

TGF β /activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells

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Summary

Human embryonic stem cells (hESCs) self-renew indefinitely and give rise to derivatives of all three primary germ layers, yet little is known about the signaling cascades that govern their pluripotent character. Because it plays a prominent role in the early cell fate decisions of embryonic development, we have examined the role of TGF β superfamily signaling in hESCs. We found that, in undifferentiated cells, the TGF β /activin/nodal branch is activated (through the signal transducer SMAD2/3) while the BMP/GDF branch (SMAD1/5) is only active in isolated mitotic cells. Upon early differentiation, SMAD2/3 signaling is decreased while SMAD1/5 signaling is activated. We next tested the functional role of TGF β /activin/nodal signaling in hESCs and found that it is required for the maintenance of markers of the

undifferentiated state. We extend these findings to show that SMAD2/3 activation is required downstream of WNT signaling, which we have previously shown to be sufficient to maintain the undifferentiated state of hESCs. Strikingly, we show that in *ex vivo* mouse blastocyst cultures, SMAD2/3 signaling is also required to maintain the inner cell mass (from which stem cells are derived). These data reveal a crucial role for TGF β signaling in the earliest stages of cell fate determination and demonstrate an interconnection between TGF β and WNT signaling in these contexts.

Key words: TGF β signaling, Human embryonic stem cells (hESCs), SMAD2/3

Introduction

Embryonic stem cells are a population of multipotent, self-renewing cells that are derived from the epiblast of mammalian blastocyst embryos and that retain this developmental identity even after prolonged culture *in vitro* (Rossant, 2001). ES cells can be induced to differentiate to functional cell types of all three primary germ layers *in vitro*, and upon integration into host animals, they have the capacity to contribute to all cell types of the embryo, including the germline (Smith, 2001). These qualities have made ES cells valuable resources for the introduction of complex genetic modifications into mice; and the recent isolation and culture of human embryonic stem cells (hESCs) (Thomson, 1998) has drawn a great deal of attention to their biology and the potential they offer for regenerative medicine, as well as the study of early human development.

Little is known about the signaling pathways that govern the unique properties of hESCs. Maintenance of mouse embryonic stem cell (mESC) identity was initially found to be dependent on extrinsic factors that were produced by a feeder layer of mouse embryonic fibroblasts (MEFs). Subsequently, it was found that leukemia inhibitory factor (LIF) was produced by MEFs and was sufficient to maintain mESC identity in the absence of a feeder layer. LIF, however, is not the lone factor responsible for the maintenance of stem cell identity in the mouse, as null mutants in which LIF/Stat3 signaling is eliminated show no defect in the establishment of the stem cell compartment (reviewed by Smith, 2001). Furthermore, LIF is

incapable of maintaining stem cell identity in hESCs (Thomson et al., 1998; Reubinoff et al., 2000; Sato et al., 2004), suggesting a contribution from other signaling pathways to the establishment and/or maintenance of stem cell identity in mammalian development.

Models of early vertebrate development have described a role for multiple signaling cascades in the emergence of pattern and cell identity (Harland and Gerhart, 1997; Gilbert, 2003). These models have suggested a prominent role for TGF β signaling in the earliest cell fate decisions of embryogenesis, including neural induction and mesendoderm specification in *Xenopus* (reviewed by Munoz-Sanjuan and Brivanlou, 2002), and primitive streak and mesoderm formation in the mouse (reviewed by Goumans et al., 2000). The TGF β superfamily of ligands, which contains ~40 potential ligands in the human genome, signals through two main branches: the SMAD1/5 branch, which transduces on behalf of BMP and GDF ligands via the type I receptors ALK1, ALK2, ALK3 and ALK6 (ACVERL1, ACVER1, BMPR1A and BMPR1B, respectively – Mouse Genome Informatics); and the TGF β /activin/nodal branch involves the activation of SMAD2/3 via ALK4, and ALK5 and ALK7 (TGFBR1 and ACVR1C – Mouse Genome Informatics) (reviewed by Shi and Massague, 2003). There are also two inhibitory SMADs – SMAD6, which selectively inhibits SMAD1/5; and SMAD7, which inhibits both branches of TGF β signaling – that provide a repressive input on the pathway. Upon activation by phosphorylation and association with a common SMAD4, the receptor-activated SMADs

translocate to the nucleus and, in concert with other transcription factors, regulate gene expression (Shi and Massague, 2003).

Here we show that activation of the TGF β /activin/nodal branch through SMAD2/3 is associated with pluripotency and is required for the maintenance of the undifferentiated state in hESCs and in ex vivo mouse blastocyst outgrowths. hESCs can be maintained undifferentiated through unknown factors in conditioned medium or through activation of the WNT pathway by BIO, a GSK3 β inhibitor. We show that activin/nodal signaling is required downstream of both conditions, in that inhibition of this pathway with a small molecule inhibitor or soluble receptors results in a loss of the undifferentiated state. Accordingly, we find that exogenous activin A is supportive of the undifferentiated state. In our study of hESCs, we have revealed a crucial role for TGF β signaling in the regulation of ES cell identity.

Materials and methods

Reagents

Human activin A, activin RIB/ALK-4/Fc chimera, activin RIB/Fc chimera, cripto 1 and BMP4 were purchased from R&D. Mouse LIF (Esgro[®]) was purchased from Chemicon International. The TGF β inhibitor SB431542 (SB) was purchased from Tocris. The GSK3 β inhibitor, 6-bromoindirubin-3'-oxime (BIO) was generously provided by Laurent Meijer. Primary antibodies used were rabbit polyclonal anti-phospho SMAD1/5, anti-phospho SMAD2 and anti-phospho Ser CDKs substrate (Cell Signaling); rabbit polyclonal anti-SMAD1/5 (Upstate); mouse monoclonal anti-SMAD2/3 and anti-OCT3/4 (Transduction Laboratories); goat polyclonal anti-human NANOG (R&D). Secondary antibodies were donkey anti-mouse or anti-rabbit conjugated to AlexaFluor488, AlexaFluor555 and AlexaFluor647 (Molecular Probes). DNA nuclear counterstains used were SytoxGreen and ToPro3-iodide (Molecular Probes). Pronase and Demecolcine solution were purchased from Sigma.

Cell culture

hESC line H1 was obtained from WiCell research institute and BGN1 and BGN2 lines were obtained from BresaGen. Human embryonic stem cells were maintained as previously described (Sato et al., 2003).

hESC synchronization

BGN1 cell cycle progression was blocked at metaphase by incubation overnight in conditioned medium with 100 ng/ml demecolcine solution. Cells were washed four times with PBS to release them from metaphase block and then harvested for immunoblotting at 15 minutes and 4 hours post-release.

Immunoblotting

Cells were lysed with 100 μ l 1 \times lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerophosphate, 1 mM Na₃VO₄ and complete mini protease inhibitor cocktail (Roche)]. Total protein (20 μ g) was loaded for each lane. Membranes were blocked in TBS with 0.1% Tween and 5% milk. Antibodies used were anti-phospho-SMADs (Cell Signaling); anti-SMAD2/3 (Transduction Laboratories; 1:1000); anti-SMAD1 (Upstate); anti-tubulin (Sigma); anti-OCT3/4 (Transduction Laboratories); anti-human nanog (R&D). Primary antibodies were incubated overnight and secondary antibodies for 2 hours. Proteins were detected with ECL (Amersham Biosciences).

RT-PCR

Cells were lysed directly with 100 μ l RNasee (Tel-Test) and total

RNA was extracted. Total RNA (1 μ g) was reverse transcribed to cDNA and 1/20 of the RT reaction was used as PCR template. Radioactive amplification was according to the following conditions: β -actin, TGGCACCACACCTTCTACAATGAGC (forward) and GCACAGCTTCTCCTTAATGTCACGC (reverse) (21 cycles); OCT3/4, GAAGGATGTGGTCCGAGTGT (forward) and GTGACAGAGACAGGGGAAA (reverse) (19 cycles); NANOG, ACCAGAAGTGTGTTCTCTTCCACC (forward) GGTTGCTCCAGGTTGAATTGTTC (reverse) (21 cycles).

Immunofluorescence

BGN2 cells were plated on matrigel coated poly-D lysine/laminin Biocoat coverslips (Becton-Dickinson) and cultured for 5 days in the described conditions. Following culture, cells or mouse embryos were fixed in 4% paraformaldehyde in PBS, washed in PBS + 0.2% bovine serum albumin (BSA), permeabilized with 0.1% Triton X-100 in PBS/BSA for 20 minutes, and then blocked in 5% donkey serum in PBS/BSA for 2 hours at room temperature. Cells were incubated overnight at 4°C with combinations of either rabbit anti-phospho-SMAD1/5 (1:100 dilution), anti-SMAD2/3 (1:500) or anti-OCT3/4 (1:500). After three 3 washes in PBS/BSA, cells were incubated with combinations of AlexaFluor488-, AlexaFluor555- or AlexaFluor647-conjugated donkey anti-rabbit/anti-mouse secondary antibodies for 2 hours at room temperature. Cells were then washed with PBS/BSA and stained with either 25 nM SytoxGreen or ToPro3-iodide nucleic acid stain in PBS/BSA. Coverslips were mounted in glycerol and imaged using a Zeiss LSM 510 confocal microscope.

Blastocyst outgrowth

Pregnant Swiss-Webster mice were sacrificed at embryonic day 3.5 and the uterus was isolated and flushed with warm culture medium to obtain blastocyst stage embryos as described previously (Nagy, 2003). Embryos were incubated in culture medium containing 1 mg/ml pronase and observed continuously until the zona pellucida was digested. Embryos were then rinsed three times in warm culture medium and incubated on gelatin-coated tissue culture plastic in mouse embryonic stem cell medium (Betts et al., 2001) containing either 20 μ M SB431542 or an equivalent dilution of DMSO. Embryos were not disturbed during culture in order to allow attachment and outgrowth. Embryos were fixed in 4% paraformaldehyde in PBS at 4 days post extraction and processed for immunofluorescence in the same manner as the hESCs. Treated embryos and outgrowths were imaged using a Zeiss LSM 510 confocal microscope.

Embryoid bodies formation

Embryoid body formation for BGN2 cells was carried out as previously described (Sato et al., 2004). BGN2 cells grown for 5 days in described conditions, harvested using dispase (Invitrogen) and plated on bacterial culture plates on which the cells are unable to attach. Embryoid bodies were allowed to form by growth in suspension for 7 days. Embryoid bodies were then plated on gelatin-coated tissue culture grade plastic and further cultured for 7 days in order to allow for terminal differentiation of cell types. Reattached and terminally differentiated embryoid bodies were harvested and processed by RT-PCR in order to assay for the presence of derivatives of primary germ layers.

BrdU incorporation and TUNEL assays

The In Situ Cell Proliferation, FLUOS (cat. No. 1810740) and In Situ Cell Death Detection, Fluorescein (cat. No. 1684795) kits were obtained from Roche. H1 cells were cultured for 2 days in CM and then further cultured in the indicated conditions for 3 days. Following culture, BrdU incorporation and TUNEL staining was assayed according to the protocols described by the manufacturer. Cells were imaged and quantified using a Zeiss LSM Pascal confocal microscope.

Results

The undifferentiated state of hESCs is characterized by activation of SMAD2/3 signaling and inhibition of SMAD1/5 signaling

Although TGF β signaling has been shown to play a role in primary cell fate decisions in many developmental models, its role in embryonic stem cell fate determination remains uncertain. To study the involvement of the TGF β superfamily pathway in embryonic stem cell fate decisions, we first analyzed the activation status of both arms of this family (TGF β /activin/nodal signaling and BMP/GDF signaling) in human embryonic stem cells, both in the undifferentiated state and during the early phase of differentiation in response to certain modulators of TGF β superfamily activation.

In the undifferentiated state that is maintained by growth in MEF-conditioned medium (CM), we found that SMAD2/3 was phosphorylated and localized to the nucleus of hESCs, indicating activation of the TGF β /activin/nodal pathways.

Furthermore, the phosphorylation and nuclear localization were reduced in cells that were allowed to differentiate by growth in non-conditioned medium (nCM) (Fig. 1A,B). We have previously shown that BIO is also capable of maintaining hESCs in the undifferentiated state, even in the absence of CM, through activation of canonical WNT signaling (Sato et al., 2004; Meijer et al., 2003). Accordingly, BIO maintained phosphorylation of SMAD2/3 above levels seen in hESCs grown in nCM alone, and this effect was accompanied by maintenance of OCT3/4, a marker of pluripotency. To test the ability of hESCs cultured in nCM to activate SMAD2/3 signaling, we cultured the cells in nCM supplemented with activin A. Under these conditions, the cells maintained high levels of SMAD2/3 phosphorylation as well as increased OCT3/4 relative to cells cultured in nCM alone. This suggests that SMAD2/3 activation in the absence of extrinsic factors present in CM may be supportive of the undifferentiated state, though not entirely sufficient to maintain typical hESC morphology nor markers of pluripotency through extended culture (Fig. 1A-C).

We also examined the state of SMAD1/5 phosphorylation in hESCs grown in the above conditions. SMAD1/5 activation in these cells showed the opposite character of SMAD2/3 activation; in the undifferentiated state, SMAD1/5 phosphorylation was barely evident and upon differentiation, phosphorylation was globally increased and localized to the nucleus (Fig. 1A; Fig. 2A). Addition of activin A to nCM reduced phosphorylation of SMAD1/5 to levels comparable with those of hESCs grown in nCM, demonstrating an input of SMAD2/3-mediated signaling on the suppression of SMAD1/5 activation. To study the effect of induced SMAD1/5 activation in undifferentiated hESCs, we cultured cells in CM supplemented with BMP4. As previously reported (Xu et al., 2002), these conditions resulted in a decrease in OCT3/4 levels and a concomitant change in morphology

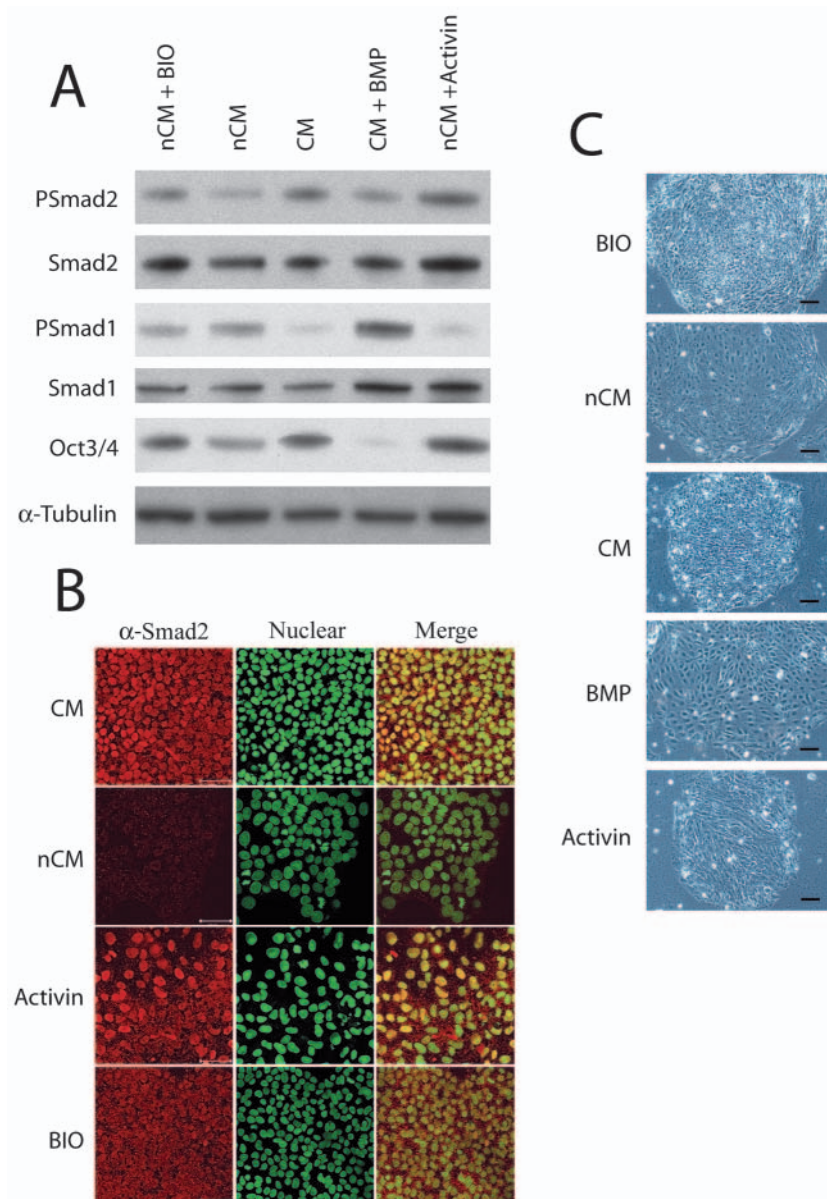


Fig. 1. The undifferentiated state of hESCs is characterized by activation of SMAD2/3-mediated signal transduction and inhibition of SMAD1/5-mediated signal transduction. (A) Western blot analysis of H1 hESCs cultured under various conditions. Cells were cultured for 4 days in the presence of: nCM supplemented with 2 μ M BIO, non-conditioned medium (nCM), MEF-conditioned medium (CM), CM supplemented with 25 ng/ml BMP4, and nCM supplemented with 25 ng/ml activin A. Membranes were probed with antibodies specific for phosphorylated (P) SMAD2, SMAD2/3, phosphorylated SMAD1/5, SMAD1/5, OCT3/4 and α -tubulin (as a control for protein loading). (B) Immunofluorescence microscopy of BGN2 hESCs in the undifferentiated and differentiated state. BGN2 cells were cultured for 5 days in CM, in nCM, in nCM supplemented with 25 ng/ml activin A, and in nCM supplemented with 2 μ M BIO. Cells were decorated with an antibody specific for SMAD2/3, as well as SytoxGreen nuclear counterstain. (C) Bright-field images of H1 hESCs cultured in conditions described in A. Scale bars: 50 μ m.

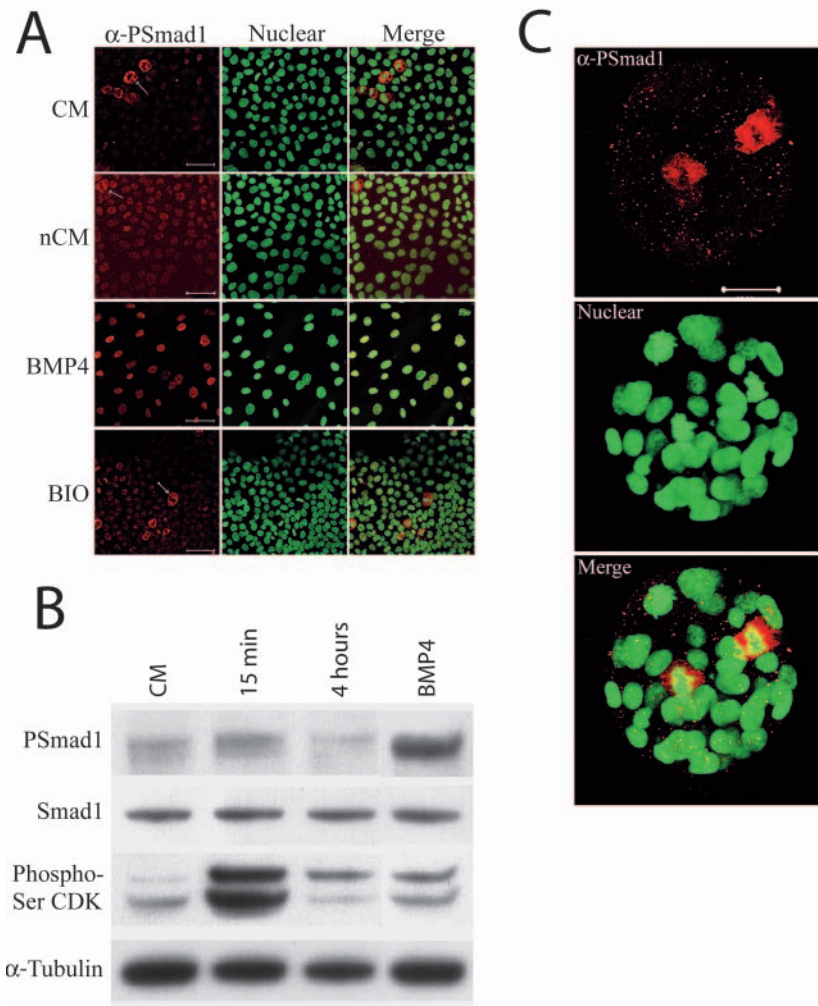


Fig. 2. Global SMAD1/5 phosphorylation is increased under differentiation conditions and is evident in mitotic cells in the undifferentiated state. (A) Immunofluorescence microscopy of BGN2 hESCs in the undifferentiated and differentiated state. BGN2 cells were cultured for 5 days in CM, in nCM, in CM supplemented with 25 ng/ml BMP4 (as a control for SMAD1/5 activation) and in nCM supplemented with 2 μ M BIO. Cells were decorated with an antibody specific for phosphorylated SMAD1/5, as well as SytoxGreen nuclear counterstain. Arrows indicate mitotic cells. Scale bars: 50 μ m. (B) Western blot analysis of colcemide synchronized BGN1 hESCs. Cells were blocked at metaphase by incubation with 100 ng/ml demecolcine solution and harvested at 15 minutes and 4 hours post-release. Cells grown in CM alone and CM supplemented with 25 ng/ml BMP4 were used as controls for asynchronous and SMAD1/5-activated cells, respectively. Membranes were probed with antibodies specific for phosphorylated (P) SMAD1/5, SMAD1/5, phosphorylated Ser CDKs substrate and α -tubulin (as a control for protein loading). (C) Immunofluorescence microscopy of blastocyst stage embryo containing mitotic cells. Mouse blastocyst embryos were fixed and labeled with anti-phospho SMAD1/5 antibody and SytoxGreen nuclear counterstain, and then imaged by confocal microscopy. Scale bars: 20 μ m.

(Fig. 1A,C). Thus, we have shown that SMAD2/3 activation correlates with stemness in hESCs descriptively and functionally, while SMAD1/5 activation correlates with differentiation.

Minimal global levels of SMAD1/5 phosphorylation in undifferentiated hESCs may be accounted for by the result that phosphorylation was only evident in the cytoplasm of cells undergoing mitosis (Fig. 2A). In order to assess the relationship between SMAD1/5 phosphorylation and mitotic index, hESCs were synchronized by incubation with colcemide, which arrests cells at the onset of metaphase. SMAD1/5 phosphorylation was compared between hESCs that had been released from the colcemide block for different lengths of time and asynchronous hESCs (Fig. 2B). hESCs that were released from colcemide block for 15 minutes showed higher levels of SMAD1/5 phosphorylation than did hESCs that had been released for 4 hours. Asynchronous hESCs show slightly reduced levels relative to cells that had been blocked and released for 15 minutes. To measure the efficacy of colcemide in hESC synchronization, we used an antibody that is specific for phosphoserines on cyclin dependent kinases (CDKs) that are phosphorylated only in the context of mitosis. BMP-stimulated hESCs were used as a control for SMAD1/5 phosphorylation. Interestingly, a correlation between SMAD1/5 phosphorylation and mitosis was also apparent in

dividing cells of preimplantation mouse embryos (Fig. 2C).

SMAD2/3 activation is necessary for maintenance of the undifferentiated state in hESCs

Having defined the nature of SMAD1/5 and SMAD2/3 activation in undifferentiated and differentiating hESCs, and having discovered a correlation between increased SMAD2/3 activation and the undifferentiated state, we set out to determine whether active SMAD2/3 signaling is necessary for the maintenance of the pluripotent state (Fig. 3). The TGF β /activin/nodal branch of TGF β signaling can be efficiently inhibited by SB431542, a synthetic compound that precludes SMAD2/3 phosphorylation by type 1 TGF β receptors (Laping et al., 2002). We challenged hESCs cultured in the presence of CM or BIO with SB431542 and phosphorylation of SMAD2/3 was reduced. Furthermore, upon challenge with SB431542, the ability of CM or BIO to maintain protein levels of the pluripotency marker, OCT3/4, was lost. In addition to reducing SMAD2/3 phosphorylation and OCT3/4, SB431542 also has the effect of increasing SMAD1/5 phosphorylation to the levels seen in differentiating hESCs (Fig. 3A).

Prompted by the result that intact SMAD2/3 signaling was necessary for the maintenance of high OCT3/4 protein levels in hESCs, we examined the expression of known markers of pluripotency in hESCs with the variable levels of SMAD2/3 activation described above (Fig. 3B). Upon withdrawal of CM and the subsequent reduction of SMAD2/3 phosphorylation, hESCs show reduced expression of OCT3/4 and of NANOG, another established marker of pluripotency. Inhibition of SMAD2/3 activation by SB431542 in the presence of CM or

Fig. 3. Intact SMAD2/3 signaling is required for the maintenance of the undifferentiated state in hESCs and the formation of embryoid bodies.

(A) Western blot analysis of H1 hESCs cultured in conditions in which SMAD2/3 signaling is intact or inhibited. Cells were cultured for 4 days in CM, in nCM, in nCM supplemented with 2 μ M BIO, in CM supplemented with 10 μ M SB431542, and in nCM supplemented with 2 μ M BIO and 10 μ M SB431542.

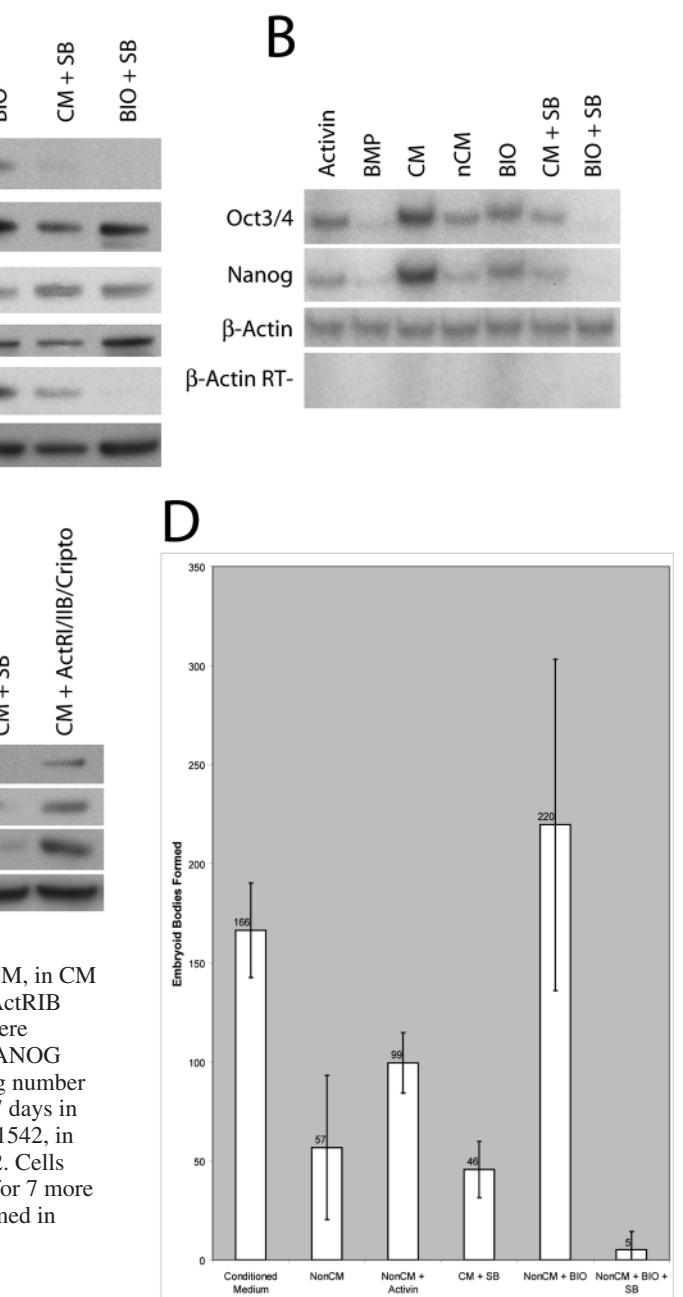
Membranes were probed with antibodies specific for phosphorylated (P) SMAD2, SMAD2/3, phosphorylated SMAD1/5, SMAD1/5, OCT3/4 and α -tubulin (as a control for protein loading). (B) Expression analysis of H1 hESCs.

Cells were cultured for 4 days in nCM supplemented with 25 ng/ml activin A, in CM supplemented with 25 ng/ml BMP4, in CM, in nCM, in nCM supplemented with 2 μ M BIO in CM supplemented with 10 μ M SB431542, and in nCM supplemented with 2 μ M BIO and 10 μ M SB431542. RT-PCR was performed on these cells using primers for human OCT3/4, nanog, β -actin (as a loading control) and β -actin RT minus (as a control for contamination with genomic DNA).

(C) Western blot analysis of H1 hESCs cultured in the presence of a cocktail of soluble receptors specific to the activin/nodal pathway. Cells were cultured for 5 days in CM, in nCM, in CM supplemented with 10 μ M SB431542, and in CM supplemented with hrActRIB (5 μ g/ml), hrActRIIB (5 μ g/ml) and hrCripto (250 ng/ml). Membranes were probed with antibodies specific for phosphorylated SMAD2, OCT3/4, NANOG and α -tubulin (as a control for protein loading). (D) Histogram describing number of embryoid bodies formed from BGN2 hESCs. Cells were cultured for 7 days in CM, in nCM, in nCM with 25 ng/ml activin A, in CM with 10 μ M SB431542, in nCM with 2 μ M BIO, and in nCM with 2 μ M BIO and 10 μ M SB431542. Cells were then detached from substrate and cultured in a suspension of nCM for 7 more days. Histograms and error bars (s.e.m.) represent the experiment performed in triplicate and on two separate passages of BGN2 cells.

BIO resulted in significantly reduced expression of both markers. Consistently, SB431542 promoted differentiation by morphological criteria, inducing cells to assume a flat, spread out morphology (data not shown). Stimulation of TGF β /activin/nodal signaling by activin A in the presence of nCM restored the expression of both markers to levels above those exhibited by hESCs cultured in nCM alone. Hence, by measure of two independent markers of pluripotency, SMAD2/3 activation is not only necessary to, but also supportive of the undifferentiated state of hESCs.

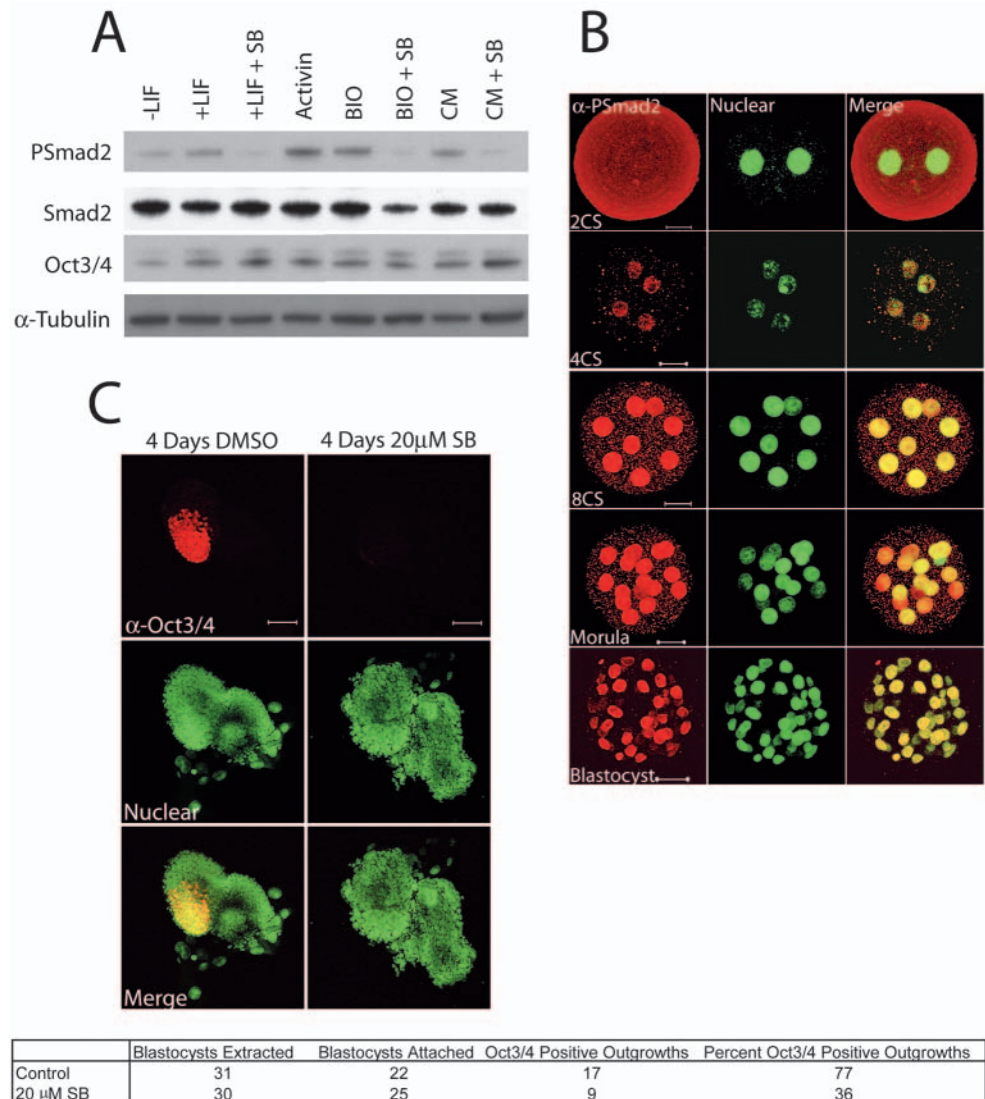
We next set out to confirm that our observations concerning the effect of SB431542 were specific to its inhibition of TGF β /activin/nodal signaling through SMAD2/3, as the use of a small molecule inhibitor could potentially affect unrelated signal transduction pathways. In order to rule out this



possibility, we used an independent means of inhibiting TGF β /activin/nodal signaling by culturing hESCs in CM supplemented with a combination of human recombinant ActRIB, hrActRIIB and hrCripto (Fig. 3C). These three proteins form a complex and bind TGF β /activin/nodal ligands in canonical TGF β signaling, so soluble extracellular domains provide an alternative to SB431542 by competing for TGF β /activin/nodal in the CM. Both OCT3/4 and NANOG were significantly reduced in hESCs cultured in the presence of soluble receptors (Fig. 3C), thus providing corroborative data demonstrating the requirement for this pathway in pluripotent hESCs.

TGF β signaling is involved in many of the more fundamental aspects of cell biology, including cell viability, adhesion, migration and proliferation (reviewed by Massague

Fig. 4. Intact SMAD2/3 signaling is not required for the maintenance of the undifferentiated state of mESCs, but is required for the maintenance of the stem cell compartment of blastocyst outgrowths. (A) Western blot analysis of 129/SVJ mESCs. mESCs were cultured for 3 days in mESC medium with (+) and without (-) LIF, in mESC medium with LIF plus 10 μ M SB431542, in mESC medium with 25 ng/ml activin A, in mESC medium with 2 μ M BIO, in mESC medium with 2 μ M BIO plus 10 μ M SB431542, in CM, and in CM with 10 μ M SB431542. Membranes were probed with antibodies specific for phosphorylated SMAD2, SMAD2/3, OCT3/4 and α -tubulin (as a control for protein loading). (B) Whole-mount immunofluorescent confocal microscopy of pre-implantation stage mouse embryos. Mouse embryos were extracted and fixed at two-cell, four-cell, eight-cell, compacted morula and blastocyst stages. Embryos were decorated with an antibody specific for phosphorylated SMAD2 and SytoxGreen nuclear counterstain. Scale bars: 20 μ m. (C) Confocal immunofluorescent microscopy of blastocyst outgrowths. Mouse blastocyst stage embryos were extracted and cultured in the presence of mESC medium supplemented with DMSO or 20 μ M SB431542 for 4 days. Outgrowths were decorated with an antibody specific for OCT3/4 and SytoxGreen nuclear counterstain. Table gives percentage of embryos exhibiting OCT3/4-positive cells. Scale bars: 100 μ m.



et al., 2000). Thus, the global inhibition of ALK4/ALK5/ALK7 activation by SB431542 could be affecting hESCs in a manner independent of differentiation. Apart from its role in activating the TGF β /activin/nodal branch of TGF β signaling, ALK4/ALK5/ALK7 has also been implicated in activation of MAP kinases, including p38, Jnk and Erk, although the only one of these kinases to be significantly affected by SB431542 in previously reported in vitro assays was p38 (Inman et al., 2002). In order to address whether SB431542 acts through means other than inhibition of SMAD2/3 activation, we performed BrdU incorporation and TUNEL assays to exclude the possibility that SB431542 was affecting cell viability or proliferation (see Fig. S1A in the supplementary material) and found no significant effect of SB431542 on these parameters. It has previously been shown that, at the doses we have used, SB does not significantly affect activation of other pathways in cell culture (Inman et al., 2002). To confirm this in hESCs, we assessed the phosphorylation of MAP kinases p38, JNK and Erk in the presence and absence of SB431542, and found that the phosphorylation of these effector molecules was not regulated upon addition of SB431542 (data not shown).

Owing to the high passage number of the H1 hESC line used, it is possible that the cells may have acquired an abnormal karyotype that endowed it with an atypical character. In order to exclude this possibility, the cells used in these experiments were karyotyped and found to have a normal complement of chromosomes (data not shown).

Embryoid body formation is inhibited in SB431542 treated hESCs

Embryonic stem cells are defined functionally by their pluripotency – the ability to give rise to cell types representing all three primary germ layers of the embryo. In examining the necessity for SMAD2/3 signaling in the maintenance of the pluripotency, it is important to assay the properties of hESCs in conditions that more closely approximate in vivo conditions. There are currently two in vivo assays for hESCs: teratoma formation in the mouse; and formation of embryoid bodies in culture (Thomson et al., 1998). Embryoid bodies are formed from undifferentiated hESC aggregates cultured in suspension and they typically contain differentiated cell types of ectodermal, mesodermal

and endodermal lineages. Differentiated hESCs do not form embryoid bodies.

We examined the efficiency of embryoid body formation in BGN2 hESCs in which SMAD2/3 signaling is activated or inhibited (Fig. 3D). Addition of the inhibitor SB431542 to cells grown in CM significantly reduced the formation of embryoid bodies. Cells grown in the presence of BIO were able to form more embryoid bodies than cells grown in nCM alone, and these embryoid bodies were similar morphologically to those formed of hESCs grown in CM (data not shown). This effect was drastically reduced in the context of SMAD2/3 inhibition and the few embryoid bodies that did form were of an atypical, multi-cystic morphology (data not shown). Activation of SMAD2/3 alone by activin A conferred upon the cells a marginally increased ability to generate embryoid bodies, which is consistent with the ability of activin A to restore OCT3/4 levels in cultured hESCs. These data support the notion that SMAD2/3 activation is necessary but only partially sufficient for maintenance of pluripotency.

In order to assess the character of embryoid bodies formed of hESCs cultured in the above conditions, we assayed the differentiated cell types contained within them by RT-PCR and found that markers of all three primary germ layers were expressed (data not shown).

SMAD2/3 activation is not necessary for maintenance of the undifferentiated state in mESCs

Having found the same requirement for active SMAD2/3 signaling in three independent lines of hESCs (data not shown), we next assayed the relevance of SMAD2/3 activation to mouse embryonic stem cell identity. mESCs are typically cultured in medium containing leukemia inhibitory factor (LIF), a protein that has been shown to maintain mESCs (Smith, 2001), but not hESCs (Thomson et al., 1998; Reubinoff et al., 2000; Sato et al., 2004), in the undifferentiated state. However, a role for LIF in the establishment and maintenance of the stem cell compartment *in vivo* is uncertain, as mouse null mutants for components of the LIF/Stat3 pathway have no stem cell defect (Smith, 2001). We examined the nature of SMAD2/3 signaling in mESCs with the expectation that the necessity for SMAD2/3 signaling in mESCs cultured in medium containing LIF would be similar to that observed for hESCs cultured in MEF conditioned medium (Fig. 4A).

mESCs were cultured in defined medium with (mESC) or without (CM) a contribution of growth factors from serum. Upon withdrawal of LIF, levels of SMAD2/3 phosphorylation in mESCs were reduced, but the SB431542-mediated inhibition of SMAD2/3 signaling in the context of LIF resulted in no significant change in OCT3/4 levels. We have previously shown that BIO is able to maintain mESCs as well as hESCs in the undifferentiated state (Sato et al., 2004). In support of this result, mESCs grown in the presence of BIO were able to maintain levels of OCT3/4 relative to mESCs cultured in the presence of LIF, but unexpectedly, inhibition of SMAD2/3 signaling in the context of BIO had no effect of OCT3/4 levels (Fig. 4A). Taken together, these results suggest that mESCs are dissimilar to hESCs in that they have no requirement for active SMAD2/3 signaling in the maintenance of pluripotency. These results suggest a striking difference between hESCs and mESCs, whereby the maintenance of the undifferentiated phenotype requires an active SMAD2/3 signaling pathway in

hESCs but not in mESCs. Furthermore, the ability of BIO to maintain the undifferentiated phenotype of these cells is dependent on active SMAD2/3 signaling in hESCs but not in mESCs.

Activation of SMAD2/3 is required for maintenance of pluripotency in the ICM of mouse blastocyst outgrowths

The character of embryonic stem cells *in vitro* does not necessarily recapitulate their behavior in an endogenous, *in vivo* context and a recent report showing that loss of both SMAD2 and SMAD3 result in reduced epiblast and OCT3/4 levels (Dunn et al., 2004) prompted us to investigate the role of SMAD2/3 activation in the mouse blastocyst. Mouse blastocyst outgrowths are used to assess the character and potency of two cell types present in blastocyst stage embryos: the trophoblast and inner cell mass (ICM). *Ex vivo*, the trophoblast cells of the blastocyst adhere to the matrix substrates and migrate across them, while the inner (ICM) maintains a compact morphology. It is the inner cell mass from which pluripotent embryonic stem cells are derived, and only these cells maintain OCT3/4 expression upon outgrowth.

TGF β ligands and receptors are expressed very early in mouse preimplantation embryos (Albano et al., 1993; Mummery et al., 1993; Paria and Dey, 1990; Roelen et al., 1994; Roelen et al., 1998; Slager et al., 1991). In order to assess SMAD2/3 activation in early mouse development, we performed confocal immunofluorescence microscopy on mouse embryos from two-cell to late blastocyst stages (Fig. 4B). SMAD2/3 is phosphorylated and localized to the nucleus beginning at the four-cell stage and remains phosphorylated in both the trophoectoderm and ICM up to the blastocyst stage.

To assess the requirement for SMAD2/3 activation in preimplantation mouse embryos, we cultured blastocyst stage embryos for 4 days in the presence or absence of SB431542. We assayed the presence of OCT3/4 in outgrowths cultured from the blastocyst stage for 4 days, both in the presence and absence of SB431542 and found that 77% of control outgrowths were positive for OCT3/4 staining, while only 36% of SB431542-treated blastocysts showed OCT3/4 staining; typical control and SB431542 treated embryos in which the OCT3/4 compartment is maintained and lost, respectively, are represented in Fig. 4C. Strikingly, although OCT3/4 protein is present in a majority of outgrowths cultured for 4 days in the presence of DMSO, most of those cultured in SB431542 display a complete loss of OCT3/4 staining. Hence, SMAD2/3 activation is required not only for maintenance of pluripotency of hESCs cultured *in vitro*, but it is also required for the maintenance of the OCT3/4-positive compartment of the ICM upon blastocyst outgrowth. Mouse blastocyst outgrowths cultured *ex vivo* in the presence of the soluble receptors hrActRIB, hrActRIIB and hrCripto showed a similar trend towards loss of the OCT3/4 positive compartment, though less consistently than outgrowths cultured in the presence of SB431542 (data not shown).

Discussion

For embryonic stem cells to realize their potential in clinical applications, it is first necessary to address fundamental questions regarding their biology and the molecular nature of

'stemness'. We demonstrate here a requirement for the TGF β /activin/nodal branch of the TGF β signaling pathway in the maintenance of human embryonic stem cell identity. Either in the context of unknown extrinsic factors secreted by MEFs (CM) or the GSK3 inhibitor BIO, inhibition of SMAD2/3 activation results in significantly reduced expression of markers of pluripotency. Although it may contribute to the maintenance of the undifferentiated state, SMAD2/3 activation alone does not confer upon hESCs the stem cell identity. Indeed, WNT and TGF β /activin/nodal signaling collaborate in the maintenance of pluripotency, and the WNT signaling pathway impinges, directly or indirectly, on SMAD2/3 activation. We also found a similar requirement for SMAD2/3 signaling in the maintenance of the stem cell compartment of ex vivo mouse blastocyst outgrowths, though activated SMAD2/3 does not seem to be necessary for the ability of LIF to maintain the undifferentiated state of mESCs.

The primary cell fate decision of mammalian development occurs in morula stage embryos when the outer cells of the embryo form trophoectoderm, which mediates attachment and implantation into uterine tissue, and the inner cells form the inner cell mass, which contributes to all the tissues of the embryo and from which embryonic stem cells are derived (reviewed by Rossant, 2001). Our results indicate a role for TGF β signaling in the maintenance of pluripotency in the cellular derivatives of the inner cell mass. Although TGF β superfamily ligands have been shown to contribute to primary cell fate determination in other vertebrate models, such as the establishment of the dorsal organizer in *Xenopus* (Harland and Gerhart, 1997), an input for TGF β s in mammalian embryogenesis has not been well described at pre-gastrula stages. Studies of ligand and receptor expression suggest that the TGF β cascade is activated in pre-implantation mouse embryos (Albano et al., 1993; Paria et al., 1992; de Sousa Lopes et al., 2003) and our analysis of SMAD2 phosphorylation at these stages showed SMAD2/3 activation as early as the four-cell stage. Yet, the role of the TGF β superfamily in early mammalian embryogenesis is not well understood.

Previous analyses of the role of TGF β signal transduction in early mammalian embryogenesis have mostly arisen from the study of knockout mice. An array of null mutations of TGF β signaling components have been made in the mouse, but among these, very few have an effect before gastrulation and none affect the establishment and/or maintenance of the stem cell compartment at peri-implantation stages (reviewed by Goumans and Mummery, 2000). For example, null mutants of SMAD2 or the TGF β /activin/nodal receptor ActRIIB result in failure of mesoderm formation and malformed primitive streak (Song et al., 1998). Recently, mice were created with null mutations for both SMAD2 and SMAD3 (Dunn et al., 2004). These mice displayed a similar, though more severe, developmental phenotype to SMAD2-null mutants, with complete failure to form mesoderm or gastrulate. A striking character of these embryos was the loss of pluripotent epiblast by E7.5, as measured by OCT3/4 expression, while the formation of extra-embryonic ectoderm was retained. As extra-embryonic ectoderm arises from trophoectoderm (Nagy et al., 2003), this double mutant phenotype supports the notion that proper maintenance of the ICM/epiblast requires an intact SMAD2/3 signaling pathway, because derivatives of this

compartment are lost while trophoectodermal derivatives are able to form. The double mutant phenotype also agrees with our finding that the OCT3/4-positive compartment of blastocyst outgrowths is lost, at the equivalent of E7.0, when SMAD2/3 signaling is globally inhibited (Fig. 4C). From these data, in combination with our results describing the necessity for SMAD2/3 signaling in maintenance of the undifferentiated state of hESCs, a paradigm emerges in which SMAD2/3 signaling plays a role in the maintenance of pluripotent cell types in vivo as well as ex vivo and in vitro.

Mice deficient for SMAD4, the common SMAD that mediates translocation of effector SMADs to the nucleus, display a similar phenotype to the SMAD2/3 double knockout, failing to gastrulate or form mesoderm, though OCT3/4 expression in the epiblast of these embryos has not been examined (Sirard et al., 1998). It will be interesting to see whether markers of pluripotency are affected for this and related phenotypes, both in vivo and upon blastocyst outgrowth. Indeed, many of the null mutations of TGF β superfamily ligands and receptors should be reconsidered with respect to their effect on the epiblast.

TGF β signaling has recently been shown by many studies to figure prominently in the maintenance of the undifferentiated state of hESCs. Among the factors found to be specifically expressed at high levels in undifferentiated hESCs are *Nodal* (Rosler et al., 2004), *Cripto*, *Lefty1* and *Lefty2* (Sato et al., 2003), all components of TGF β signal transduction. *Cripto* encodes an EGF-CFC co-receptor that is essential for responsiveness to nodal, and LEFTY1 and LEFTY2 are both inhibitors of nodal signaling. Expression of nodal, LEFTY1 and LEFTY2 has been shown to be high in undifferentiated hESCs and reduced upon differentiation (Besser, 2004), and hESCs cultured in recombinant nodal exhibit prolonged expression of pluripotency markers (Vallier et al., 2004). Furthermore, TGF β has recently been shown to contribute to a cocktail of growth factors that maintain the undifferentiated state of hESCs in feeder-free culture (Amit et al., 2004) and SMAD1/5 activation by BMP4 is known to induce trophoblast in the context of CM (Xu et al., 2002). Our results extend the role of the TGF β pathway in the maintenance of the undifferentiated state of hESCs, demonstrating a requirement for the TGF β /activin/nodal branch downstream of canonical WNT activation or extrinsic factors present in CM.

The correlation between SMAD1/5 phosphorylation and mitosis we have described suggests a compelling role for ligands of the BMP/GDF branch of the TGF β superfamily in cell proliferation. This pathway has been linked to cell proliferation in other developmental contexts. For example, in *Drosophila*, overexpression of the BMP4 homologue *dpp* promotes primordial germ cell (PGC) proliferation and causes accumulation of more PGCs in the *Drosophila* gonad (Xhu and Zie, 2003). In mice, null mutations of *Alk3*, the type I TGF β receptor that mediates SMAD1/5 activation, exhibit reduced cell proliferation in the epiblast (Mishina et al., 2002). In light of these phenotypes, it is not surprising to see that mitosis in two further developmental contexts (pre-implantation mouse embryos and undifferentiated hESCs) is coincident with SMAD1/5 phosphorylation, yet there is no evidence to support any causal link between the two.

That SMAD2/3 signaling is not required for the LIF or BIO-mediated maintenance of the undifferentiated state of mESCs

underscores the dissimilarity between hESCs and mESCs. LIF is sufficient to maintain pluripotency and self-renewal of mESCs, but these cells differentiate when cultured with LIF in the absence of serum (Ying et al., 2003), suggesting a necessary input from other extrinsic factors. It has recently been shown that mESC identity can be maintained in cells cultured in the absence of serum, as long as LIF is supplemented with either BMP4 or the forced expression of the downstream BMP4 target, *Id* (Ying et al., 2003). This suggests an essential contribution of the TGF β superfamily to the maintenance of mESCs, though this contribution activates the SMAD1/5 signaling cascade that induces differentiation to trophoblast when it is activated in hESCs (Xu et al., 2002). Considering the disparity between our results describing the necessity for SMAD2/3 activation in the maintenance of pluripotency in ex vivo blastocyst outgrowths versus mESCs, it is possible that one or both of these paradigms of 'stemness' may not recapitulate the behavior of embryonic stem cells in vivo. Indeed, this contradiction raises questions about whether ES cells in culture are an adequate tool for the study of embryonic development.

We have previously demonstrated the ability of BIO to maintain pluripotency of mESCs and hESCs (Sato et al., 2004), and our results describing the character of SMAD1/5 and SMAD2/3 activation in hESCs extend these findings to implicate a combinatorial role for the TGF β and WNT signal transduction pathways in the molecular events that underlie the undifferentiated state. In other model organisms, namely *Xenopus* and zebrafish, WNT and TGF β signaling are believed to converge on the induction of primary cell types. In *Xenopus* embryos, for example, the establishment of the organizer has been shown to result from the dorsally localized coincidence of three events: stabilization of β -catenin; SMAD2/3 activation by Xnr proteins; and inhibition of SMAD1/5 activation by the BMP inhibitors *chordin*, *noggin* and *cerberus* (Scialli, 2003). Input from both SMAD2/3 and WNT signaling has been shown to be required for the expression of the BMP inhibitory organizer genes (Xanthos et al., 2002). Undifferentiated hESCs exhibit the same reciprocal character with respect to SMAD activation shown here, with SMAD2/3 signaling being active and SMAD1/5 signaling being inhibited. In light of the ability of BIO to maintain the undifferentiated state of hESCs and the dependence of this ability on active SMAD2/3 signaling, it is tempting to speculate that the molecular basis of pluripotent hESC identity may be rooted in a conserved mechanism of primary cell fate specification evident in lower vertebrates.

Embryonic stem cells are defined by their ability to self renew indefinitely and give rise to all cell types of the embryo, yet they are present for a relatively narrow window of the mammalian life cycle. Maintenance of pluripotency in cultured hESCs results from the integration of multiple signaling inputs to retain this identity through indefinite passages. In demonstrating a requirement for TGF β signaling, we have defined one of the necessary inputs for the maintenance of pluripotency of hESCs. However, the means by which SMAD2/3 activation has its effect are unclear. It remains to be seen what targets of SMAD2/3 activation are involved in mediating the maintenance of the undifferentiated state; and the manner in which WNT signaling and SMAD2/3 activation collaborate to mediate pluripotency, if at all.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/6/1273/DC1>

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