. Overexpression of Nanog obviates the requirement for extrinsic stimulation but also enhances the efficiency of self-renewal in the presence of gp130 stimulation. This co-operative interaction between Nanog and STAT3 suggests that when LIF is withdrawn from genetically unmodified ES cells, there is a decline in the ability of Nanog to function. Interestingly, similar differentiation to that observed when STAT3 function is impaired is seen when Oct4 is overexpressed in the presence of gp130 stimulation. This may indicate an imbalance in the co-dependent Oct4- and Nanog-mediated aspects of the self-renewal machinery. The most parsimonious explanation of these observations is that a co-factor that Oct4 requires for function is present at limiting amounts and that this activity is stimulated by LIF/STAT3. The fact that Nanog overexpression enhances the efficiency of self-renewal in the presence of STAT3 indicates that it is in fact limiting. A prediction of this model would be that when Nanog is overexpressed it could act to restrict the prodifferentiative effect of elevated Oct4 levels. These considerations are particularly interesting in light of a previously propounded model of ES cell self-renewal based on the response of Oct4 target genes to alterations in the Oct4 level (Niwa, 2001). This model also predicts the existence of a partner of Oct4, which is present in limiting amounts in ES cells. This factor is proposed to act on a subset of Oct4 target genes including Rex1 and to be downstream of gp130 stimulation. If we modify this model to say that the missing factor acts in co-operation with rather than downstream of gp130, then could the missing factor be Nanog? The Rex1 promoter is regulated by Oct4 and by an unknown factor(s) termed Rox that binds at a site adjacent to the Oct4-binding site (Ben-Shushan *et al.*, 1998; Mitsui *et al.*, 2003). It has been proposed that this site may actually bind Nanog (Mitsui *et al.*, 2003). In this case, the

sequences corresponding to the Rox site are quite far removed from the *in vitro* determined Nanog DNA-binding site, although it is possible that the Nanog DNA recognition site could be altered through interaction with other adjacently binding factors.

Clearly, these suggestions can be tested experimentally. An area that is likely to provide particular insight into the mechanics of ES cell self-renewal is a determination of whether or not there are direct biochemical interactions between Nanog, Oct4 and STAT3. If so, it will be important to understand how these interactions are brought about and how they are perturbed following alterations in the levels of the active forms of the various factors.

A further aspect of self-renewal that needs to be accommodated into a current model of ES cell self-renewal is the dual roles of BMP. Smads can activate non-neural differentiation in serum free cultures, whereas STAT3 acts to repress the activation of these pathways (Figure 4). Whether this occurs directly and/or via an effect upon Smads is not clear. An interaction between STAT3 and Smads has previously been demonstrated in neural cells (Nakashima *et al.*, 1999). In this case, STAT3 and Smads coexist in a complex bridged by p300/CBP. Such a complex may exist in ES cells (Figure 4). It should be noted that the presence of LIF switches BMP from a differentiative signal to a self-renewal signal. An extreme hypothesis is that all that STAT3 is required for in ES cells is to tie up Smads in a p300/CBP bridged complex and by doing so reduce the pool of free Smad available to activate differentiation determining genes. This raises the question of whether LIF would be required at all if cells could be removed from all differentiation stimuli. In this respect, it may be fruitful to enquire how nullipotent EC lines acquired their resistance to differentiation stimuli. Finally, since overexpression of Nanog liberates ES cell self-renewal from the requirement for BMP signalling (Ying *et al.*, 2003), this places Nanog at centre stage in our current attempts to unravel the mechanisms of ES cell self-renewal.

In summary, we note that some components of ES cell identity are unique to pluripotent cells (Nanog, Oct4),

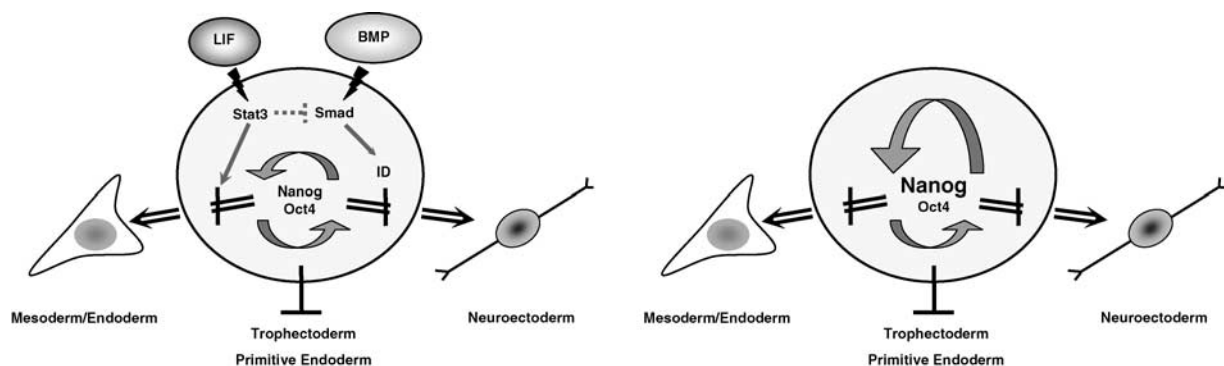


Figure 4 Model of ES cell self-renewal signalling. Left, LIF and BMP act together to block differentiation. LIF activates STAT3 and blocks non-neural differentiation. BMP blocks neural differentiation by induction of Id's. The intrinsic transcriptional regulators Nanog and Oct4 act to maintain an undifferentiated phenotype. Right, upon overexpression of Nanog, the requirement for extrinsic signals to block differentiation is alleviated and ES cells self-renew constitutively

whereas others (BMP, LIF) are generic and not stem cell specific. We hypothesize that the intrinsic factors Nanog and Oct4 are required to maintain epiblast identity and that extrinsic factors (BMP, LIF) are required to block germ-layer differentiation. It remains to be seen whether illuminating the circuitry of ES cell self-renewal will be informative in detail for tissue stem cell self renewal. Perhaps, a conservation in principle is more likely: unique intrinsic determinants imposing cell identity and

extrinsic signals suppressing activation of differentiation pathways.

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