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Cell, Volume 113, Issue 5 , 30 May 2003, Pages 643-655

doi:10.1016/S0092-8674(03)00392-1

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Functional Expression Cloning of Nanog, a Pluripotency Sustaining Factor in Embryonic Stem Cells

Ian Chambers, Douglas Colby, Morag Robertson, Jennifer Nichols, Sonia Lee, Susan Tweedie and Austin Smith

Institute for Stem Cell Research, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JQ, Scotland, United Kingdom

Abstract

Embryonic stem (ES) cells undergo extended proliferation while remaining poised for multilineage differentiation. A unique network of transcription factors may characterize self-renewal and simultaneously suppress differentiation. We applied expression cloning in mouse ES cells to isolate a self-renewal determinant. Nanog is a divergent homeodomain protein that directs propagation of undifferentiated ES cells. Nanog mRNA is present in pluripotent mouse and human cell lines, and absent from differentiated cells. In preimplantation embryos, Nanog is restricted to founder cells from which ES cells can be derived. Endogenous Nanog acts in parallel with cytokine stimulation of Stat3 to drive ES cell self-renewal. Elevated Nanog expression from transgene constructs is sufficient for clonal expansion of ES cells, bypassing Stat3 and maintaining Oct4 levels. Cytokine dependence, multilineage differentiation, and embryo colonization capacity are fully restored upon transgene excision. These findings establish a central role for Nanog in the transcription factor hierarchy that defines ES cell identity.

Introduction

Mouse embryonic stem (ES) cells are permanent cell lines derived from the preimplantation embryo (Evans and Kaufman 1981 and Martin 1981). These cells have three hallmarks: they undergo symmetrical self-renewing divisions, they are pluripotent with capacity to differentiate into all fetal and adult cell lineages, and they can incorporate into embryos and contribute to functional tissue generation. Surprisingly, however, the intrinsic biology of these extraordinary cells remains poorly described (Smith, 2001). In particular, the molecular foundations of cellular pluripotency have not been defined, much less integrated, to provide a mechanism that can explain the robust preservation of stem cell identity and potency during ES cell propagation.

At each ES cell division, the alternative outcomes of self-renewal and differentiation are decided by the interplay between intrinsic factors and extrinsic instructive or selective signals. Propagation of mouse ES cells is mediated via coculture with a feeder layer of embryo fibroblasts or provision of a cytokine such as leukemia inhibitory factor (LIF) that acts through the LIF-R/gp130 complex (Yoshida et al. 1994 and Burdon et al. 1999a). LIF-deficient fibroblasts are reported to be incapable of supporting ES cell self-renewal (Stewart et al., 1992), indicating that supply of LIF

is a key attribute of feeders. However, although no self-renewal factors have been identified other than gp130 cytokines, there is evidence for operation of a gp130-independent pathway that can maintain ES cell identity (Dani et al., 1998). Furthermore, self-renewal of human blastocyst-derived stem cells appears not to be maintained by LIF (Thomson et al. 1998 and Reubinoff et al. 2000). Finally, gp130 signaling is dispensable for pluripotent cells in the mouse embryo because null mutants undergo gastrulation and organogenesis. A requirement for gp130 is only revealed if the schedule of embryo implantation is delayed (Nichols et al., 2001). Thus, the gp130 pathway serves a facultative role *in vivo*, and is not fundamental to pluripotency.

Recent studies have begun to define key players in the transcriptional specification of the mouse ES cell phenotype. Essential roles have been assigned to gp130-mediated activation of Stat3 (Niwa et al. 1998 and Matsuda et al. 1999) and to the intrinsic activity of the POU factor Oct4 (Niwa et al., 2000). However, Stat3 is not ES cell-specific but is found in a variety of other cell types, often associated with differentiation (Hirano et al., 2000). Oct4 is restricted to pluripotent and germ line cells (Pesce and Schöler, 2001), but forced constitutive expression of Oct4 is not sufficient to prevent ES cell differentiation (Niwa et al., 2000). Oct4 and Stat3 each interact with various cofactors and regulate expression of multiple target genes. Which of these interactions and targets are important for the ES cell phenotype is presently unknown. Nor do the existing data preclude a central role for another transcriptional regulator. Indeed, the finding that overexpression of Oct4 induces differentiation similar to withdrawal of LIF is suggestive of an intersection between Stat3 and Oct4 that could be mediated by a "master" transcriptional organizer (Niwa et al. 2000 and Niwa 2001).

We sought to identify gene functions capable of directing mouse ES cell propagation. Functional screening of an ES cell cDNA library resulted in isolation of a divergent homeodomain protein that acts as an intrinsic effector of ES cell self-renewal. This homeobox gene is also expressed in the founder cells of the early embryo. Accordingly, we have named the gene *nanog*, after the mythological Celtic land of the ever young, Tir nan Og.

Results

A Functional Screen for ES Cell Self-Renewal Determinants

To undertake a functional library screen in ES cells, we exploited a system for episomal transduction and expression of cDNAs. This is based on the replicative functions of the polyoma virus (Gassmann et al., 1995). ES cells that express polyoma large T antigen can routinely be "supertransfected" with plasmids carrying a polyoma origin of replication (*ori*) at a frequency of 1%, three orders of magnitude greater than DNA integration efficiencies in ES cells. A cDNA expression library of 10⁵ independent plasmids can therefore be screened by episomal transfection of 10⁷ cells. Provided selection is maintained, episomal propagation of the transfected DNA can be sustained for several weeks, allowing facile recovery of DNA conferring a phenotype of interest. Additionally, robust cDNA expression is assured by avoidance of epigenetic effects arising from chromosomal integration.

Of various episomal constructs we have tested, the pPyCAGIP plasmid (Figure 1A) gives the highest levels of cDNA expression. Puromycin selection rapidly eliminates untransfected ES cells removing a source of potentially confounding biological activities. However, in the absence of added LIF, supertransfection with empty pPyCAGIP vector yields mixed colonies containing undifferentiated ES cells. This is due to production of LIF by differentiated cells (Rathjen et al., 1990). In order to eliminate this background, the *lifr* gene was deleted. LRK1 cells are maintained as undifferentiated ES cells by activation of gp130 using IL6/sIL6R. Compared to parental E14/T cells, they produce negligible stem cell colonies when transfected with empty vector in the absence of cytokine (Figure 1B).

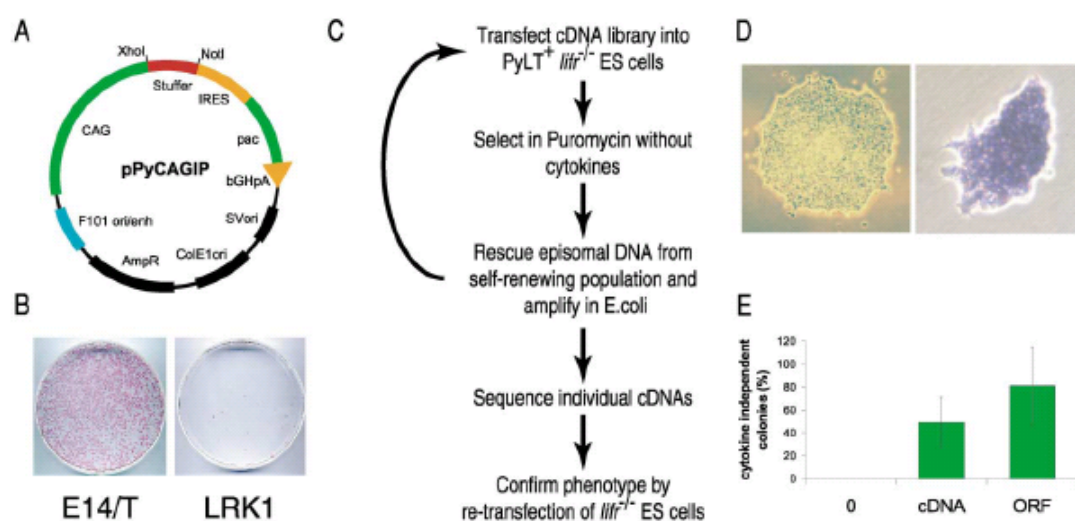


Figure 1. Components of the Expression Cloning Strategy(A) pPyCAGIP episomal expression vector. The plasmid carries a polyoma origin with the F101 mutation allowing episomal replication in ES cells. cDNA is cloned directionally in place of the stuffer fragment within a transcription unit linked to the puromycin resistance gene (*pac*) through an IRES.(B) Reduced background of self-renewal in ES cells deleted for the *lifr* gene. E14/T or the *lifr* targeted subclone, LRK1, were transfected with pPyCAGIP and plated at 106 per 9 cm petri dish. Selection was applied 30 hr later and plates stained for alkaline phosphatase after 12 days.(C) Logic of the expression strategy. Plasmid directing ES cell self-renewal amplifies during ES cell propagation and can be recovered and enriched by further rounds of selection in ES cells.(D) Colonies of LRK1 cells expressing Nanog cDNA from the pPyCAGIP episome in the absence of cytokine; left, colony morphology; right, in situ hybridization for Oct4 mRNA.(E) Quantitation of stem cell colonies formed following transfection of LRK1 cells with pPyCAGIP derivatives carrying no insert (0), Nanog cDNA, or Nanog ORF. Data are the average of at least three independent experiments; bars indicate standard deviations.

When LIFR-negative ES cells were plated onto MEF feeder cells, a number of small, undifferentiated colonies reproducibly emerged. Thus, while the major self-renewal activity derived from MEFs acts via LIFR, another mechanism(s) of sustaining ES cell propagation is operative. Consequently, we prepared a cDNA library from ES cell/MEF cocultures.

LRK1 cells were transfected with library DNA and cultured in the absence of cytokine according to the strategy outlined in Figure 1C. Two pools were identified that yielded morphologically undifferentiated colonies. Further analysis indicated that the active cDNA in both pools encoded the same cDNA. Transfection of purified plasmid DNA produced self-renewing ES cell colonies that expressed Oct4 (Figure 1D). The biological activity of the cDNA resides within the open reading frame (Figure 1E).

Characterization of the Nanog cDNA

A search for recognizable domains in the ORF using SMART (<http://smart.embl-heidelberg.de>) revealed the presence of a homeodomain between amino acid residues 96 and 155, with no other obvious relationship to previously characterized proteins. A maximum of 50% amino acid identity over the homeodomain is found with Barx1, Msx1, and members of the NK2 family of homeoproteins, with no conserved motifs outside the homeodomain (Figure 2). The protein thus appears to be a unique variant homeoprotein, which we named Nanog. Orthologs of Nanog were identified by BLAST in the rat (87% identity) and human (58% identity). Sequence conservation was most pronounced over the homeodomain where identities were 94% (rat) and 87% (human) (Figure 2B). Outwith the homeodomain are 4 conserved regions of 5 or more contiguous amino acids. A serine-rich motif is N-terminal to the homeodomain, the others C-terminal. In addition, the mouse sequence from residues 198–243 has a tryptophan at every fifth position. Despite a short insertion (rat) or deletion (human), the spacing of tryptophans is conserved, although one of the tryptophans in the human is replaced by a glutamine. The first 31 amino acids of the tryptophan repeat in the two rodent sequences represent a simple reiteration of WnsQTWTNPTW ($n = G, S, N$ and $S = S, N$).

A B2 repetitive element is present in the 3' UTR of the Nanog mRNA (Figure 2C). This could contribute to regulation of Nanog gene expression, since B2 elements are expressed at high levels in embryonic cells (Ryskov et al., 1983).

The ability of the putative human Nanog ortholog to function in mouse ES cells was tested by transfecting LRK1 cells. Although less effective than mouse Nanog, the human sequence was capable of directing cytokine-independent self-renewal (Figure 2D). We also tested the ORF of Nkx2.5, one of the mouse homeobox genes most closely related to Nanog. No cytokine-independent stem cell colonies formed; in fact, Nkx2.5 transfectants appeared morphologically differentiated even in the presence of LIF. Thus, cytokine independence is specifically conferred by Nanog and is not a common attribute of homeodomain proteins.

Nanog mRNA Appears Confined to Pluripotent Tissues and Cell Lines

Nanog mRNA is expressed in ES cell/MEF cocultures but not by MEFs alone (Figure 3A). We eliminated hybridization of the Nanog cDNA to small B2 transcripts using a probe restricted to the Nanog ORF and employed this in all subsequent analyses. In situ hybridization confirmed that ES cells were the source of expression in coculture (Figure 3B). Nanog mRNA was not detected in parietal endoderm, fibroblast, and hematopoietic cell lines, but was abundant in ES cells, EG cells, and in both LIF-dependent and LIF-independent embryonal carcinoma cell lines (Figure 3C). P19 EC cells showed reduced Nanog expression. Human Nanog mRNA was detected in embryonal carcinoma cells but not lymphoid cells (Figure 3D). During ES cell differentiation, Nanog mRNA declined markedly (Figure 3E). In situ hybridization of partially differentiated ES cell cultures showed that Nanog expression was retained only in undifferentiated cells (Figure 3B, right). Nanog transcript was undetectable in various adult mouse tissues by Northern hybridization of total RNA (Figure 3F).

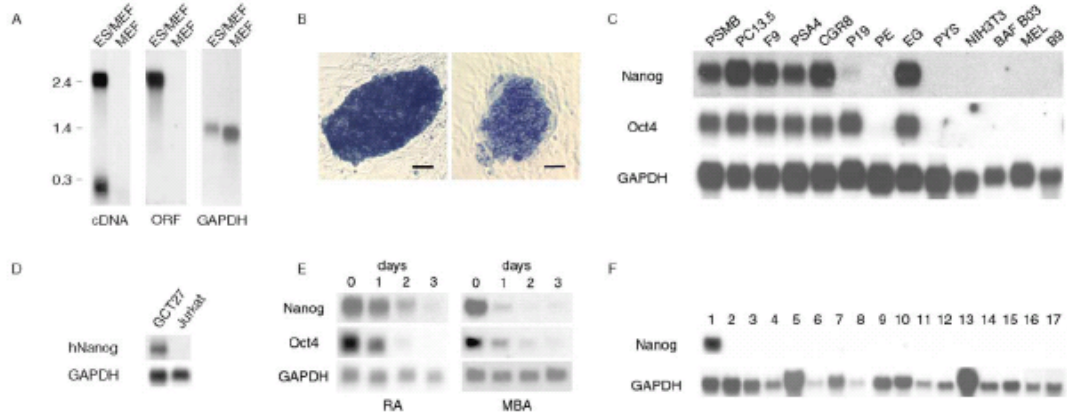


Figure 3. Expression of Nanog in Pluripotent Cell Lines (A) Hybridization of RNA from MEFs and from MEF/ES cell cocultures used for library construction. 1 μ g pA⁺ RNA was loaded per lane and hybridized with probes for Nanog cDNA (left), GAPDH (right), and Nanog ORF (middle). Positions of RNA markers (kb) are shown to the left. (B) Nanog in situ hybridization of a MEF/ES cell coculture (left) and a feeder-free culture in which an undifferentiated cluster of ES cells is surrounded by differentiated cells (right); bars are 50 μ m. (C) Nanog expression in cell lines. RNAs were from PSMB, PC13.5, F9, PSA4 and P19 EC cells; CGR8, ES cells; PE, D7-A3 parietal endoderm-like; EG, embryonic germ cells; PYS, parietal yolk sac; NIH3T3, fibroblasts; BAFB03, pro-B cells; MEL, erythroleukaemia; B9, plasmacytoma. (D) Human nanog RNA is expressed in EC cells. RNAs were from embryonal carcinoma (GCT27) (Pera et al., 1989) and lymphoid (Jurkat) cells. (E) Nanog is downregulated during ES cell differentiation. E14Tg2a cells were induced to differentiate by application of retinoic acid (RA) or 3-methoxybenzamide (MBA) for the number of days shown. (F) Lack of detectable Nanog mRNA in adult tissues. RNAs were: 1, CGR8 ES cells; 2, adipose; 3, kidney; 4, liver; 5, heart; 6, spleen; 7, brain; 8, bone marrow; 9, tongue; 10, eye; 11, oviduct; 12, thymus; 13, skeletal muscle; 14, skin; 15, ovary; 16, seminiferous vesicle; 17, lung. Northern analysis was performed by sequential hybridization with probes for nanog, GAPDH, and oct4 (C and E), hnanog and GAPDH (D), and nanog and GAPDH (F).

We investigated distribution of Nanog mRNA in the mouse embryo (Figure 4). No expression is evident during early cleavage stages. The first sign of Nanog mRNA is in compacted morulae. Strikingly, the hybridization signal is localized to interior cells, the future inner cell mass (ICM). Blastocyst expression is confined to the ICM and absent from the trophectoderm. In later blastocysts, Nanog mRNA is further restricted to the epiblast and excluded from the primitive endoderm. By implantation stage, Nanog mRNA is downregulated.

We also examined Nanog expression in primordial germ cells, which can be converted into pluripotent EG cells (Matsui et al. 1992 and Resnick et al. 1992). Expression is readily detectable in the genital ridges of E11.5 embryos (Figure 4B).
ES Cell Identity Is Faithfully Maintained by Nanog Expression

We introduced nanog into ES cells that do not contain polyoma LT. A loxP-containing construct was employed such that the nanog cDNA could subsequently be excised by Cre recombinase simultaneously bringing GFP under CAG promoter control (Figure 5A). Excision of the nanog cassette controls for any genetic or epigenetic changes associated with either the transfection and selection process or directly with the transgene. We first tested the ability of cells overexpressing Nanog to be propagated clonally in the absence of gp130 signaling and then examined whether pluripotency and embryo colonization capacity were sustained.

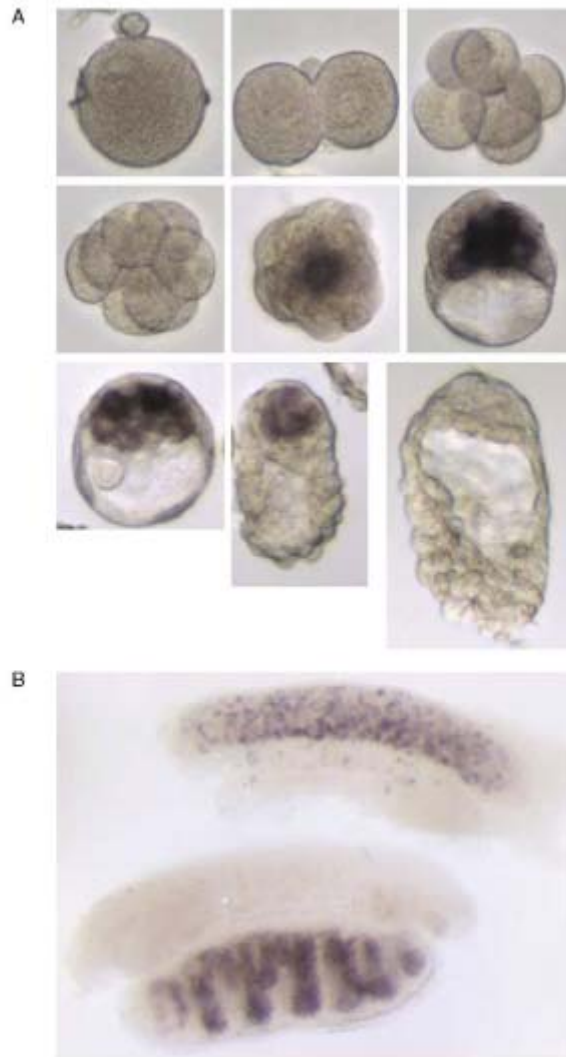


Figure 4. Expression of Nanog In Vivo(A) Preimplantation embryos. Top: embryos of 1, 2, and 6 cells. Middle: 8-cell embryo, late morula and early blastocyst. Bottom: blastocysts at expanded, hatched, and implanting stages. Embryos were hybridized in the same reaction and stained for the same time. All panels are shown at equal magnification.(B) E11.5 genital ridges from female (top) and male (bottom) embryos. Hybridization appears localized to the primordial germ cells overlying the somatic tissue.

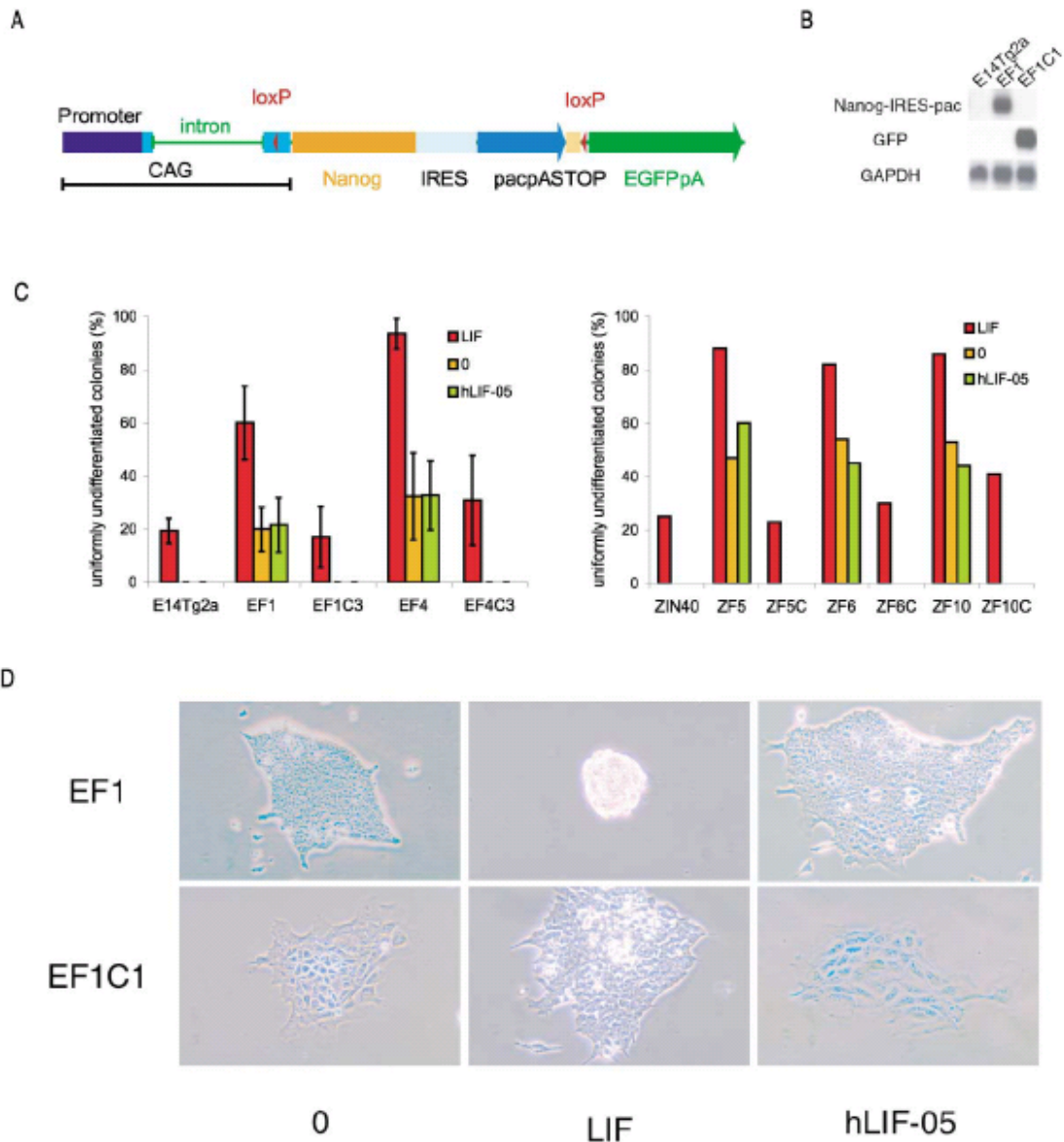


Figure 5. Reversibility of gp130 independent self-renewal(A) Schematic of floxed Nanog expression vector. The LoxP sites are positioned in the second exon of the CAG cassette and between the terminator sequence and egfp such that following Cre-mediated recombination CAG directs expression of egfp.(B) Northern analysis before and after Cre excision. The blot was hybridized sequentially with the indicated probes. EF1, E14Tg2a subclone carrying the floxed transgene; EF1C1, EF1 subclone following Cre-mediated excision.(C) Removal of Nanog cassette restores LIF dependence. Parental ES cells, transfectants expressing the nanog transgene, and their Cre-excised derivative lines were analyzed following plating at clonal density in the indicated culture conditions; 0, no addition; LIF, 100 U/ml LIF, hLIF-05, LIF antagonist. After 6 days culture, pure alkaline phosphatase positive colonies were quantitated; left, E14Tg2a and derivatives, data are the means of at least three determinations; right, Zin40 and derivatives, single determinations from a representative experiment.(D) Morphology of Nanog-expressing cells and Cre-excised derivative in the absence of cytokine (0), in 100 U/ml LIF (LIF) or in the presence of hLIF-05.

The following procedure was performed with two independent ES cell lines, E14Tg2a and ZIN40. Clonal transfectants were isolated and expression of transgene mRNA confirmed (Figure 5B). Cells were then seeded at low density and expanded in the presence of the LIF antagonist, hLIF-05 (Vernallis et al., 1997) for at least 7 days. hLIF-05 blocks the activity of all known LIF-R ligands. Parental cells treated in parallel underwent complete differentiation, yielding no passageable colonies. In contrast, cells expressing the nanog-IRES-pac cassette continued to proliferate as

undifferentiated stem cells. Subsequently, cells were transiently transfected with Cre in the presence of LIF. Colonies in which the Nanog expression cassette had been eliminated were identified by expression of GFP and restoration of puromycin sensitivity. Chromosomal spreads were examined for several clones and in all cases found to be predominantly 40XY.

Colony assays were used to evaluate the phenotype of Nanog transfectants and their Cre-treated derivatives. From 25%–50% of colonies produced by parental ES cells in the presence of LIF can be classified as pure stem cell colonies, consisting solely of alkaline phosphatase positive undifferentiated cells, by the end of this 6 day assay. In the absence of LIF, parental ES cells form only differentiated cell colonies. Nanog transfectants, however, generated appreciable numbers (30%) of pure stem cell colonies even in the presence of hLIF-05, which blocks juxtacrine LIF activity. Cre-treated cells reverted fully to LIF dependence, demonstrating that this phenotype is directly attributable to Nanog overexpression. Similar observations were made for multiple different clones (Figure 5C).

In the presence of LIF, differentiation of Nanog-expressing cells was further reduced and the proportion of pure stem cell colonies rose to >60% (Figure 5C). This was accompanied by a change in the colony growth characteristics of the cells (Figure 5D). Without LIF, the Nanog expressers formed flat colonies of adjoining small cells with a scant cytoplasm that were indistinguishable from parental or Cre-reverted ES cell colonies in the presence of LIF. Addition of LIF caused the Nanog colonies to adopt a more tightly packed morphology in which the cells preferentially adhered to one another rather than growing out as an even monolayer over the substratum. These compact colonies resembled ES cells cultured on feeder layers or on uncoated plastic.

We then challenged Nanog expressers with agents that promote differentiation. Following exposure to 3-methoxybenzamide (MBA) or all-trans retinoic acid (RA), most Nanog expressing cells remained morphologically undifferentiated, whereas cultures of both Cre revertants and parental cells underwent widespread differentiation within 4 days. This was reflected at the molecular level in continued expression of Oct4 by Nanog expressers compared with downregulation in Cre derivatives (Figure 6A). Therefore, Nanog transfectants are resistant, though not completely refractory, to chemical induction of differentiation in monolayer cultures. Normal differentiation responsiveness was restored following transgene excision.

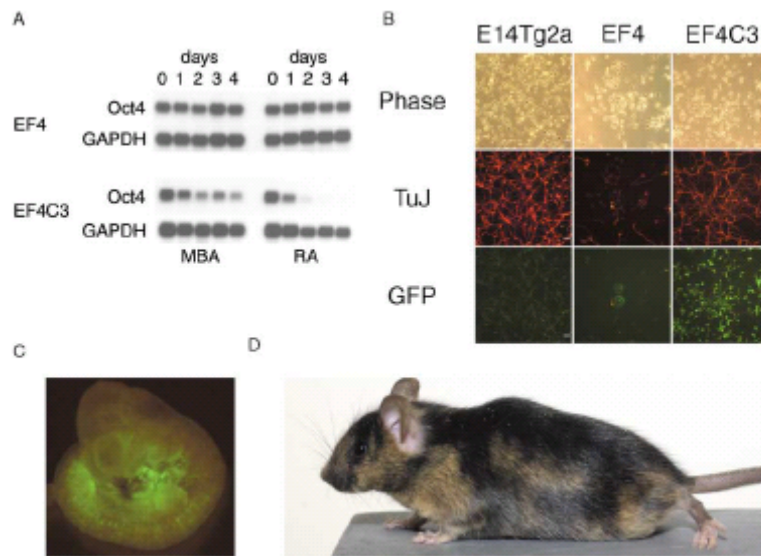


Figure 6. Nanog Transfectants Retain ES Cell Identity(A) Oct4 Northern analysis of RNA from cultures of E14Tg2a derivatives expressing the floxed transgene (EF4) or a Cre-excised subclone (EF4C3) prepared at 0, 1, 2, 3, or 4 days following exposure to RA or MBA.(B) Nanog suppresses neuronal differentiation. E14Tg2a, transfectant EF4 expressing the floxed Nanog transgene, and Cre-excised subclone EF4C3 were assessed by TuJ immunohistochemistry 2 days after plating retinoic acid treated aggregates.(C) Contribution of Cre-deleted cells to mid-gestation embryo. Fetus generated from an MF1 blastocyst injected with EF1C1 cells and examined at E9.5 for green fluorescence.(D) Adult chimera generated by injection of EF4C3 cells into C57BL/6 blastocyst.

We examined the capacity for neuroectodermal differentiation. Cells were aggregated and exposed to retinoic acid, then plated on laminin-coated plastic (Li et al., 1998). E14Tg2a parental cells gave rise to abundant neuronal cells that expressed neuron-specific type III β -tubulin (TuJ). In Nanog-expressing EF4 cells, the incidence of TuJ-positive cells was greatly reduced, but neuronal differentiation was fully restored in Cre revertants (Figure 6B). Similar results were observed in three separate trials.

These observations indicate that differentiation of ES cells expressing Nanog is significantly suppressed. Crucially, the Cre derivative cell lines appear unaffected by the period of clonal expansion driven by enhanced Nanog expression, since under all conditions examined, they displayed comparable differentiation efficiency to parental ES cells.

Finally, we investigated whether forced expression of Nanog bypasses the requirement for LIF-R/gp130-mediated signaling in maintenance of embryo colonization properties. EF1C and EF4C Cre-reverted Nanog transfectants were injected into mouse blastocysts. Examination at mid-gestation demonstrated widespread contribution of ES cell derivatives to tissues of the developing fetuses as revealed by GFP expression (Figure 6C). Injected embryos were also brought to term and resulted in the production of live chimeras from both clones (Table 1 and Figure 6D).

Table 1. Live-Born Chimeras Obtained from Cre Derivatives of Nanog Transfectants

Line	Mice born	Chimeras
EF1C1	7	3
EF1C3	21	10
EF1C5	11	11
EF4C3	3	3

Table 1. Live-Born Chimeras Obtained from Cre Derivatives of Nanog Transfectants

These results establish: (1) that cells expressing the Nanog transgene self-renew in a cytokine-independent manner, (2) that LIF can combine with the Nanog transgene to confer enhanced self-renewal capacity, and (3) that the effects of Nanog transgene expression are fully reversed following transgene excision.

Relationship of Nanog with Other ES Cell Regulators

ES cells normally express Nanog, yet this is not sufficient to sustain self-renewal upon LIF withdrawal, suggesting that the dosage of Nanog may be critical. We transfected LRK1 cells with episomal constructs directing differing levels of cDNA expression (Figure 7A). The proportion of self-renewing colonies in the absence of cytokine correlated with the relative expected level of expression per cell based on similar studies measuring GFP production (data not shown). Furthermore, as observed for cells carrying integrated Nanog transgenes, the addition of LIF to cultures of cells carrying Nanog episomes augmented self-renewal assessed in terms of both the number and morphology of self-renewing colonies. Similar results were obtained by transfection of the same DNAs into the CCE-derived polyoma large T-expressing cell line MG1.19 (Gassmann et al., 1995).

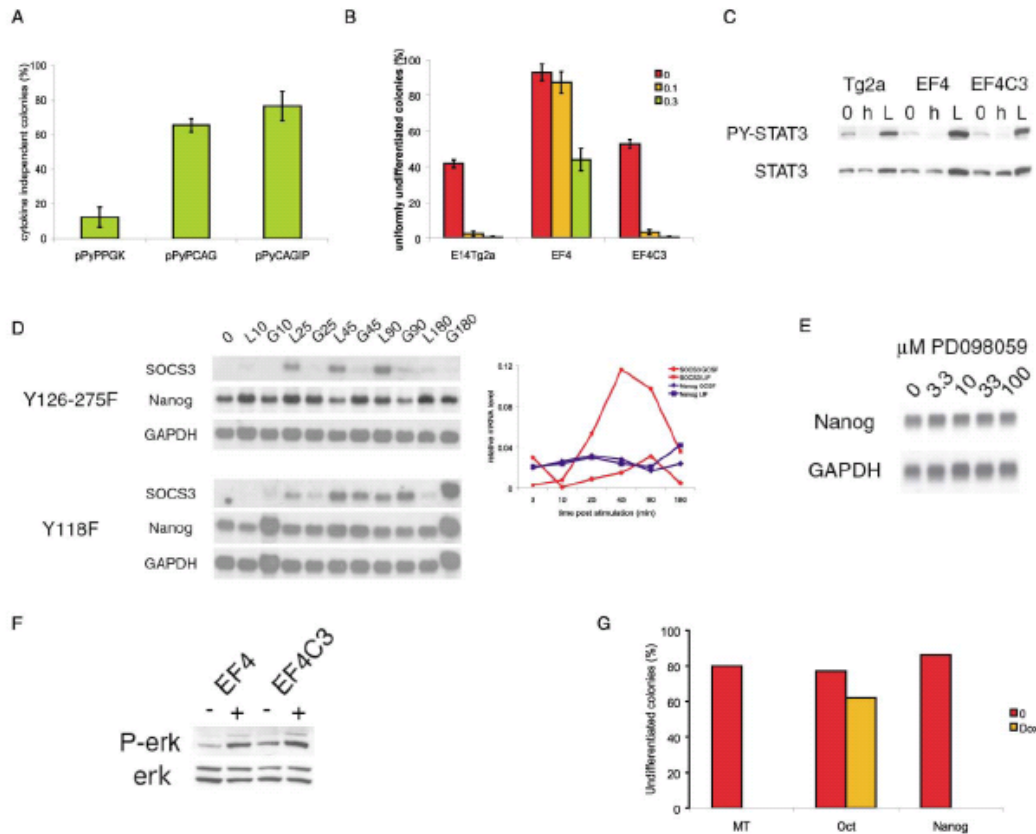


Figure 7. Relationship of Nanog to Other Known Mediators of Self-Renewal(A) Generation of stem cell colonies correlates with level of Nanog expression. LRK1 cells were transfected with episomal vectors expected to direct increasing levels of expression of Nanog (pPyPPGK < pPyPCAG < pPyCAGIP). Following selection, the proportion of stem cell colonies in the absence of cytokine is expressed relative to the number in the presence of IL6/sIL6R. Data are the averages and standard deviations of three determinations.(B) Nanog sustains self-renewal in the presence of Jak inhibitor. Parental ES cells, transfectants expressing the nanog transgene, and Cre-excised derivatives were plated at clonal density in medium containing 100 U/ml LIF with D6665 at 0, 0.1, or 0.3 μ M. After 6 days culture, the percentages of pure alkaline phosphatase-positive colonies were quantitated.(C) Nanog does not activate Stat3. Parental ES cells, transfectants expressing the nanog transgene, and Cre-excised derivatives were plated at 105 cells/cm2 and incubated overnight in medium containing no addition (0), hLIF-05 (h), or 100 U/ml LIF (L). Cells were then lysed and analyzed by sequential Western blotting for P-Tyr (705) Stat3 and total Stat3.(D) Nanog is not a Stat3 target. ES cells expressing chimeric GCSFR-gp130 variant molecules in which the Stat3 binding sites are mutated (Y126-275F) or in which the negative regulatory tyrosine is mutated (Y118F) were stimulated with LIF (L) or GCSF (G; 30 ng/ml) for the indicated time (min). Northern analysis was performed by sequential hybridization with probes against Nanog, GAPDH, and SOCS3. Histogram shows phosphoimager quantitation of the data for Y126-275F cells, with signals normalized to GAPDH.(E) Nanog expression is not upregulated by MEK inhibitor. OKO160 ES cells carrying oct4 β -geo (Mountford et al., 1994) were cultured for 4 days in LIF, G418, and the indicated concentration of PD98059. RNA was analyzed by sequential Northern analysis with probes for nanog and GAPDH.(F) Nanog does not suppress Erk activation. ES cells expressing the nanog transgene and Cre-excised derivatives were plated at 105 cells/cm2 and incubated overnight in LIF prior to cytokine withdrawal for 6 hr and stimulation with LIF (+) or mock (-). Cell lysates prepared 10 min post stimulation were analyzed by sequential Western blotting for P-erk and erk.(G) Nanog action requires maintenance of Oct4 expression. ZHBTc4.1 ES cells containing a doxycycline-responsive oct4 transgene and with both alleles of endogenous oct4 deleted were transfected with pPyCAGIP derivatives carrying no insert (MT), Oct4, or Nanog. Cells were cultured in the presence of LIF and absence (0) or presence (Dox) of doxycycline, which represses the Oct4 transgene. Following puromycin selection, the percentage of undifferentiated colonies was determined.

We investigated whether Nanog might act through the Jak/Stat pathway. First, we used the specific inhibitor D6665. Treatment with concentrations (0.1–0.3 μ M) previously shown to block Jak activity and Stat phosphorylation (Thompson et al., 2002) caused complete differentiation of parental ES cells in the presence of LIF. In contrast, Nanog expressers formed undifferentiated colonies though the additive effect of LIF was reduced or abolished (Figure 7B). We then examined activation of Stat3 directly. Steady-state levels of active Stat3 in parental ES cells, Nanog expressers, and Cre-reverted cells were determined by immunoblotting. Stat3 activation in the presence of LIF is not substantially increased in Nanog expressers (Figure 7C). More

significantly, on withdrawal of LIF, the low basal level of active Stat3 is not enhanced. Furthermore, addition of hLIF-05 to block autocrine LIF eliminated tyrosine phosphorylated Stat3 in parental and reverted ES cells and equally effectively in Nanog expressers. Thus, active Stat3 is undetectable under conditions in which Nanog-expressing ES cells can be clonally propagated.

The combined effect of LIF and Nanog suggests that Nanog is not simply a downstream effector of gp130. We investigated directly whether Nanog is a transcriptional target of LIF/gp130 signaling, taking advantage of chimeric receptors in which Stat3 activation is either abolished or enhanced (Burdon et al., 1999a). SOCS3 provides a control Stat3 target gene that is induced in ES cells by LIF. SOCS3 induction is eliminated when the four Stat binding sites in a chimeric receptor (Y126-275F) are mutated (Figure 7D). Hyperactivation of Stat3 via stimulation of a chimeric receptor (Y118F) lacking the negative regulatory tyrosine (Burdon et al., 1999b) results in enhanced and sustained induction of SOCS3. In contrast to SOCS3, no change in Nanog expression is evident upon LIF stimulation of native receptor. Most persuasively, Nanog expression is unaltered upon stimulation of the hypersensitized chimeric receptor (Figure 7D). These data argue that Nanog expression is not directly regulated by gp130 stimulation.

LIF-dependent ES cell self-renewal is enhanced by inhibition of the Erk mitogen activated kinase pathway (Burdon et al., 1999b). The Y118F chimeric receptor is incapable of engaging the adaptor protein SHP2 and thence coupling to the Ras-Erk pathway (Takahashi-Tezuka et al., 1998). The absence of any change in Nanog expression upon stimulation of this receptor suggests that Erk signaling does not repress Nanog expression. To confirm this point, ES cells were cultured in the presence of the MEK inhibitor PD98059 (Dudley et al., 1995). To ensure comparability with untreated cultures, differentiating cells were eliminated from the analysis by selection for Oct4 expression (Mountford et al., 1994). Nanog expression was then evaluated (Figure 7E). Steady-state Nanog mRNA levels are unaltered in the presence of MEK inhibitor, indicating that Erk1/2 signaling does not repress Nanog expression.

We examined whether Nanog overexpression influences Erk activation. Immunoblotting for phospho-Erk shows that basal levels of activation of Erk1 and Erk2 are indistinguishable between Nanog expressing ES cells and their Cre revertants, and that Erks remain activatable in response to LIF stimulation in Nanog expressers (Figure 7F).

We explored the relationship between Nanog and Oct4. Blastocysts from intercross matings of oct4 heterozygous mice were analyzed for nanog mRNA by in situ hybridization. We examined a total of 26 embryos, one in four of which are predicted to be homozygous. All expressed nanog in the ICM as in Figure 4A. Oct4 protein is undetectable in homozygous embryos from the 8-cell stage (Nichols et al., 1998). This result therefore indicates that nanog transcription is induced in the complete absence of Oct4.

The preceding observation also suggests that Nanog is not sufficient to sustain pluripotency if Oct4 is deleted. We therefore tested the requirement for continued presence of Oct4 for function of Nanog in ES cells. ZHBTc4.1 ES cells carry two null

oct4 alleles and are sustained by a doxycycline-responsive Oct4 transgene (Niwa et al., 2000). When the Oct4 transgene is repressed by administration of doxycycline, the cells differentiate. ZHBTc4.1 cells were transfected with linearized pPyCAGIP sequences carrying no insert, Oct4, or Nanog. As previously documented, the Oct4 plasmid prevented the differentiation caused by doxycycline repression (Niwa et al., 2002). In contrast, the Nanog expression vector could not complement elimination of Oct4 and cells transfected with this plasmid differentiated in doxycycline (Figure 7G). Therefore, continued expression of Oct4 is essential for Nanog-mediated self-renewal.

We conclude that Nanog can bypass completely the requirement for Stat3 activation and that maintained expression of Oct4 is integral to its ability to sustain self-renewal.

Discussion

A Variant Homeodomain Protein Mediates ES Cell Self-Renewal

Through functional screening of an ES cell cDNA library, we identified a previously unrecognized player in the stem cell self-renewal theater. Nanog is a homeodomain protein whose enhanced expression confers constitutive self-renewal on ES cells. nanog expression vectors support cytokine-independent ES cell propagation both upon episomal transfection of polyoma T-containing stem cells and following chromosomal integration. These results were obtained with several ES cell clones originating from three independent ES cell derivations, CCE, CGR8, and E14Tg2a, and are therefore likely to be representative for ES cells generally.

The effect of Nanog is highly specific and is not reproduced by a panel of candidate cDNAs introduced into ES cells, including Oct4 and two transcription factors, Sox2 and FoxD3, identified as Oct4 partners and implicated in the pluripotency of epiblast by *in vivo* deletion (Hanna et al. 2002 and Avilion et al. 2003).

Forced expression of Nanog not only liberates ES cells from the requirement for gp130 stimulation but also reduces differentiation in response to retinoic acid, 3-methoxybenzamide, and, to a lesser extent, aggregation. Cre-mediated excision of a floxed nanog transgene restores cytokine dependence and multilineage differentiation capacity of clonally expanded nanog transfectants. This phenotype reversal, observed with multiple independent clones, establishes a causal relationship with Nanog expression. Introduction of the Cre-reverted cells into mouse blastocysts results in embryo colonization, contribution to fetal tissues, and the generation of healthy chimeric mice. We conclude that elevated expression of nanog is sufficient to sustain the cardinal attributes of ES cell identity.

Expression and Function of Nanog

nanog mRNA is found in pluripotent ES and EG cells and in both mouse and human EC cells. Expression is downregulated early during ES cell differentiation, consistent with an intimate association with pluripotent stem cell identity. A recently reported homeodomain gene selectively expressed in ES cells is identical to Nanog (Wang et al., 2003). nanog is also represented in an ES cell gene set described by microarray analysis (Ramalho-Santos et al., 2002). Indeed, Nanog is readily detectable in ES cell RNA by hybridization to the Affymetrix mouse U74Av2 genechip (T. Era, personal

communication; our unpublished data), but has not been reported in any other cell samples.

Low expression of *nanog* in P19 cells, which are derived from and postulated to resemble cells of the egg cylinder rather than the ICM, is consistent with the dynamic profile of *nanog* mRNA in the embryo (Figure 4). *Nanog* transcripts appear as a temporal wave with maximal levels between the late morula and the mid blastocyst and downregulation just prior to implantation. The restricted expression of *Nanog* coincides with the transient potential for ES cell generation, which arises in the ICM and is lost around the time of implantation. Epiblast expression of *Nanog* persists in implantation-delayed blastocysts (data not shown), a favored source for ES cell derivation (Brook and Gardner, 1997). Additionally, *nanog* mRNA is present in primordial germ cells in E11.5 gonadal ridges, the very cells that can be reprogrammed to generate EG stem cells (Resnick et al., 1992). Thus, *nanog* is expressed in cells from which it is possible to establish pluripotent stem cell lines. Expression of *Oct4* overlaps with that of *nanog* but also includes oocytes, early cleavage embryos and egg cylinders (Pesce and Schöler, 2001); that is, both before and after the window for stem cell derivation (Smith, 2001).

Cytokine stimulation of LIF-R/gp130 and consequent activation of Stat3 is the only previously characterized pathway for sustaining self-renewal of mouse ES cells. The isolation of *Nanog* using LIF-R deficient ES cells and clonal expansion of parental ES cells in the presence of the LIF receptor blocking agent hLIF-05 demonstrates that *Nanog* bypasses LIF-R/gp130 stimulation. Furthermore, *Nanog* supports stem cell colony formation in the presence of the Jak inhibitor D6665, which abolishes cytokine-mediated activation of Stats. The possibility that *Nanog* might itself regulate Stat3 is ruled out by the finding that Stat3 activation is unaltered and can be completely abolished in *Nanog* transfectants. We conclude that *Nanog* can function independently of Stat3.

However, in the absence of gp130 stimulation, endogenous *Nanog* expression is insufficient to sustain self-renewal. As *nanog* appears not to be a transcriptional target of gp130, this implies that ES cells are normally propagated by collaboration between cytokine-activated Stat3 and an otherwise limiting amount of *Nanog*. Consistent with this interpretation, dosage of *Nanog* is the critical determinant of cytokine-independent colony formation (Figure 7). Although increased *Nanog* renders LIF-R/gp130 signaling dispensable, maximal self-renewal is obtained by combination of *Nanog* overexpression and LIF stimulation. Under these conditions, peripheral differentiation is eliminated almost entirely and ES cell colonies adopt a highly compacted morphology, suggestive of a subtle change in cell adhesion properties (Figure 5D). Thus, *Nanog* can act combinatorially with the gp130 signal.

The Transcription Factor Network for ES Cell Identity

The presence of a homeodomain indicates that *Nanog* is highly likely to act as a transcriptional regulator. How then does *Nanog* fit into the transcription factor hierarchy that dictates ES cell phenotype (Niwa 2001 and Smith 2001)?

The dosage effect indicated for *Nanog* is also a feature of *Oct-4* (Niwa et al., 2000) and Stat3 (Niwa et al., 1998) activity in ES cells. This suggests that the stoichiometry

of these factors is critical to provision of a robust framework for self-renewal. The analysis in ZHBTc4 cells establishes that Nanog action requires continued presence of Oct4, implying that Nanog can determine Oct4 expression. This effect is likely to be indirect, however, since Oct4 expression is not noticeably increased in Nanog overexpressers. Oct4 appears to be expressed constitutively in ES cells and only downregulated upon commitment to differentiate (Fuhrmann et al., 2001). Likewise, Oct4 seems not to be essential for Nanog expression. Thus, these two factors act in concert rather than in series (Figure 8). However, increased expression of Oct4 causes ES cells to differentiate (Niwa et al., 2000) whereas increased Nanog prevents differentiation. Therefore, Nanog may act to restrict the differentiation-inducing potential of Oct4. A key question is whether Nanog interacts directly with Oct4 or acts in parallel to determine expression/repression of key genes.

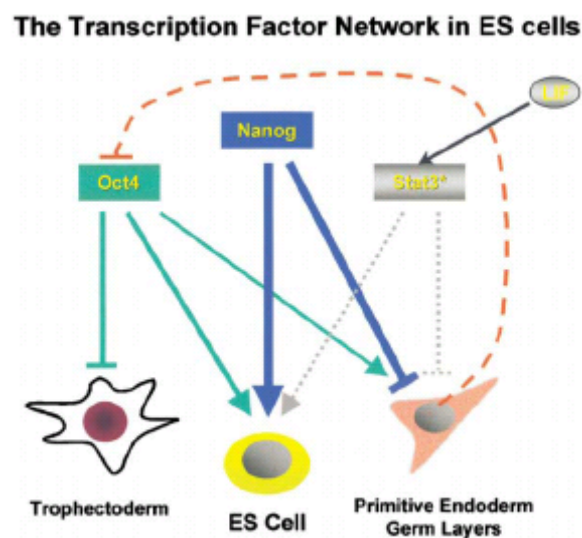


Figure 8. Model of the Transcription Factor Hierarchy in Pluripotent Cells Both Nanog and Oct4 are essential to sustain ES cell identity, whereas Stat3 has an accessory function. Oct4 serves a specific role in blocking differentiation into trophoblast but tends to promote differentiation into primitive endoderm and germ layers. Nanog (and activated Stat3) may block this differentiation effect of Oct4. Suppression of differentiation by Nanog also maintains Oct4 transcription, which is not downregulated unless the cells commit to differentiate (dashed red line).

The activity of Nanog may explain the apparent paradox that ES cells rely on cytokine stimulation while development of the ICM/epiblast proceeds normally in the absence of gp130 or Stat3 (Yoshida et al. 1996 and Takeda et al. 1997). In an accompanying manuscript in this issue of Cell, Mitsui et al. (2003) show that Nanog is required in the epiblast, indicating that it fulfils a similar role in vivo as in ES cells. The downregulation of Nanog mRNA expression at implantation (Figure 4A) may therefore be critical to restrict expansion of the epiblast and initiate egg cylinder formation. An obligate requirement for Nanog in the early embryo compared with the dispensability of Stat3 is further evidence that Nanog expression is not directed by, nor its function dependent on, gp130/Stat3. However, the data do not rule out the possibility of crosstalk between these two factors, and indeed this seems likely given their collaborative effect on ES cell self-renewal.

Currently, efforts are underway to identify a core program of so-called "stemness" genes (Ramalho-Santos et al., 2002). It is striking, however, that the functional identity of mouse ES cells depends on two master transcription factors, Oct4 and

Nanog, that are not detected in hematopoietic or neural stem cells (Ivanova et al. 2002 and Ramalho-Santos et al. 2002), nor in trophoblast stem cells (our unpublished data). It will be of interest to determine whether Nanog is expressed in multipotent adult progenitor cells (Jiang et al., 2002) and may therefore be generally associated with pluripotency, or whether it is truly unique to the ES cell phenotype. More broadly, the regulation and interactions of Nanog and the nature of its target genes now emerge as central issues in pluripotent stem cell biology.

Experimental Procedures

ES Cell Culture

ES cell lines used and their culture and differentiation are described elsewhere (Smith 1991 and Li et al. 1998). To prepare LRK1 cells, E14/T cells (Aubert et al., 2002) were subjected to two rounds of gene targeting aimed at the *lifr* locus (Li et al., 1995). The selectable marker cassette was IRESBSDrpA and IRES ϕ hpA for the first and second rounds of targeting, respectively. LRK1 cells were maintained in the presence of IL6 and sIL6R (Yoshida et al., 1994).

cDNA Library Construction and Characterization

We prepared total RNA from ES cells cultured on γ -irradiated mouse embryonic fibroblasts. Polyadenylated RNA (Oligotex, Qiagen) was evaluated by examination for 11 kb LIFR transcripts (Chambers et al., 1997). cDNA was synthesized by oligo d(T) priming (Superscript II, Life Technologies). cDNA > 1 kb, recovered by electrophoretic fractionation and electroelution, was ligated into XhoI/NotI-digested pPyCAGIP to produce a library of 7.4×10^5 primary recombinants. Of 48 recombinants examined, 40 (83%) contained inserts, ranging in size from 0.5–3.0 kb (average 1.5 kb). cDNAs for 16/27 well-characterized genes extended 5' to the known initiator methionine codon.

cDNA Library Transfection and Screening

We introduced library (25 μ g) or empty vector into LRK1 cells (6×10^6) by electroporation and seeded cells at 10^6 or 5×10^4 per 9 cm dish and cultured in IL6/sIL6R. After 2 days, puromycin was added and cytokine was withdrawn from the high-density plates. Cytokine was maintained on the low-density plates to monitor transfection efficiency. (We estimate that 7,200 clones were screened. We subsequently screened a second independent library, and of an estimated 66,000 clones, the only active cDNA isolated encoded Nanog.) Medium was renewed every 2 days until 9 days posttransfection. Mock transfected plates and plates transfected with empty vector contained no cells and only differentiated cells, respectively. In contrast, colonies with undifferentiated cells were present on some of the library transfection plates. Extrachromosomal DNA was prepared from these cells and transferred to *E. coli*. DNA prepared from pools of 104 bacterial colonies was reintroduced into LRK1 cells and the selection process repeated. Of 10 pools screened, 2 yielded undifferentiated colonies, and extrachromosomal DNA was prepared from these 14 days posttransfection. This cycle was repeated once more. Plasmid DNA was then prepared from individual colonies and retransfected into LRK1 cells. Active cDNA was sequenced and submitted to GenBank (accession number AY278951).

Preparation of Episomal Constructs

The Nanog ORF was amplified by PCR, cloned into pCR2.1, and verified mutation-free by sequence analysis. We then subcloned Nanog ORF into three episomal vectors that direct differing levels of expression as judged by analysis of populations of ES cells maintaining egfp derivatives (I.C., unpublished data). pPyCAGIP gives the highest expression. pPyPCAG, in which the IRESpac cassette has been deleted and a PGKpacpA gene placed upstream of the CAG cassette in the same transcriptional orientation, directs an intermediate level of expression. pPyPPGK is derived from pPyPCAG by replacement of the CAG cassette with the PGK-1 minimal promoter and gives the lowest expression. hNanog ORF was amplified by PCR from IMAGE clone 664153 and Nkx2.5 ORF from IMAGE clone 1315616. Both PCR products were cloned into pCR2.1, verified mutation-free and subcloned into pPyCAGIP. Episomal DNA was transfected either by electroporation or using Lipofectamine 2000. Selective medium was renewed every 2–3 days until 12 days posttransfection when colonies were fixed and stained for alkaline phosphatase.

Colony Forming Assays

ES cells were trypsinized to obtain a single cell suspension and 600 cells plated per 10 cm² well. After 6 days, culture plates were stained for alkaline phosphatase and colonies scored in three categories: pure stem cell, mixed, and fully differentiated. hLIF-05 was prepared in COS cells and used at a concentration that blocked 10 U/ml LIF.

RNA Analysis

Unless otherwise stated, we carried out Northern hybridizations on 10 µg of total RNA. We used a digoxigenin-labeled ORF probe for in situ hybridization on cells and embryos.

Preparation of Cells Expressing a loxP Flanked Nanog Transgene

We prepared a cassette in which a synthetic polyadenylation sequence and a MAZ site from the C2 gene (Ashfield et al., 1994) precede a loxP site upstream of egfpA. This was introduced into the pPyCAG-Nanog-IP vector, replacing the pA sequence 3' to the pac gene. We inserted a loxP site within the rabbit β-globin exon of the CAG cassette to create the floxed Nanog vector. ES cells were transfected with linearized plasmid and selected in puromycin at 1 µg/ml. Resistant colonies were picked and expanded. We then plated cells at 100 cells/cm² in nonselective medium supplemented with hLIF-05 to block endogenous LIF. After 4 days, cells were trypsinized to a single cell suspension and replated at 100 cells/cm². After a further 4–5 days, colonies of transfected ES cells had expanded, whereas similarly treated parental cells generated only differentiated cells. LIF was then added, cells expanded, and stocks frozen. For Cre excision, cells were transiently transfected using Lipofectamine 2000. Approximately 10 days later, colonies were examined by fluorescent microscopy for GFP and expressing clones expanded. Complete excision of the Nanog cassette was confirmed by puromycin sensitivity.

Immunoblotting

Steady state levels of activated Stat3 were measured by plating 106 cells onto a 10 cm² dish in medium alone or supplemented with LIF (100 U/ml) or hLIF-05. Approximately 20 hr later, cells were lysed in 0.2 ml SDS sample buffer and 10 µl aliquots analyzed for tyrosine phosphorylated Stat3 (NEB antibody 9131) and total Stat3 (Transduction labs antibody S21320). Erk1/2 activation was determined as described (Burdon et al., 1999b).

Acknowledgements

We thank Shinya Yamanaka and colleagues for sharing data and discussion. Thanks to Azim Surani and Susan Hunter/Martin Evans for EG and PSA4 cells, to Max Gassmann/Greg Donoho/Paul Berg for MG1.19 cells and pMGDneo, Anne Vernallis for the hLIF-05 construct, and Dario Alessi for D6665. Frances Stenhouse, Jamie Marland, and Derek Rout provided excellent technical support, and Val Wilson, assistance with imaging. Finally, we thank Helen Wallace, Joe O'Donoghue, William Gillies, Shinya Yamanaka, and Hung Li for discussions leading to the use of the Tirnan Og legend in naming this gene. Our research is supported by the Biotechnology and Biological Sciences Research Council and the Medical Research Council of the UK.

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