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BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3

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Summary

The cytokine leukemia inhibitory factor (LIF) drives self-renewal of mouse embryonic stem (ES) cells by activating the transcription factor STAT3. In serum-free cultures, however, LIF is insufficient to block neural differentiation and maintain pluripotency. Here, we report that bone morphogenetic proteins (BMPs) act in combination with LIF to sustain self-renewal and preserve multilineage differentiation, chimera colonization, and germline transmission properties. ES cells can be propagated from single cells and derived de novo without serum or feeders using LIF plus BMP. The critical contribution of BMP is to induce expression of Id genes via the Smad pathway. Forced expression of Id liberates ES cells from BMP or serum dependence and allows self-renewal in LIF alone. Upon LIF withdrawal, Id-expressing ES cells differentiate but do not give rise to neural lineages. We conclude that blockade of lineage-specific transcription factors by Id proteins enables the self-renewal response to LIF/STAT3.

Introduction

Stem cells may be defined as cells that must choose between alternative fates of self-renewal and differentiation at each division. The decisive instructive and permissive signals that govern this choice are provided by growth factors in the microenvironment or "stem cell niche" (Schofield, 1978). Identifying these growth factors and defining their respective inputs are critical to understanding the developmental and physiological regulation of stem cell-mediated tissue generation, turnover, and repair. Furthermore, extending such knowledge to control the expansion and differentiation of stem cells ex vivo holds promise for applications in regenerative medicine and biopharmaceutical discovery.

Embryonic stem (ES) cells provide the paradigm for a mammalian stem cell with broad differentiation capacity that undergoes symmetrical self-renewal in culture (Smith, 2001a), apparently without limit (Suda et al., 1987). ES cells are derived from pre-implantation embryos and retain the developmental potency of fetal founder cells, being able to generate cell and tissue types of all three germ layers in vitro and in vivo (Beddington and Robertson, 1989; Bradley et al., 1984). Mouse ES cells were originally isolated and maintained by coculture on a feeder layer of mitotically inactivated mouse embryo fibroblasts (Evans and Kaufman, 1981; Martin, 1981). The essential function of the fibroblast feeder layer is provision of the cytokine leukemia inhibitory factor (LIF). LIF null fibroblasts are deficient at supporting self-renewal (Stewart et al., 1992) and LIF can replace the requirement for feeders in both routine propagation (Smith et al., 1988; Williams et al., 1988) and de novo derivation (Nichols et al., 1990) of mouse ES cells.

LIF and related cytokines that engage the gp130 receptor provide the only molecularly defined pathway that will sustain long-term self-renewal of mouse ES cells with retention of the cardinal attributes of undifferentiated phenotype, pluripotency, and embryo colonization capacity (Smith, 2001b). LIF directs ES cell self-renewal through activation of the latent transcription factor STAT3 (Matsuda et al., 1999; Niwa et al., 1998). Paradoxically gp130 cytokines also stimulate the prodifferentiative Ras-erk pathway (Burdon et al., 1999b). A steroid hormone activatable STAT3-ER fusion has been employed to bypass gp130 stimulation and support ES cell expansion (Matsuda et al., 1999). A conclusion that STAT3 activation is sufficient to effect self-renewal is premature, however, because the presence of fetal calf serum in these studies masks the possible requirement for other inputs. ES cells can be propagated in a commercial serum substitute supplemented with LIF, but this is only effective at moderate to high cell densities and colony formation from single cells requires the presence of either serum or a feeder layer. Furthermore, for human ES cells, even in the presence of serum, LIF is not adequate to support self-renewal (Thomson et al., 1998).

These considerations suggest that growth factors in serum or produced by feeders and/or in an autocrine fashion by ES cells could contribute to ES cell self-renewal. We investigated this possibility by culturing mouse ES cells in the absence of serum. Under these conditions, neural differentiation is only partially inhibited by LIF and self-renewal is attenuated. Suppression of differentiation and concomitant efficient self-renewal requires an additional signal, provided by bone morphogenetic proteins (BMPs), that induces *I*nhibitor of *d*ifferentiation (*Id*) genes.

Results

Bone Morphogenetic Protein Supports ES Cell Self-Renewal in the Absence of Serum

Fetal calf serum is important for viability of undifferentiated ES cells in minimal media (Wiles and Johansson, 1999). However, in enriched basal media containing N2 and B27 supplements, ES cell viability remains high (Ying and Smith, 2003). This allowed us to examine whether LIF is capable of driving continuous cycles of self-renewal in the absence of serum factors.

In N2B27 medium alone, adherent ES cells efficiently convert into *Sox1* positive neural precursors (Ying et al., 2003). LIF reduces but does not eliminate neural

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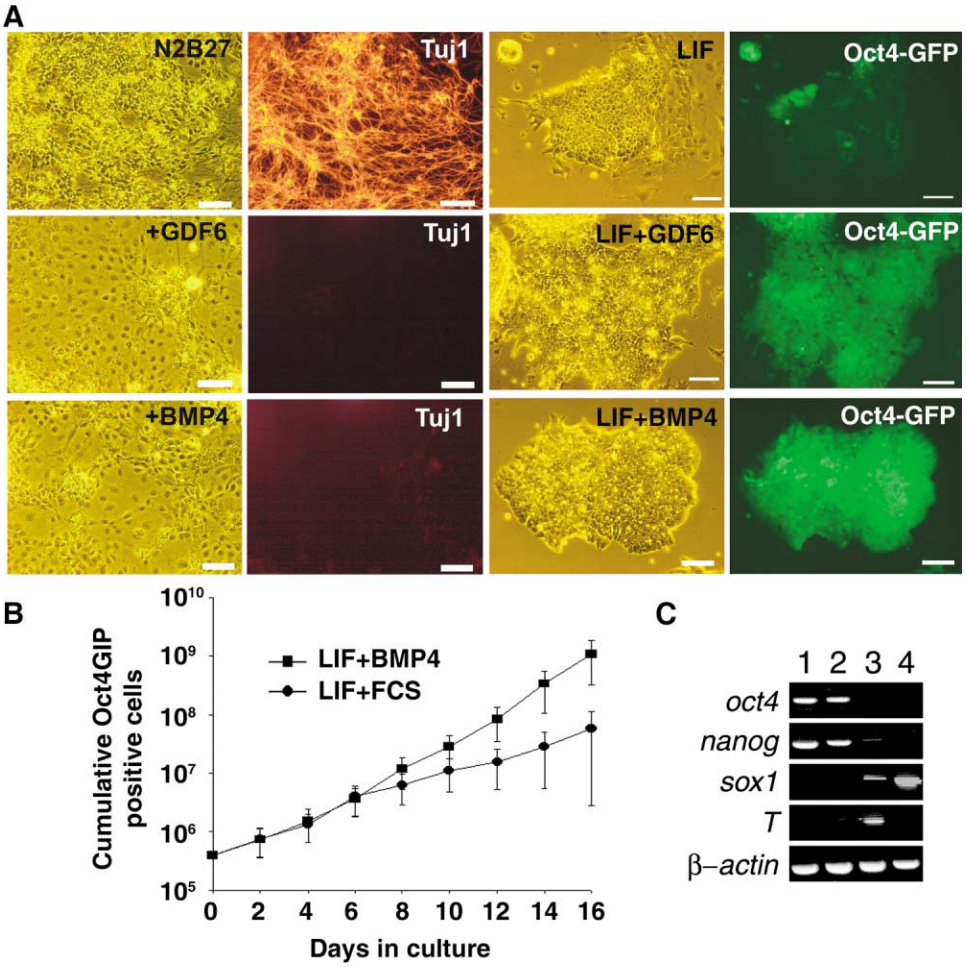

Figure 1. LIF Plus BMP Sustain ES Cell Self-Renewal in Serum-Free Medium

(A) Phase contrast and fluorescent images of *Oct4*-GiP cells cultured in N2B27 with the indicated factors. TuJ1 immunostaining detects neuronal differentiation; green fluorescence reflects activity of the Oct4 promoter in undifferentiated ES cells. Bar: 50 μm.

(B) Plot of cumulative *Oct4*-GFP positive undifferentiated ES cell numbers during progressive passaging in conventional medium with FCS plus LIF or in N2B27 with LIF (10 ng/ml) plus BMP4 (10 ng/ml). Cultures were passaged every 48 hr using cell dissociation buffer and replated at 4×10^5 cells per 10 cm^2 well. The number of GFP-positive cells was determined by FACS analysis at each passage.

(C) RT-PCR analyis of Oct4, Nanog, T (brachyury), and Sox1 mRNAs in (1) ES cells in N2B27 with LIF plus BMP for 6 passages, (2) ES cells cultured in serum with LIF, (3) day 8 embryoid bodies, and (4) day 8 embryoid bodies with retinoic acid treatment.

differentiation under these conditions. Upon successive passaging in N2B27 medium plus LIF, we found that following an initial increase, the number of undifferentiated ES cells reached a plateau and then began to decline after 2–3 passages. This finding was reproduced with several different ES cell lines. Many cells in these cultures had morphology of neural precursors or immature neurons. Neural differentiation was confirmed by activation of the *Sox1*-GFP neural reporter in 46C ES cells (Ying et al., 2003). These observations indicate that additional signaling pathways to LIF/STAT3 are required to promote ES cell self-renewal and in particular to suppress neural determination.

BMPs are well known anti-neural factors in vertebrate embryos (Wilson and Hemmati-Brivanlou, 1995; Wilson and Edlund, 2001) and have been shown to antagonize neural differentiation of ES cells (Tropepe et al., 2001; Ying et al., 2003). BMP alone promotes differentiation of ES cells into non-neural fates (Johansson and Wiles, 1995; Wiles and Johansson, 1999; Ying et al., 2003) and therefore initially appears unlikely as a candidate self-renewal factor. However, we examined whether addition of BMP might contribute to an inhibition of differentation in conjunction with costimulation by LIF. We found that the combination of LIF plus BMP4 (or BMP2) enhanced self-renewal, resulting in highly pure populations of undifferentiated ES cells after 2 or 3 passages in N2B27 (Figure 1A). These cultures could subsequently be expanded for multiple passages with no deterioration in growth rate or viability and no neural differentiation (Figures 1A and 1B). This response was observed in each of 11 different ES cell lines, originating from three independent derivations. The representation of Oct4 positive undifferentiated cells and the population doubling time were slightly higher than obtained in serum plus LIF (Figure 1B). ES cell status was confirmed by expression of SSEA-1 and alkaline phosphatase (not shown), and of mRNAs for ES-cell-specific transcription factors Nanog

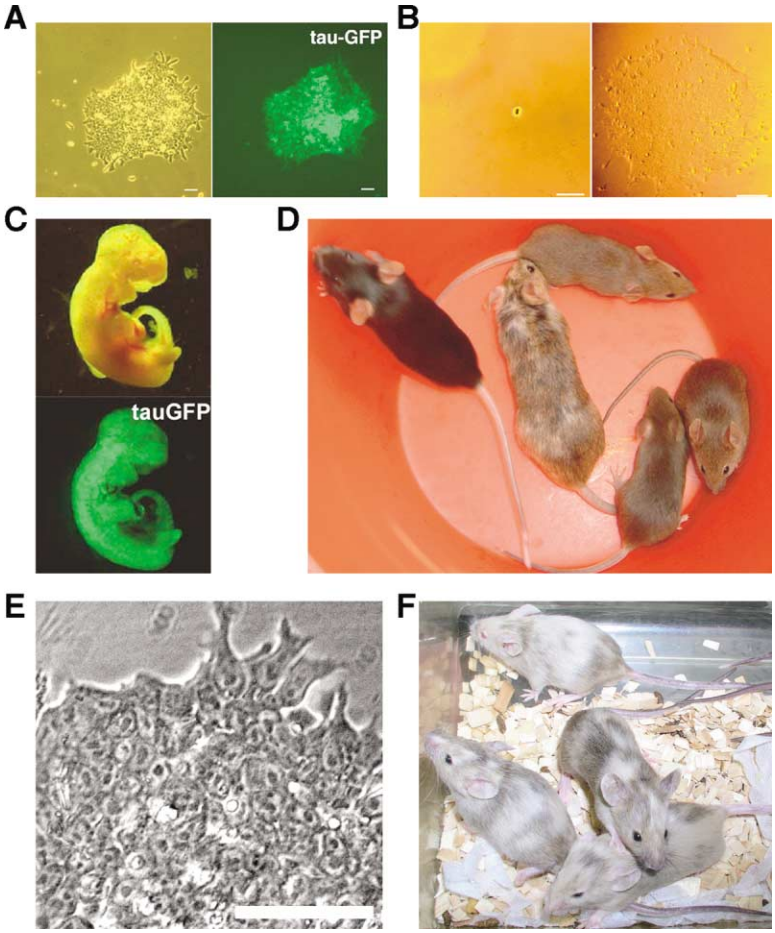

Figure 2. Clonogenicity, Potency, and Derivation of ES Cells in N2B27 with LIF Plus BMP

(A) CAG-*taugfp* transfectant colony isolated by electroporation of E14Tg2a cells and selection in puromycin.

(B) Single CAG-*taugfp* transfectant ES cell and derivative colony.

(C) Mid-gestation fetal chimera produced from TP6.3 ES cells after 6 passages in N2B27 with LIF plus BMP. GFP fluorescence marks ES cell progeny.

(D) Male chimera from CAG-*taugfp* transfected ES cell with C57Bl/6 mate and offspring. Agouti coat color denotes ES cell origin of offspring.

(E) Colony of first passage SF1 ES cells derived in N2B27 with LIF plus BMP.

(F) Chimeras generated from SF1 ES cells

Bar: 50 μm.

and Oct4 with absence of markers of mesoderm (T) and neuroectoderm (Sox1) (Figure 1C).

The N2 and B27 components improve viability but are not essential for self-renewal. In basal medium supplemented only with transferrin, self-renewal and undifferentiated ES cell expansion can be sustained for multiple passages by LIF plus BMP, but not by LIF alone. The requirement for BMP is therefore not induced by a component in B27.

We tested the BMP relative growth and differentiation factor-6 (GDF-6) and found that it similarly supported ES cell self-renewal in the presence of LIF (Figure 1A). This is not a general feature of the TGF-β superfamily, however, but is restricted to BMP receptor ligands. TGF-β1 had no discernible effect on ES cells, while activin increased viability and/or proliferation but did not suppress differentiation.

Clonogenicity and Potency of ES Cells Cultured in LIF Plus BMP

To test the efficiency of ES cell propagation supported by LIF plus BMP, we undertook electroporation and selection of stable transfectants. Colonies stably expressing *tauGFP* were readily isolated (Figure 2A) and could be amplified into bulk cultures demonstrating the feasibility of using this serum-free system in genetic manipulation protocols.

Self-renewal of isolated ES cells was then investigated. Single ES cells were transferred to 96-well plates in N2B27 with addition of LIF only or of LIF plus BMP4 (Figure 2B). A single colony that formed in the presence of LIF alone contained a high proportion of differentiated cells and could not be expanded further. In contrast, undifferentiated colonies formed in 12/192 wells in LIF plus BMP4 and 10 of these were amplified without serum (Table 1).

ES cells cultured in LIF plus BMP maintained a diploid chromosome complement after multiple generations. They also retained differentiation potential. Withdrawal of both LIF and BMP resulted in neural differentiation. Removal of LIF with retention of BMP caused differentiation into sheets of flattened epithelial-like cells. Thus, the self-renewal response to BMP remains dependent on continuous LIF signaling.

The definitive functional attribute of mouse ES cells is their capacity to re-enter embryonic development and contribute to the full repertoire of differentiated tissues in chimeric mice. We injected GFP reporter ES cells into mouse blastocysts after propagation in N2B27 with LIF plus BMP for 3 weeks. Analysis at mid-gestation identified several chimeras with high ES cell contributions to a range of tissues (Figure 2C). As a more rigorous test, we used ES cells transfected with *taugfp* and selected and expanded in LIF plus BMP. Liveborn chimeras were obtained and two male animals transmitted the ES cell genome (Figure 2D).

Table 1. Propagation of Single ES Cells in Serum-Free Medium with LIF plus BMP or with LIF Alone after Id Transfection

	Parental ES cells		Id1 transfectants	
	LIF	LIF+BMP4	LIF	LIF+BMP4
Number of single cells picked	96	192	192	192
Number of colonies formed at day 8	1	12	19	22
Number of colonies expanded	0	10	16	20

Derivation of ES Cells without Feeders or Serum

We investigated whether the response to BMP may be an adaptation of established ES cells to culture or is manifest during the initial stages of ES cell derivation. We plated blastocysts in N2B27 supplemented with BMP plus LIF. After several days, expanded inner cell masses (ICMs) were dissociated and replated in the same culture conditions. In initial trials, ES cell colonies were not obtained following ICM dissociation after 5–6 days in culture, the standard timing for ES cell derivation (Nichols et al., 1990; Robertson, 1987). However, in the absence of serum and presence of BMP, the ICM exhibits reduced growth and more rapid onset of overt differentiation. Therefore, we subsequently dissociated the ICM after only 4 days of blastocyst culture in LIF only and added BMP4 on replating. Under these conditions, primary ES cell colonies did form (Figure 2E). These could be passaged and expanded as morphologically undifferentiated ES cells. One line (SF1) was characterized further. Upon withdrawal of LIF and BMP, SF1 ES cells underwent neural differentiation in vitro. Moreover, SF1 cells produced extensively chimeric mice (Figure 2F). Twelve chimeras were all male, indicative of sex conversion by highly contributing XY ES cells (Bradley et al., 1984).

Undifferentiated ES Cells Express Functional BMP Signaling Machinery

Single cell cloning and the near-complete absence of differentiation in LIF plus BMP cultures suggested that the effect of BMP is likely to be directly on ES cells rather than mediated via differentiated progeny. However, previous studies reporting BMP receptor expression and BMP responsiveness during ES cell differentiation (Adelman et al., 2002; Hollnagel et al., 1999) have not established whether ES cells in the undifferentiated state can actually respond to BMP. To confirm this we used selection for activity of an *Oct4* transgene (Ying et al., 2002) to purify undifferentiated cells for RNA and protein analyses.

BMPs act through heterodimers of type 1 and type II serine/threonine kinase receptors (Shi and Massague, 2003). Undifferentiated ES cells show little or no type I *Bmprlb* mRNA, but express both type I *Bmprla* and type II *BmprII* receptor mRNAs (Figure 3A). BMP4 and GDF6 transcripts are also readily detectable in undifferentiated ES cells. The principal effectors downstream of the BMP receptors are the Smad transcription factors (Attisano and Wrana, 2002; von Bubnoff and Cho, 2001). R-Smads 1, 5, and 8 are recruited to and phosphorylated by the active BMP receptor complex and then combine with Smad4 and translocate to the nucleus. We investigated Smad activation by immunoblotting using antibody specific for the active serine phosphorylated form of Smad1. Increased phosphorylation of Smad1 in undifferentiated ES cells is apparent after BMP4 addition (Figure 3B). BMP stimulation also enhances the basal activation of p38 and, by one hour, of erk mitogen-activated protein kinases (Figure 3B).

These data establish that undifferentiated ES cells possess the signal transduction machinery for responsiveness to BMP stimulation and furthermore that they may have the potential for autocrine stimulation via BMP4 and GDF production.

BMP Supports Self-Renewal through Smad Activation

The self-renewal action of LIF is mediated via the transcription factor STAT3 (Matsuda et al., 1999; Niwa et al., 1998). BMP alone does not activate STAT3 measured by phosphorylation of tyrosine 705 (Figure 3C), nor does it increase STAT3 activation by LIF. Erk activation downstream of gp130 is not required for ES cell self-renewal but appears to be a pro-differentiative signal (Burdon et al., 1999a). Thus,reduced erk activity facilitates ES cell derivation (Buehr and Smith, 2003) and promotes self-renewal (Burdon et al., 1999b). Erk activation in response to LIF was not appreciably inhibited by the presence of BMP, however (Figure 3B). These data indicate that BMP does not modulate gp130 signal transduction in ES cells, implying that a BMP signaling pathway contributes directly to self-renewal.

We introduced the inhibitory Smad family members, Smad6 and Smad7 (Shi and Massague, 2003; von Bubnoff and Cho, 2001) into ES cells to antagonize BMP signaling. Cells were transfected and grown under puromycin selection in the presence of serum and LIF. Smad6 or Smad7 expression vectors yielded fewer and smaller ES cell colonies relative to transfections with empty vector. Furthermore, Smad6 and even more so Smad7 transfectants expanded poorly after passaging. A high level of differentiation was evident in the transfected cell populations. Neural differentiation is normally suppressed by serum in adherent cultures, but was readily apparent after Smad7 transfection (Figure 3D).

In addition to blocking Smad activity, Smad6/7 can also inhibit the TAK/p38 pathway downstream of BMPR (Kimura et al., 2000). To assess the potential contribution of p38 in ES cells, we used the specific inhibitor SB203580 (Cuenda et al., 1995). This reagent had no noticeable effect on the capacity of BMP to support self-renewal (Figure 3E). In LIF only, SB203580 did not alter the balance between self-renewal and neural differentiation, but appeared to enhance overall cell viability, suggesting that in ES cells as in other cell types, p38 is proapoptotic (Kimura et al., 2000). The Smad pathway is therefore the likely transducer of the self-renewal signal.

A mechanism of cooperative transcriptional regula-

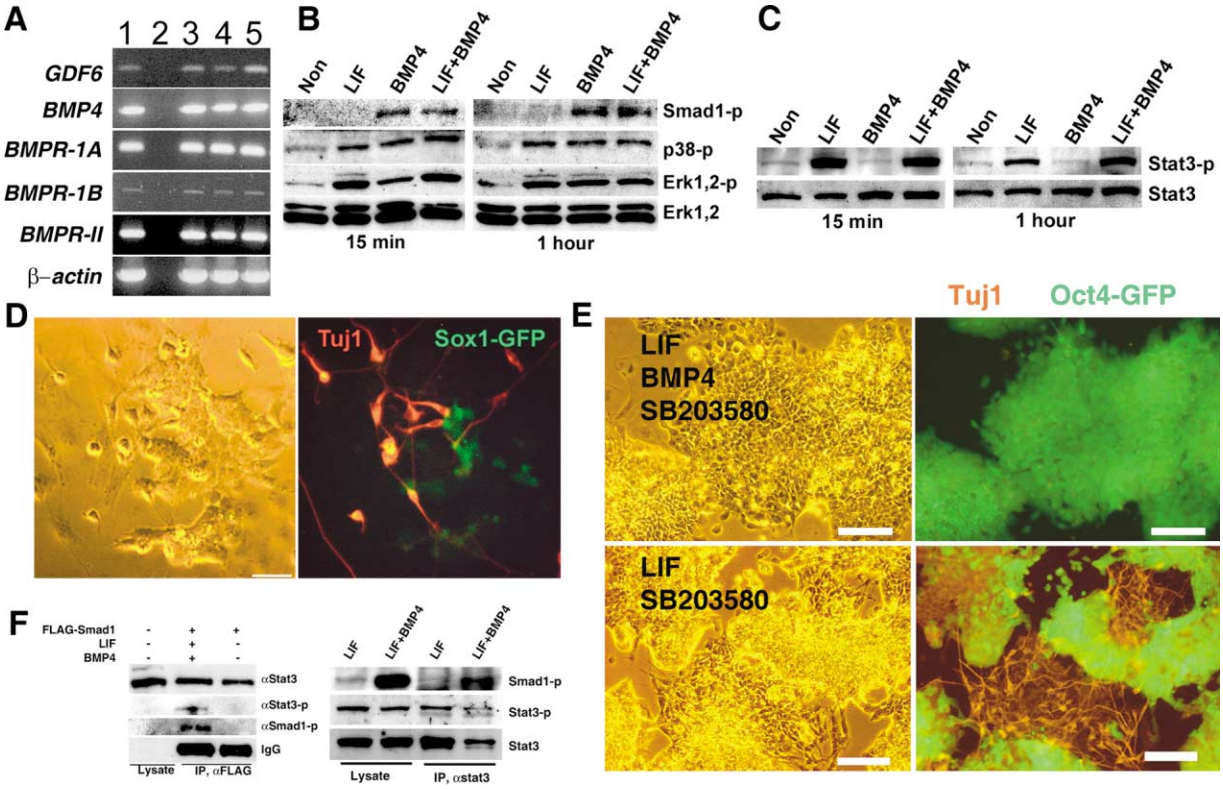

Figure 3. BMP Signaling in ES Cells

(A) Reverse transcription-PCR analysis of RNA samples from Oct-GiP cells (1) in N2B27 with LIF plus BMP, passage 6, (2) in serum plus LIF, no reverse transcriptase control, (3) in serum plus LIF, (4) day 1 after plating in N2B27 without LIF or BMP, and (5) day 5 without LIF or BMP.

(B) Immunoblots showing Smad1, erk and p38 response to mock treatment (non) or stimulation with LIF, BMP, or LIF plus BMP for 15 min or 1 hr after overnight culture in N2B27.

(C) Immunoblot showing STAT3 tyrosine phosphorylation response to LIF, BMP, and LIF plus BMP.

(D) Smad7 episomal transfectants differentiate and express neural precursor (S*ox1*-GFP) and neuronal (TuJ) markers in the presence of serum and LIF.

(E) SB203580 (30 μM) p38 inhibitor does not suppress either self-renewal in LIF plus BMP or neural differentiation in LIF alone. Oct4-GFP marks undifferentiated ES cells and TuJ1 immunostaining identifies neurons.

(F) Coimmunoprecipitation of active Smad1 and STAT3 in ES cells. Left panel: FLAG immunoprecipitates following transfection with FLAG-tagged Smad1. Right panel: STAT3 immunoprecipitates from non-anipulated ES cells. Cells were stimulated as indicated for 1 hr.

Bar: 50 μm.

tion between Smad and STAT3 has been characterized in neuroepithelial cells (Nakashima et al., 1999; Sun et al., 2001). This involves formation of a ternary complex bridged by the ubiquitous transcriptional coactivator p300 and results in synergistic activation of glial-specific promoters. We investigated whether a complex containing STAT3 and Smads may be formed in ES cells stimulated with LIF plus BMP. Immunoprecipation following transfection with FLAG-tagged Smad1 indicated that activated STAT3 and Smad1 may colocalize (Figure 3F). This conclusion was corroborated by coimmunoprecipitation of endogenous phosphorylated Smad1 and STAT3 following LIF plus BMP stimulation (Figure 3F).

BMP Target Genes in ES Cells

To effect ES cell self-renewal, BMP/Smad and LIF/STAT3 signaling could operate in parallel on distinct target genes and/or may converge on common target genes, for example via the ternary complex with p300. We used real-time RT-PCR to survey candidate genes for induction by LIF, BMP, or LIF plus BMP in Oct4-selected ES cells (Figure 4A). Two known LIF targets, *tis11* and *c-fos*, showed no response to BMP. Two others, *junB* and in particular *socs3*, appeared to be more highly induced by LIF in the presence of BMP. These data suggest that a subset of STAT3 target genes may be responsive to costimulation with BMP. However, neither JunB nor Socs3 are candidates for effectors of self-renewal: *junB* null ES cells show no defects (Schorpp-Kistner et al., 1999), and SOCS3 functions as a negative feedback regulator of gp130 signaling (Schmitz et al., 2000) that blocks self-renewal when overexpressed (I.C., unpublished data).

We also examined expression of *Id* genes, which encode negative bHLH factors and have been shown to be induced by BMP/Smad in neuroepithelial cells (Nakashima et al., 2001) and C2C12 myoblasts (Lopez-Rovira et al., 2002). *Id* mRNA induction by BMP has also been reported in differentiating ES cell cultures (Hollnagel et al., 1999). We found that *Id1* and *Id3* are strongly induced by BMP (and GDF, data not shown), but not by LIF (Figure 4A). Northern hybridization confirmed these find-

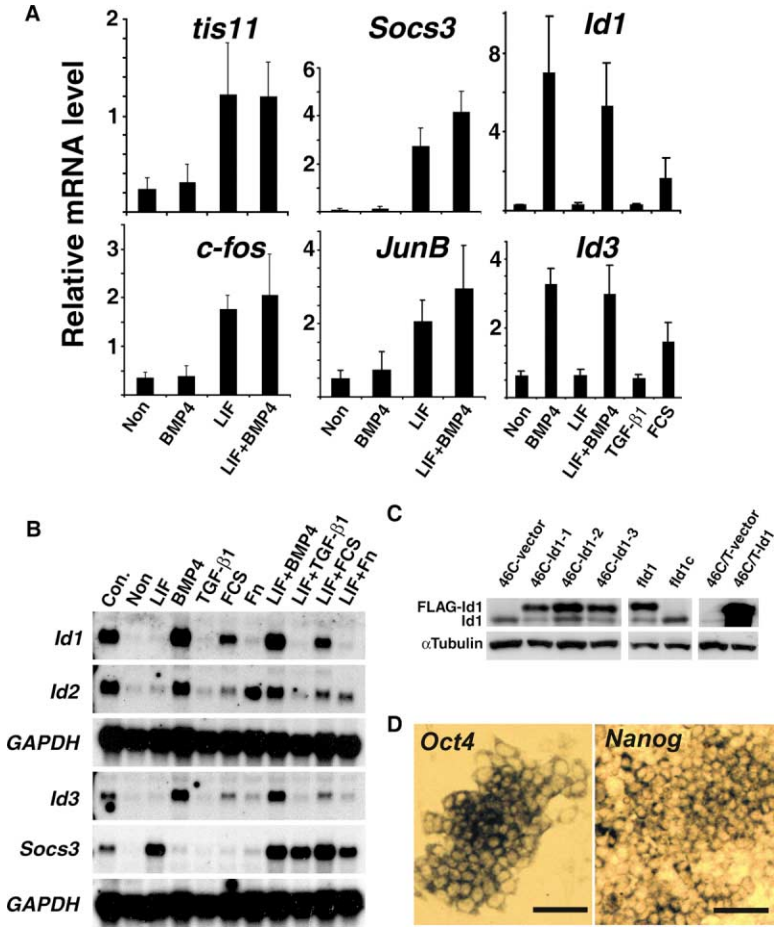

Figure 4. Expression and Function of Ids in ES Cells

(A) LightCycler reverse transcription PCR analyses of gene induction in response to LIF, BMP, or LIF + BMP. ES cells were cultured overnight in N2B27 alone, then stimulated for 45 min.

(B) Northern hybridization of Id mRNA expression in Oct4-GiP cells. Con: steady state ES cells maintained in serum containing medium plus LIF. Lanes 2–11: Cells cultured overnight in N2B27 without factors then stimulated as indicated for 45 min. Fn, fibronectin.

(C) Steady-state level of Id1 protein in 46C ES cells transfected with vector alone and cultured in serum-containing medium with LIF, and overexpression in Id1 and fId1 stable integrant clones and after episomal supertransfection of 46C/T cells. The latter blot was exposed for only 10 s. Transfected Id1 is FLAG tagged and therefore has retarded migration compared with endogenous Id1.

(D) In situ hybridization of Nanog and Oct4 mRNA in Id1 stable integrant ES cell colonies cultured in N2B27 plus LIF. Equivalent results were obtained with Id2 and Id3 transfectants. Bar: 50 μm.

ings and extended them to Id2 (Figure 4B). Neither activin (data not shown) nor TGF-β1 induce *Id* gene expression indicating that this response is specific to Smads downstream of the BMP receptor.

The *Id* genes are also induced by fetal calf serum and by fibronectin, although to a lesser extent than by BMP (Figure 4B). ES cells cultured in serum show readily detectable steady state amounts of Id mRNAs. We examined whether fibronectin, which induces Id2 and Id3, could replace BMP in N2B27 cultures. Soluble fibronectin in combination with LIF could expand undifferentiated Oct4-GiP cells for at least 10 passages, although with more differentiation and slower population expansion than in BMP.

Constitutive Id Bypasses BMP or Serum Requirements for ES Cell Self-Renewal

We hypothesized that Id induction may provide a specific restriction of neural differentiation to complement the self-renewal activity of STAT3. Accordingly, we prepared expression constructs for Id1, Id2, and Id3 and introduced these into ES cells. Colonies were readily recovered by both episomal supertransfection and conventional stable integration. For Id1, elevated protein expression was confirmed by immunoblotting (Figure 4C). Overexpression of the transgene appears to be associated with a reduction in endogenous Id1 protein, implying operation of a feedback or autoregulatory loop.

Forced Id expression did not impair ES cell self-renewal nor block differentiation in the presence of serum. Under these conditions, the transfectants were not overtly different from parental ES cells or empty vector transfectants. In contrast, in serum-free N2B27, Id transfectants, while remaining LIF-dependent, were liberated from requirement for BMP. These cells proliferated in LIF alone as rapidly and with as little differentiation as parental ES cells in LIF plus BMP. The cultures could be passaged multiple times with no change in undifferentiated morphology or factor dependence. The ES cell phenotype was confirmed by expression of *Oct4* and *Nanog* mRNAs (Figure 4D). As a rigorous test of the capacity of *Id* expression to substitute for serum or BMP/GDF, we plated single cells in N2B27. Undifferentiated passageable colonies formed in LIF alone with comparable frequency (10%) to colony formation from isolated cells in LIF plus BMP (Table 1).

Id Proteins Exert a Lineage-Specific Block on ES Cell Differentiation

LIF is essential for self-renewal of Id transfectants because Ids do not impose a complete block on ES cell differentiation. If LIF is withdrawn in serum-containing medium, Id transfectant cells differentiate as parental ES cells. In adherent culture they produced mostly flattened epithelial-like cells with some fibroblasts. On aggregation they formed embryoid bodies with activation of me-

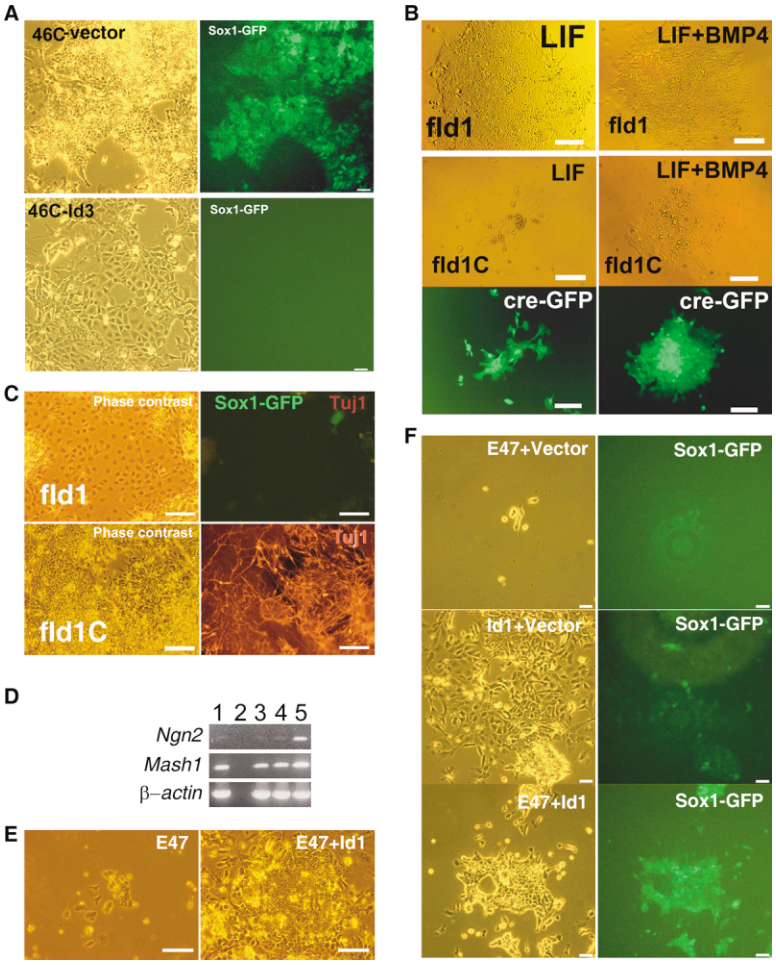

Figure 5. Id Suppresses Neural Differentiation and Is Required for ES Cell Self-Renewal

(A) Phase contrast and GFP fluorescence images of vector and Id3 stable integrant 46C clones after 6 days differentiation in N2B27 without added factors. Id1 and Id2 transfectants showed similar suppression of neural differentiation.

(B) Upper panels: fld1 transfectant 46C cells form self-renewing colonies in N2B27 with LIF alone. Middle panels: after Cre excision, fld1C cells differentiate in LIF and require LIF plus BMP for ES colony formation. Lower panels: GFP expression in fld1C colonies driven by the constitutive CAG unit after excision of the floxed Id1-STOP cassette.

(C) fld1 cells undergo non-neural differentiation on withdrawal of LIF in N2B27 and do not activate *Sox1*-GFP or express TuJ. After Cre excision, fld1C cells show restored differentiation of TuJ positive neuronal cells. (*Sox1*-GFP cannot be specifically detected in fldC cells due to the constitutive activation of GFP)

(D) Reverse transcription PCR analysis of mash1 and ngn2 expression in ES cells and during neural differentiation. Samples as in Figure 3A.

(E) Overexpression of E47 blocks ES cell self-renewal, which can be rescued by increased Id1. 46C/T ES cells were supertransfected with E47 or cosupertransfected with E47 plus Id1 episomal expression vectors and cultured for 6 days under dual puromycin and zeocin selection in serum-containing medium with LIF.

(F) Increased E47 overcomes Id1 suppression of neural differentiation. 46C/T ES cells were supertransfected as in (E), then 24 hr after transfection transferred into N2B27 without added factors and cultured for 6 days under dual selection.

Bar: 50 μm.

sodermal (T) and endodermal (Hnf4) marker expression (data not shown) and developed spontaneous contractility indicative of cardiomyocyte differentiation. However, in N2B27 in the absence of LIF, Id transfectants behaved differently from other ES cells. Neural differentiation, assessed by morphology and by activation of *Sox1*-GFP was minimal (Figure 5A). Instead, the transfectants differentiated into sheets of flattened epithelioid cells, similar to parental ES cells exposed to BMP alone (cf. Figure 1A).

We prepared a revertible expression construct to test whether self-renewal and blockade of neural differentiation are dependent on continuous Id expression. We generated 46C ES cells expressing floxed Id1 (fld1 cells) and subsequently a Cre-treated derivative clone (fld1C) in which the *Id1* transgene had been excised. After Cre excision, fld1C cells show absence of FLAG-Id1 and restored levels of endogenous Id1 (Figure 4C). fld1 and fld1C cells were plated at clonal density in N2B27 with LIF or LIF plus BMP. fld1 cells formed stem cell colonies efficiently in LIF alone but this ability was lost in fld1C cells which produced only differentiated cells in LIF without BMP (Figure 5B). In N2B27 alone, fld1 cells underwent non-neural differentiation, whereas fld1C cells behaved in identical fashion to parental ES cells, generating a high proportion of TuJ positive neurons (Figure 5C).

These observations indicate that Id expression specifically blocks neural lineage commitment and diverts differentiating ES cells into alternative fates, much like what was observed for BMP treatment in the absence of LIF (Ying et al., 2003). Id expressing ES cells are thus wholly dependent on LIF/STAT3 for inhibition of non-neural lineage commitment and maintainance of pluripotency.

How do Ids block neural differentiation? The neurogenic bHLH transcription factors are known to be antagonized by Id proteins in the developing CNS (Lyden et al., 1999). In vivo these bHLH factors have not been reported prior to neurulation. However, cultured ES cells show expression of mRNAs expected to be found only in differentiating lineages (Ramalho-Santos et al., 2002). We therefore investigated the potential expression of two bHLH genes, *mash1* and *neurogenin2*, in Oct4 selected ES cells. While neurogenin2 mRNA is not detectable above background levels, mash1 mRNA appears relatively abundant (Figure 5D). We propose therefore that Id expression may be necessary to prevent continuous neural differentiation of ES cells triggered by preco-

cious expression of mash1 and other pro-neural bHLH factors. Such action might also encompass non-bHLH partners such as Pax and Ets factors (Norton, 2000).

Id proteins bind to ubiquitous HLH factors, the E proteins, with high affinity (Norton, 2000). Overexpression of either will sequester and block activity of the other. To assess whether Id proteins may normally be required for ES cell propagation, we overexpressed the E47 protein by episomal supertransfection either alone or in cotransfection with Id1 or Id3. E47 singly or in cotransfection with empty vector yielded few, very small, sickly colonies (Figure 5E). In contrast, healthy ES cell colonies were generated from cotransfection of *E47* and *Id* vectors. Cotransfectant colonies appeared indistinguishable in serum-containing medium from cells transfected with *Id* alone or with empty vector. This suggests that increased E47 is not intrinsically toxic but has a specific growth inhibitory action due to sequestration of Id. A certain level of free Id may by required for ES cell propagation as observed in other cell types (Norton, 2000). When transferred to N2B27 without LIF or BMP, the cotransfectants underwent neural rather than non-neural differentiation, shown by activation of *Sox1*-GFP (Figure 5F). Thus, E47 neutralizes the neural suppression effect of Id. This is consistent with the suggestion that Id acts to limit availability of E proteins for partnering with proneural bHLH factors.

Nanog Can Bypass Requirements for BMP or Serum

Increased levels of the variant homeodomain protein Nanog render ES self-renewal independent of LIF/STAT3 in the presence of serum (Chambers et al., 2003). We examined whether LIF and/or BMP are required for self-renewal of Nanog overexpressing ES cells in N2B27. Figure 6A shows that EF4 cells expressing a floxed *Nanog* transgene can be propagated in N2B27 without either LIF or BMP. This behavior is directly attributable to Nanog, since derivative EF4C cells in which the *Nanog* transgene has been excised by Cre recombinase rapidly undergo neural differentiation. Addition of BMP alone has no apparent effect on EF4 cells, unless cultures are maintained without passage for more than 6 days when some differentiation becomes apparent (see Discussion). On addition of LIF, with or without BMP, EF4 cells adhere more evenly to the culture dish (Figure 6A) and the population doubling rate increases. This accords with previous indications of combinatorial effects of LIF/STAT3 and Nanog in ES cells (Chambers et al., 2003).

Since Nanog renders BMP or serum stimulation redundant, we asked whether EF4 cells express Ids. After overnight culture in N2B27 without LIF or BMP, expression of Id1 and Id3 was markedly down-regulated in parental E14Tg2a cells. By contrast, in EF4 cells, Id1 mRNA was reduced though still appreciable, and Id3 mRNA actually increased (Figure 6B). Thus, overexpression of *Nanog* maintains a substantial level of Id expression constitutively.

Discussion

The development of robust serum-free culture conditions is essential in order to delimit the ES cell culture environment and thereby define and control signaling inputs that direct self-renewal or differentiaton (Smith, 2001b). Using this approach, we have uncovered a requirement for extrinsic induction of Id proteins to suppress ES cell differentiation and sustain pluripotency.

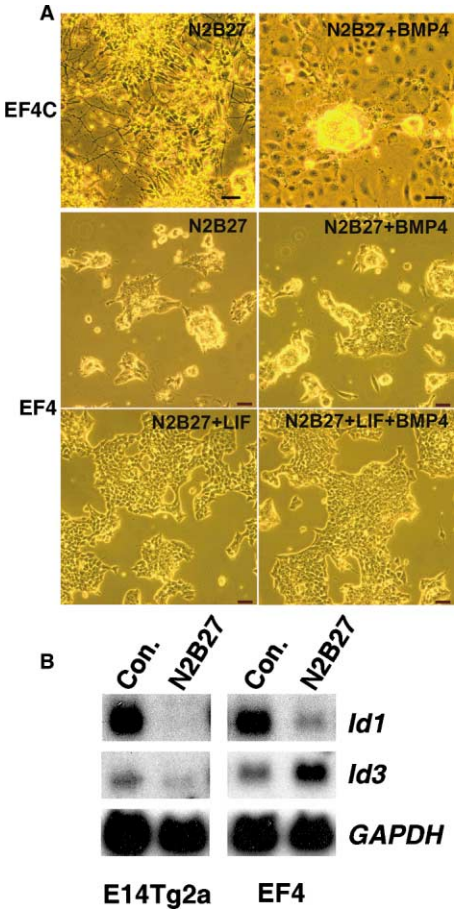

Figure 6. Nanog Bypasses Requirement for BMP/Serum to Induce Id

(A) EF4C cells were cultured for 6 days in N2B27 or in N2B27 plus BMP. EF4 Nanog transfectants were cultured under the indicated conditions for 6 passages and then photographed. Bar: 50 μm.

(B) Northern hybridization of Id1 and Id3 mRNAs in E14Tg2a parental ES cells and EF4 Nanog transfectants in serum plus LIF (Con) or overnight in N2B27 without factors.

BMPs Collaborate with gp130 Cytokines to Sustain ES Cell Self-Renewal

BMP is not an obvious candidate for an ES cell self-renewal factor. Previous studies in ES cells have focused on differentiation and reported stimulatory effects on induction of mesoderm (Johansson and Wiles, 1995; Wiles and Johansson, 1999), hematopoiesis (Adelman et al., 2002), and epidermis (Kawasaki et al., 2000), in line with the potent inductive and patterning actions of BMPs in vertebrate embryos. However, a consistent action of BMPs in various embryo models is to suppress the acquisition of neural fate (Harland, 2000). Accordingly, we investigated whether if added in combination with LIF, BMP might act to promote ES cell self-renewal.

In serum-free cultures, BMPs or GDF completely block *Sox1* activation and neural differentiation. In the absence of LIF, this leads to non-neural differentiation (Ying et al., 2003). When LIF is present, however, the ES cells remain undifferentiated and proliferative. Thus, BMP/GDF effectively substitutes for serum and supports robust ES cell propagation. This effect is wholly dependent on costimulation with LIF.

ES cells cultured in N2B27 with LIF plus BMP appear to require a short period of adaptation before self-renewal proceeds with maximum efficiency. For the initial few days there is no obvious beneficial effect of BMP addition, and differentiation can even appear more extensive than with LIF alone. Thereafter, however, the necessity for BMP becomes apparent. This delayed response may be due to ongoing effects of residual serum components and/or may reflect a resetting of intracellular signaling or transcriptional networks.

After equilibration, ES cells in N2B27 with LIF plus BMP exhibit little differentiation. Attachment after passaging is less efficient than in serum, but cell viability is otherwise comparable and population doubling times appear slightly faster. The robust character of serum-free self-renewal in LIF plus BMP is demonstrated by the isolation of stable transfectants and by single cell cloning. Chimera colonization with germ line transmission establishes that there is no compromise to developmental potential under these conditions and formally eliminates concern about selection of deviant cells.

The finding that ES cells can be derived in N2B27 using LIF plus BMP indicates that BMP responsiveness is present early during ES cell derivation. Although ES cells are a cell culture phenomenon, they are closely related to pluripotent cells in the early mammalian embryo (Brook and Gardner, 1997; Smith, 2001b). Mouse embryos lacking zygotic BMP4, BMPR-1a, or Smad4 develop normally until early egg cylinder stages, but then show reduced proliferation in the epiblast and subsequent partial or complete failure of gastrulation (Mishina et al., 1995; Sirard et al., 1998; Winnier et al., 1995). Analysis of tetraploid aggregation chimeras generated from *smad4* $^{-/-}$ ES cells suggests that the proliferative defect is not autonomous to the epiblast but arises primarily from a deficit in the visceral endoderm (Sirard et al., 1998). However, an additional direct contribution of BMP/GDF stimuli to epiblast proliferation is not ruled out. Furthermore, as with gp130 stimulation (Nichols et al., 2001), such a role may be facultative rather than obligatory in vivo.

Id Induction by Smad Mediates the Self-Renewal Action of BMP

BMP does not appear to potentiate gp130 signaling or otherwise increase STAT3 activity. Nor does BMP inhibit pro-differentiative erk activation. As in other cell types, BMP induces activation of p38 in ES cells, but this is not required for self-renewal and may even have a negative impact by promoting apoptosis. Contradictory self-renewal signaling has previously been described for gp130 activation of erk (Burdon et al., 1999a). One effect of erk may be to antagonize BMP action by inhibitory phosphorylation of Smad1 (Kretzschmar et al., 1997). Possibly, it will emerge as a general rule that self-renewal stimuli simultaneously activate counterbalancing signals that predispose for responsiveness to apoptosis- or differentiation-inducing stimuli and thus constrain unrestricted expansion of the stem cell pool.

The major effectors activated by BMP are the Smads (Shi and Massague, 2003). Transfection with inhibitory Smad6/7 induces differentiation and significantly impairs ES cell self-renewal. This occurs in the presence of serum and LIF, implying that the Smad pathway is not operative only in serum-free culture. Smad activity without addition of BMP may arise from BMPR ligands in serum and/or the autocrine effect of BMP/GDF. The I-Smads, in particular Smad7, will inhibit Smad2/3 activation downstream of TGF-β and activin receptors (Attisano and Wrana, 2002; Shi and Massague, 2003). Although activin stimulation does not suppress ES cell differentiation, it does promote overall population expansion. The reduced colony size on overexpression of I-Smads may be partly attributable therefore to blockade of an activin receptor contribution to cell viability or proliferation, for example from autocrine nodal.

Id genes are prominent targets of BMP/Smad signaling in undifferentiated ES cells. Ids are negative helix-loop-helix factors that sequester E proteins to prevent the transcriptional activity of bHLH factors such as myoD and mash1 (Jen et al., 1992; Lyden et al., 1999). They can also interact with and inhibit Pax and Ets transcription factors (Norton, 2000). The finding that ES cells transfected with *Ids* can self-renew in serum-free culture on addition of LIF alone establishes that a critical contribution of BMP/Smad is to induce *Id* expression. Although an additional contribution from autocrine BMP or GDF cannot be excluded entirely, single cell clonogenic capacity of Id1 transfectant ES cells without added BMP strongly argues that Ids are the only essential BMP/Smad targets for ES cell self-renewal.

Serum induces *Id* genes via multiple pathways, including integrin engagement by extracellular matrix molecules such as fibronectin (Benezra, 2001; Norton, 2000). Hence ES cells cultured in serum without addition of BMP show appreciable levels of *Id* expression. This is also true for human ES cells (Sato et al., 2003). Genetic analysis in *Drosophila* indicates that Ids may also be downstream of the Notch pathway (Baonza et al., 2000). Alternatives to the BMP pathway for induction of Id could account for the propagation of Smad4-deficient ES cells on feeders in the presence of serum (Sirard et al., 1998).

On LIF withdrawal, Id expressing ES cells readily differentiate but do not give rise to neural precursors. Thus, Id proteins act in a lineage-specific manner, suppressing neural determination with little or no effect on mesoderm or primitive endoderm commitment. Ids therefore contribute to self-renewal by complementing the blockade of other lineages by STAT3 (Figure 7). Precisely how Id exerts this lineage restriction on ES cell differentiation will be the subject of future investigations. Detection of mash1 in ES cells, however, prompts the speculation that at least part of Id function may be to block the action of prematurely expressed pro-neural factors. Ids may thus act to insulate the stem cell from functional consequences of lineage priming (Hu et al., 1997). A potential contribution of Ids to cell cycle progression (Norton, 2000) should also be considered.

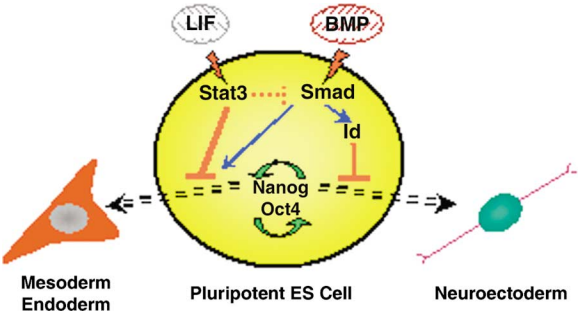

Figure 7. Cooperative Lineage Restriction by BMP/Id and LIF/STAT3

ES cell self-renewal requires suppression of lineage commitment. Id genes induced by BMP or other signals blockade entry into neural lineages, which is otherwise only partially prevented by LIF/STAT3. In parallel, the capacity of BMP to induce mesodermal and endodermal differentiation is constrained by STAT3, probably involving direct as well as indirect mechanisms. Withdrawal of LIF therefore results in a switch in BMP action from supporting self-renewal to promoting lineage commitment.

LIF and BMP: Cooperation and Competition

LIF/STAT3 and BMP/Smad act in combination to sustain ES cell self-renewal. These two pathways also mediate ventralization of the *Xenopus* embryo (Nishinakamura et al., 1999). In that case, each appears to be sufficient independent of activity of the other, with no evidence of cross-regulation between STAT3 and Smad1. The situation in ES cells is more complex, because withdrawal of LIF causes a switch in BMP action from support of self-renewal to promotion of differentiation. How does LIF/STAT3 restrain the potential for Smads to activate differentiation programs? Overexpression of Smad1/4 or introducing constitutively active BMP receptor into ES cells overrides the effect of LIF and causes non-neural differentiation (data not shown). This suggests that STAT3 and Smad inputs should be appropriately balanced. STAT3 can directly or indirectly repress genes that induce mesodermal and endodermal lineage commitment while Smad transcriptional complexes may have the capacity to activate these same genes (Figure 7). These two actions may proceed in parallel, with STAT3 dominant. It is also likely that STAT3 directly modulates Smad1 activity. In neuroepithelial cells, LIF and BMP signaling interact cooperatively via formation of a ternary complex between STAT3, Smad, and p300/CBP (Nakashima et al., 1999; Sun et al., 2001). This complex may be active in ES cells and induce particular target genes. However, such targets seem unlikely to be critical for self-renewal given the apparent sufficiency of Id to reproduce the effect of BMP. Nonetheless, formation of the STAT3/Smad1 complex may play a key role by limiting the availability of active Smad1 for partnering with other cofactors (Figure 7). Effective Smad action may be restricted by STAT3 to a subset of targets, notably *Id* genes, that are either receptive to the STAT3/Smad complex or are inducible by low levels of Smad. In this scenario, withdrawal of gp130 stimulation would release active Smad to complex with transcriptional coactivators that drive recruitment to differentiation genes.

The homoedomain protein Nanog can bypass the requirement for activation of STAT3 in serum-containing medium (Chambers et al., 2003). Nanog can also replace the requirement for BMP/serum stimulation, at least in part by conferring constitutive expression of Id. It remains to be determined whether this effect is direct or indirect. Nanog does not have unlimited capacity to inhibit the Smad differentiation pathway, however, and addition of BMP can promote differentiation of Nanog expressing ES cells under certain conditions. If EF4 cultures are left for 6 or more days without passaging in N2B27, cells around the periphery of colonies begin to differentiate and this is markedly increased in the presence of BMP. Such differentiation may be driven by cooperation between BMP and a second intercellular signal. This observation reinforces the notion of conflicting potentials of BMP both to support self-renewal and to direct non-neural differentiation (Figure 7).

BMPR and gp130 stimulation serve a range of functions during development and in adult tissues and neither can be considered as a dedicated stem cell pathway. Nor is there much evidence that they act as generic stem cell self-renewal stimuli. However, key roles in stem cell niches in *Drosophila* have been defined for the BMP2/4 homolog *dpp* in ovarian germline stem cell self-renewal (Xie and Spradling, 1998), and for activation of the STAT pathway in male germ cells (Kiger et al., 2001; Tulina and Matunis, 2001). It will be interesting to see if neutralization of the differentiation inducing capacity of BMPs by gp130/STAT to reveal anti-differentiation Id activity is specific to ES cells or may be reiterated in other stem cells.

Experimental Procedures

ES Cell Culture

ES cells were maintained without feeder cells. For serum-free culture, ES cells were plated onto gelatin-coated plates in N2B27 medium (Ying and Smith, 2003) supplemented with 10 ng/ml LIF (Sigma) and 10 ng/ml BMP4 or 200 ng/ml GDF6 (R&D Systems). Cells were passaged every 2–4 days using either enzyme-free cell dissociation buffer (Invitrogen) or 0.025% trypsin/1% chicken serum. Dissociated cells were harvested in N2B27 and pelleted. Supernatant was aspirated and the cell pellet resuspended in N2B27 and replated directly. For single cell cloning, a finely drawn Pasteur pipette preloaded with N2B27 was used to pick individual cells into 10 μl drops. Drops were then singly transferred to 96-well plates preloaded with 150 μl N2B27 per well with LIF or LIF plus BMP4. After 8 days, ES cell colonies were identified and passaged. To produce chimeras, ES cells were injected into C57Bl/6 blastocysts. Germline transmission was tested by mating male chimeras with C57Bl/6 females.

Derivation of ES Cells in Serum-Free Medium

Strain 129 mice were ovariectomized on the third day of pregnancy and embryos in diapause flushed 4 days later (Nichols et al., 1990). Intact blastocysts were plated on gelatin-coated plastic in N2B27 supplemented with LIF (10ng/ml). After 3–6 days, the central mass of each explant was picked, rinsed in PBS, and placed in a drop of trypsin for a few minutes. The cell mass was picked up in a finely drawn-out Pasteur pipette preloaded with medium, ensuring minimal carryover of trypsin, and expelled with gentle trituration into a fresh well in N2B27 supplemented with LIF and BMP4 (10 ng/ml). Resultant primary ES cell colonies were individually passaged into wells of a 96 well plate. Thereafter, cells were expanded by trypsinization of the entire culture with centrifugation and aspiration before replating.

RNA Analyses

Oct4GiP ES cells (Ying et al., 2002) were cultured in the presence of puromycin for 4–6 days to eliminate differentiated cells. Purified ES cells were cultured in complete medium plus LIF for 24 hr, then washed once with PBS and transferred to N2B27 medium overnight prior to stimulation for 45 min with 20 ng/ml LIF, 50 ng/ml BMP4, LIF plus BMP4, 10 ng/ml TGF-β1 (all R&D Systems), or 15% FCS. Quantitative RT-PCR was carried out using the LightCycler Instrument (Roche). Data were normalized relative to Oct4 amplification. Primer pairs and reaction conditions are available upon request. Northern hybridizations were carried out on 5 μg aliquots of total RNA.

Plasmid Construction and Transfection

Smad6 and Smad7 plasmids were kindly provided by H. Niwa and FLAG-tagged Id1 by T. Taga. Mouse Id2, Id3, and E47 open reading frames (ORFs) were amplified by PCR, cloned into pCR2.1, and verified mutation-free by sequence analysis. Expression vectors were introduced into ES cells episomally or by stable integration. Floxed Id1 and Cre-excised derivative ES cell lines were derived using the strategy described by Chambers et al. (2003).

Immunochemistry

Preselected Oct4GiP ES cells were transferred to N2B27 medium overnight prior to stimulation with LIF (20 ng/ml), BMP4 (50 ng/ml), or LIF plus BMP4 for 15min or 1 hr. Phosphorylated stat3, smad1, erk1/2, and p38 were detected by immunoblotting (Cell Signaling Technology). Cell lysis and immunoprecipitation (Nakashima et al., 1997) employed anti-FLAG (Sigma) or anti-Stat3 (Transduction Labs). Immunostaining was performed as described (Ying et al., 2003)

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