Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells

Ludovic Vallier*, Morgan Alexander and Roger A. Pedersen

Department of Surgery and Cambridge Institute for Medical Research, Addenbrooke's Hospital, University of Cambridge, Hills Road, Cambridge, CB2 2XY, UK

*Author for correspondence (e-mail: lv225@cam.ac.uk)

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Summary

Maintenance of pluripotency is crucial to the mammalian embryo's ability to generate the extra-embryonic and embryonic tissues that are needed for intrauterine survival and foetal development. The recent establishment of embryonic stem cells from human blastocysts (hESCs) provides an opportunity to identify the factors supporting pluripotency at early stages of human development. Using this in vitro model, we have recently shown that Nodal can block neuronal differentiation, suggesting that TGFβ family members are involved in cell fate decisions of hESCs, including preservation of their pluripotency. Here, we report that Activin/Nodal signalling through Smad2/3 activation is necessary to maintain the pluripotent status of hESCs. Inhibition of Activin/Nodal signalling by follistatin and by overexpression of Lefty or Cerberus-Short, or by the Activin receptor inhibitor SB431542, precipitates hESC differentiation. Nevertheless, neither Nodal nor Activin is sufficient to sustain long-term hESC growth in a chemically defined medium without serum. Recent studies have shown that FGF2 can also maintain long-term expression of pluripotency markers, and we find that inhibition of the FGF signalling pathway by the tyrosine kinase inhibitor SU5402 causes hESC differentiation. However, this effect of FGF on hESC pluripotency depends on Activin/Nodal signalling, because it is blocked by SB431542. Finally, long-term maintenance of in-vitro pluripotency can be achieved with a combination of Activin or Nodal plus FGF2 in the absence of feeder-cell layers, conditioned medium or Serum Replacer. These findings suggest that the Activin/Nodal pathway maintains pluripotency through mechanism(s) in which FGF acts as a competence factor and therefore provide further evidence of distinct mechanisms for preservation of pluripotency in mouse and human ESCs.

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Key words: Human embryonic stem cells, Pluripotency, Activin, Nodal, FGF

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of human blastocysts (Thomson et al., 1998). Their embryonic origin confers upon them unique properties. They can proliferate indefinitely in vitro (Amit et al., 2000) while maintaining their pluripotent status, and they can also differentiate into a large number of somatic cell types (Odorico et al., 2001). Their potential use to produce clinically effective somatic cells confers exceptional value on hESCs for regenerative medicine. Besides their therapeutic potential, hESCs also represent a new and unique in vitro model to study mechanisms controlling differentiation and pluripotency during early human development.

Understanding the mechanisms controlling the pluripotent status of hESCs remains a major challenge, especially because recent studies have shown that human and mouse ES cells differ in these mechanisms despite their similar embryonic origins. LIF signalling, which is essential for mESC self renewal (Chambers and Smith, 2004), is not active in undifferentiated hESCs (Daheron et al., 2004; Humphrey et al., 2004). Likewise, whereas BMP4 has been shown to block differentiation of mESCs along the neuroectoderm default pathway (Ying et al., 2003), it induces trophectoderm differentiation of hESCs (Gerami-Naini et al., 2004; Xu et al., 2002). Wnt signalling is so far the only pathway reported to be active in maintaining pluripotency in both species (Sato et al., 2004), but Wnt activity alone is not sufficient to maintain pluripotency of hESCs (James et al., 2005). Consequently, the signalling pathways involved in the maintenance of hESC pluripotent status are still unknown. Several studies suggest that FGF and TGFβ1 could be potential candidates to regulate these mechanisms, however the function and the interaction of these pathways are still largely unexplored (Amit et al., 2000; Amit et al., 2004; Beattie et al., 2005; James et al., 2005; Schuldiner et al., 2000; Wang et al., 2005a; Wang et al., 2005b; Xu et al., 2005a; Xu et al., 2005b).

Nodal, a member of the TGFβ superfamily, acts by binding to heteromeric complexes between type I (Alk4 and Alk7) and type II (ActRIIB) Activin receptors, which in turn act through the Smad2/Smad3 signalling pathway (Reissmann et al., 2001; Schier, 2003). Nodal and Activin share type I and II receptors (Alk4, ActRIIB) and have the same Smad signalling pathway (Smad 2, 3), whereas TGFβ1 preferentially uses the TGFβ1 receptors (Alk5,TβRII) and Smads 2, 3. Nodal signalling is

regulated by Cripto, an extracellular GPI-linked protein that acts as a cofactor, and by antagonists the best studied of which are Lefty1 and Cerberus (Belo et al., 1997; Meno et al., 1999; Perea-Gomez et al., 2002).

Our laboratory has recently shown that overexpression of the Nodal growth factor in hESCs can block their default neuroectoderm differentiation during the formation of embryoid bodies (EBs) in a chemically defined medium (CDM) (Vallier et al., 2004a). This blockage was also observed when Nodal overexpressing hESCs were grown in adherent conditions. Nodal itself is expressed in hESCs and quickly disappears upon differentiation, suggesting that Nodal signalling could be involved in the maintenance of pluripotency. Here we present data showing that inhibition of Nodal using Lefty and Cerberus-Short does not induce differentiation of hESCs. However inhibition of the Activin/Nodal/TGFβ signalling pathway by the Alk4/5/7 inhibitor SB431542 (Inman et al., 2002) induces differentiation of hESCs, demonstrating that the Activin/Nodal pathway is essential for maintenance of pluripotency. We also show that Activin/Nodal, but not TGFβ1, is sufficient to block differentiation of hESCs grown in CDM during short culture periods. However, these and other FGF signalling inhibitor studies performed in CDM supplemented with purified growth factors suggested a role for an additional signalling component in maintenance of hESC pluripotency markers. This complementary effect could be provided in the form of FGF, thus revealing a clear interconnection between these two pathways.

Materials and Methods

hESC culture and transfection

H9 hES cells (WiCell, Madison, WI) were routinely cultured as described (Thomson et al., 1998) in KSR medium containing KO-DMEM supplemented with 20% Serum Replacer (Invitrogen). Every 4 days, cells were harvested with 1 mg/ml collagenase IV (Gibco) and then plated onto 60 mm plates (Costar) that had been coated with 0.1% porcine gelatin (Sigma) and contained 1×10^5 irradiated mouse embryonic fibroblasts as feeder cells (feeders). For stable transfection with vectors encoding mouse Lefty2 or *Xenopus* Cerberus-Short, three confluent 60-mm plates containing around 2000 hES colonies each were plated onto one 6-well gelatin-coated plate containing 5×10^4 feeders. After 48 hours the cells were transfected using Lipofectamine 2000 (Invitrogen) as described (Vallier et al., 2004b). Three days after transfection, the cells were passed onto 60 mm gelatin-coated tissue-culture plates containing puromycin-resistant mouse fetal fibroblasts as feeders. After 3 additional days, puromycin (1 μg/ml final concentration) was added. Puromycin-resistant colonies that appeared within 12 days of selection were picked, dissociated and plated onto 24-well gelatin-coated, feeder-containing plates, and expanded for further analysis as described above.

Feeder-free culture on Matrigel was performed as described by Xu et al. (Xu et al., 2001). For feeder and Serum Replacer free culture, hESCs were grown in CDM, (Johansson and Wiles, 1995) supplemented with Activin (10 ng/ml, R&D Systems or Peprotech) and FGF2 (12 ng/ml, R&D Systems). The composition of CDM was 50% IMDM (Gibco) plus 50% F12 NUT-MIX (Gibco), supplemented with 7 μg/ml of insulin (Roche), 15 μg/ml of transferrin (Roche), 450 μM of monothioglycerol (Sigma) and 5 mg/ml bovine serum albumin fraction V (Sigma). To allow hESCs adhesion in CDM, plates were pre-coated with FBS for 24 hours at 37°C and then washed twice in PBS to eliminate any serum. The optimal amount of each cytokine used was chosen according to previous publications or by empirically testing the effect of different doses on the expression of the pluripotency marker Oct-4.

hESC differentiation was induced by the formation of EBs. This was accomplished by incubating colonies in medium containing 1 mg/ml collagenase IV and no FGF for 6 hours, after which all the colonies (but not feeder cells) had detached from the plate. The colonies were then rinsed once in CDM and grown in non-adherent conditions to generate EBs. The effect of different members of the TGFβ family on EB growth was assayed by adding 10 ng/ml Activin (R&D Systems or Peprotech), 50 ng/ml of mouse recombinant Nodal (R&D Systems), 1 ng/ml of TGFβ1 (Peprotech) or 10 μM SB431542 (Tocris).

Karyotype analyses were performed on H9 and hSF-6 cells at various passages. Abnormalities involving chromosomes 9, 5, and 19 were rarely observed at late passages (p80-p115) confirming recent reports suggesting that hESCs are susceptible to genetic anomalies (Draper et al., 2004). Consequently only hESCs from earlier passages (p50-p70) were used for these experiments.

Transcriptional response assay

DNA plasmids including the Tlx2-lux firefly luciferase reporter and CMV-Renilla (Promega) were co-transfected into hESCs to assess their transcriptional response to exogenous Nodal. The ratio between Tlx2-lux and CMV-Renilla was 10:1. Recombinant BMP4 (100 ng/ml) (R&D Systems), FGF2 (40 ng/ml) or SU5402 inhibitor (10 μM) (Calbiochem) was added 18 hours after Tlx2-lux transfection. Cells were harvested 48 hours later for luciferase essay. Luciferase activity was measured using the dual luciferase assay in cell lysates as described (Promega). Firefly luciferase activity was normalised to Renilla luciferase activity.

Flow cytometry and cell sorting

For detection of the pluripotency markers Tra-1-60, SSEA-3 and SSEA-4, adherent cells were washed twice in PBS then incubated for 20 minutes at 37°C in cell dissociation buffer (Invitrogen). Cells were then dissociated by gentle pipetting and resuspended to approximately 0.1 to 1.0×10^5 cells/ml in PBS supplemented with 3% normal goat serum that contained 0.1% azide (NGS) (Serotec). Cells were incubated for 20 minutes at 4°C with Tra-1-60 (1:200, Chemicon), SSEA-3 (MC631, Developmental Studies Hybridoma Bank), anti-SSEA-4 antibody (clone MC813, 1:200, Developmental Studies Hybridoma Bank) or the corresponding isotype control (mouse IgM isotype control, Sigma; rat IgM isotype control, Sigma; mouse IgG isotype control, Pharmingen). Cells were then washed twice in PBS + 3% NGS and incubated for 20 minutes on ice with respectively FITC-conjugated goat anti-mouse IgM antibody (1:200, Sigma); FITC-conjugated goat anti-rat IgM antibody (1:300, Jackson ImmunoResearch) and FITC-conjugated goat anti-mouse IgG antibody (1:200, Sigma). Subsequently, cells were resuspended in PBS supplemented with 3% NGS and stained with 7-aminoactinomycin D (7-AAD) viability dye (Immunotech) at 20 μl/ml for 15 minutes at room temperature. Live cells, identified by 7-AAD exclusion, were analysed for surface-marker expression by fluorescence activated cell sorting (FACS) with FACSCalibur or sorted with a Dakocytomation MoFlo cells sorter.

Western blotting

Cells were mechanically detached from the plate in PBS and each sample was divided in two. One part of the cells was lysed to obtain nuclear extract the other to obtain total protein extract. For nuclear extract, the cell pellet was resuspended in 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, with proteinase inhibitors and 0.5 mM PMSF added just before use. After 15 minutes incubation at 4°C, 1% NP40 was added and, following centrifugation (11,400 ***g*** for 5

minutes), the nuclei were lysed in 10 mM Hepes, 0.4 mM NaCl and 5 mM EDTA. After 30 minutes at 4°C and centrifugation (11,400 ***g*** for 5 minutes), the supernatant containing the nuclear extract was transferred into a fresh tube and the protein content estimated as described bellow. For total protein extract, the cell-lysis solution consisted of 50 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 0.2% NP40. Each 100 μl aliquot of cell-lysis solution was supplemented with proteinase inhibitors, 0.5 μM PMSF, 10 μl of 20% NP40 and 10 μl of 20% Triton X-100. Cell pellets were incubated in the lysis buffer for 10 minutes at 4°C and then centrifuged at 11,400 ***g*** for 10 minutes at 4°C. Quantification of protein extract was carried out using the Protein Quantification Kit-Rapid (Biochemica) according to the manufacturer's instructions. Proteins were run on a Nupage gel (Invitrogen). Gels were blotted onto nitrocellulose membrane (Amersham Biosciences) which was then stained with rabbit anti-PhosphoSmad2/3 antibody (New England Biolabs) or rabbit anti-PhosphoSmad1/5/8 (New England Biolabs) for nuclear proteins and rabbit anti-Smad2/3 (Zymed) or rabbit anti-Smad1/5/8 (New England Biolabs) for total protein followed by secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Dakocytomation). Membranes were developed using ECL western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions.

RNA extraction, reverse-transcriptase PCR and real-time PCR

Total RNAs were extracted from hESCs or EBs using the RNeasy Mini Kit and RNeasy Microkit for dissected EB layers (Qiagen). Each sample was treated with RNase-free DNase (Qiagen) to avoid DNA contamination. Test-PCR reactions were carried out for all RNA samples to verify the absence of genomic contamination. For each sample 0.5 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). PCR reaction mixtures were prepared as described (Promega protocol for Taq polymerase), were denatured at 94°C for 5 minutes and cycled at 94°C for 30 seconds, 50-65°C for 30 seconds, and 72°C for 30 seconds followed by final extension at 72°C for 10 minutes after completion of 40 cycles. Primer sequences, annealing temperatures and their expected products are (hNodal FP: 5′-AGAAGCAGATGTCCAGGGTAGC-3′, hNodal BP: 5′-AGAGGCACCCACATTCTTCC-3′, 65C; mNodal FP: 5′-CCA-GACAGAAGCCAACTGTG-3′, mNodal BP: 5′-AAGCATGCTC-AGTGGCTTGG-3′, 60C; Activin FP: 5′-GAATGAACTTATG-GAGCAGACC-3′, Activin BP: 5′-ACTGCTCACAGGCAATCC-3′, 55C; hTGFβ1 FP: 5′-GACATCAACGGGTTCACTACCG-3′, hTGFβ1 BP: 5′-GTGTCCAGGCTCCAAATGTAGG-3′, 60C; mT-GFβ1 FP: 5′-ACTACTATGCTAAAGAGGTCACCCG-3′, mTGFβ1 BP: 5′-CTGAAGCAATAGTTGGTATCCAGGGC-3′, 60C). All PCR reactions were carried out with a negative control that contained only water and a positive control that contained RNA extracted from EBs grown for 30 days in FBS supplemented medium (data not shown). The expression of the beta2 microglobulin (β2M) housekeeping gene was used to normalise PCR reactions.

Real-time (Taqman) PCR was performed using an ABI 7700, with 1× Mastermix (Eurogentec), 500 nM of each primer, 200 nM Taqman probe, and 100 ng cDNA. Cycle conditions were as recommended by Eurogentec. Sequences were: PBGD-FP: 5′-GGAGCCATGTCTG-GTAACGG-3′, PBGD-BP: 5′-CCACGCGAATCACTCTCATCT-3′, PBGD-Probe: 5′-TTTCTTCCGCCGTTGCAGCCG-3′, Oct-4-FP: 5′-AGTGAGAGGCAACCTGGAGA-3′, Oct-4-BP: 5′-ACACTC-GGACCACATCCTTC-3′, Oct-4-Probe: 5′-AAACCCACACTG-CAGCAGAT-3′, Cripto-FP: 5′-TCCTTCTACGGACGGAACTG-3′, Cripto-BP: 5′-AGAAATGCCTGAGGAAAGCA-3′, Cripto-Probe: 5′-GATGTGCGCAAAGAGAACTG-3′.

Immunofluorescence

hESCs were fixed for 20 minutes at 4°C in 4% paraformaldehyde (PFA) and then washed three times in PBS. Cells were incubated at room temperature for 20 minutes in PBS containing 10% goat serum (Serotec), and subsequently incubated at room temperature for 2 hours with primary antibody diluted in 1% goat serum in PBS (dilutions were as follows: SSEA-3, clone MC631, 1:50, Developmental Studies Hybridoma Bank; SSEA-4, clone MC813, 1:50, Developmental Studies Hybridoma Bank; Tra-1-60, Chemicon International, 1:200; Oct-4, SantaCruz, 1:100; Smad2, Zymed, 1:50; Smad3, Zymed, 1:50. Cells were then washed three times in PBS and incubated with fluorescein-isothiocyanate-conjugated anti-mouse IgG or IgM (Sigma, 1:200 in 1% goat serum in PBS) or rat IgM (Jackson ImmunoResearch, 1:300 in goat serum in PBS) or rabbit IgG (Jackson ImmunoResearch Laboratory, 1:200 in donkey serum in PBS) for 2 hours at room temperature. Unbound secondary antibody was removed in three washes with PBS. Hoechst 33258 dye (Sigma) was added to the first wash at a dilution of 1:10,000.

Results

TGFβ signalling induced by either Nodal or Activin maintains in vitro pluripotency of hESCs

We have recently shown that overexpression of Nodal can block neuroectoderm differentiation of hESCs that had been grown in adherent conditions in CDM during a prolonged period of time (Vallier et al., 2004a). Interestingly, Nodal-overexpressing hESCs showed little differentiation in general (Fig. 1D) and had increased proliferation when grown on feeders (data not shown). In addition, Nodal is endogenously expressed in hESCs and its expression decreases quickly upon differentiation (Fig. 1C and Fig. 2B). Taken together, these results suggest that Nodal could be involved in the maintenance of hESC pluripotency. The prerequisite of this hypothesis is the activation of the downstream effectors of Nodal signalling, Smad2 and Smad3, in hESCs. Immunofluoresence studies (Fig. 1A and supplementary material Fig. S1) showed that Smad2/3 proteins are expressed in the nucleus of hESCs expressing Oct-4 and SSEA-3, two specific markers for pluripotency. These results imply that Activin/Nodal/TGFβ signalling is active in hESCs. To analyse further the function of Nodal in hESCs, we diminished its activity by using the two extracellular inhibitors, Lefty (Meno et al., 1999) and Cerberus-Short (Bertocchini and Stern, 2002; Parisi et al., 2003; Piccolo et al., 1999), proposing that this would lead to differentiation by blocking Nodal-driven maintenance of pluripotency. Corresponding cDNAs were subcloned into the pTP6 expression vector (Pratt et al., 2000) and the resulting constructs were stably transfected into the H9 cell line. After selection, hESC colonies were counted, picked (n=5 for Lefty-pTP6 and n=15 for CerS-pTP6) and then amplified for additional analyses, by growing them on layers of feeder cells in Serum-Replacer (Invitrogen). The number of stably transfected colonies were similar to those generated with the hrGFP-pTP6 vector (Fig. 1B). Proper expression of Lefty and CerS proteins in transfected hESCs was validated by showing that the supernatant of an individual Lefty- or CerS-overexpressing cell line was capable of inducing differentiation of Nodal overexpressing hESCs grown in CDM (data not shown). Lefty-overexpressing hESCs were difficult to growth because they tended to differentiate in prolonged culture (Fig. 3A). Nevertheless, we were able to maintain Lefty-expressing hES cell lines for more than 20 passages. CerS-overexpressing cell lines showed little

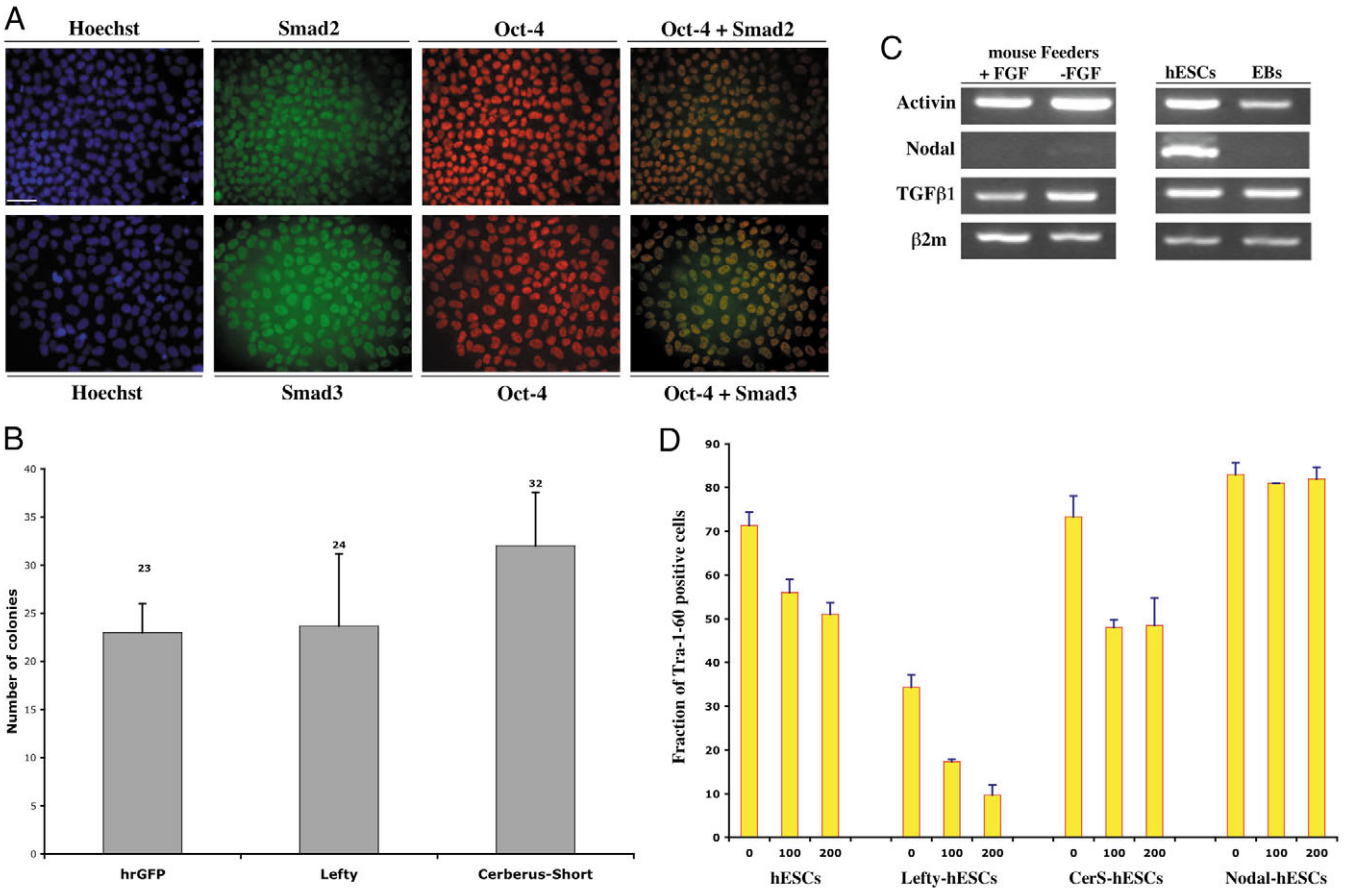

Fig. 1. Activity of the Activin/Nodal/TGFβ signalling pathway and consequences of Activin/Nodal signalling inhibition on hESC pluripotency. (A) Nuclear co-localisation of Smad2 (upper panel, green fluorescence) and Smad3 (lower panel, green fluorescence) with Oct-4 (red fluorescence) in hESCs grown on feeders. Nuclei stained with Hoechst 33258 dye (blue fluorescence). Combined Smad and Oct-4 staining (right panels). Bar, 50 μM. (B) Number of hESCs colonies generated after stable transfection of pTP6 expression vectors for the human recombinant Green Fluorescent Protein (hrGFP), Lefty2 (Lefty) and Cerberus-Short. hESCs were transfected using Lipofectamine 2000 (see Materials and Methods) and the numbers of colonies generated were determined after 10 days of puromycin selection. In the pTP6 vector, the expression of the transgene is linked to the expression of the puromycin resistance gene though an IRES (internal ribosome entry site). Therefore, all the hESC colonies resistant to puromycin expressed the relevant transgene. (C) Expression of Activin, Nodal and TGFβ1 in feeder cells, grown in the presence (+) or absence (–) of FGF2, and in hESCs and EBs that had differentiated for 14 days in CDM, using reverse-transcriptase PCR. β2 microglobulin (β2M) was used as a loading control. (D) Effect of the Activin inhibitor follistatin on hESC pluripotency. Wild-type-, Lefty-, Cerberus-Short- and Nodal-hESCs were grown for 10 days (1 passage) in the presence of 0, 100 or 200 ng/ml follistatin. FACS was used to determine the fraction of Tra-1-60-expressing cells. Values represent the mean and standard deviation of three separate experiments.

differentiation compared with wild-type hESCs (Fig. 3A). Finally, a high dose of recombinant Lefty protein (200 ng/ml) did not induce differentiation of hESCs grown on feeders (data not shown) confirming the limited effect of the Lefty transgene on pluripotency. Together, these results suggest that inhibition of Nodal function does not lead to immediate differentiation. However, the Nodal signalling pathway could be activated by other growth factors, with either Nodal or its cognate(s) alone being sufficient for pluripotency. Activin and TGFβ1 are particularly likely candidates for this role, because they can activate the same Smad signalling pathway (Amit et al., 2004; Beattie et al., 2005). Furthermore, we found by reverse-transcriptase PCR that Activin and TGFβ1 were expressed by mouse feeder cells as well as hESCs (Fig. 1C). Therefore, these growth factors could act either together with Nodal or on their own to maintain pluripotency of hESCs.

To analyse the effect of Activin on hESCs, the Activin inhibitor follistatin (Harrison et al., 2005) was added to the medium (100 ng/ml or 200 ng/ml) of wild-type cells, Lefty-hESCs, CerS-hESCs and Nodal-hESCs (NHN-hESCs) and left for 10 days, after which the fraction of cells expressing the pluripotency marker Tra-1-60 (Henderson et al., 2002) was determined by FACS (Fig. 1D). High doses of follistatin significantly reduced the number of Tra-1-60-positive cells in wild-type hESCs, Lefty-hESCs or CerS-hESCs, thereby confirming an in-vitro role for Activin in hESC pluripotency. However, follistatin had no effect on cells overexpressing Nodal, confirming that Nodal can sustaining the expression of pluripotency marker(s) independently of Activin (Fig. 1D). These results imply that Activin and Nodal can act independently from each other to maintain markers of pluripotency.

Activin and Nodal but not TGFβ1 can delay differentiation of hESCs cultured in CDM

We next determined whether TGFβ1 or other potential Activin/Nodal cognates and cofactors were sufficient to maintain pluripotency of hESCs. H9 hES cells were plated in feeder-free conditions in CDM (Johansson and Wiles, 1995). This simplified medium contains only BSA, lipids, insulin, and transferrin as complex additives, without Matrigel or Serum Replacer; thus by using CDM, unknown components that could compensate for a missing factor or mask its presence are efficiently avoided. CDM was supplemented with different growth factors either added individually [TGFβ1 (1 ng/ml), Activin A (10 ng/ml), Nodal (100 ng/ml), BMP4 (2 ng/ml), Cripto (50 ng/ml) and FGF2 (4, 12 or 40 ng/ml)] or as pairs [Nodal (100 ng/ml) + Cripto (50 ng/ml) and Nodal (100 ng/ml) + FGF (12 ng/ml)]. The effect of growth factor addition was measured by culturing plated hESC colonies for 1 week in CDM, and then assaying the extent and frequency of Oct-4 expression in the colonies by immunofluorescence (Fig. 2A). In the absence of any growth factors, most of the plated hESCs differentiated, as indicated by the high frequency of colonies with little or no Oct-4 expression (Fig. 2A). In the presence of Activin, 95% of the colonies showed extensive Oct-4 expression. This suggests that Activin alone is sufficient to prevent differentiation for short culture periods in the absence of other TGFβ ligands or FGF. Addition of Nodal or the highest dose of FGF (40 ng/ml) resulted in only 50% and 63% Oct-4-positive colonies, respectively. However, addition of Nodal and FGF together resulted in 95% Oct-4-positive colonies. Finally, TGFβ1, Cripto or BMP4 had little effect on the number of Oct-4-positive cells, showing that these factors are not efficiently maintaining the expression of pluripotency markers by hESCs grown in these conditions.

These results were confirmed by studying the effects of Activin, Nodal and TGFβ1 on hESCs and on their differentiation during EB formation (Fig. 2B). Reverse-transcriptase PCR analyses showed that the disappearance of pluripotency markers (Oct-4, FGF4 and Nodal) (Avilion et al., 2003; Vallier et al., 2004a) was blocked in the presence of these growth factors, and that the appearance of the neuroectodermal marker Neurod1 was delayed. Interestingly, Pitx2, a known target gene of the Smad 2/3 signalling pathway (Ryan et al., 1998), was only induced in hESCs by TGFβ1, suggesting that Activin/Nodal and TGFβ1 signalling have different effects on hESCs. In addition, markers of endoderm (IFABP, Sox17) and mesoderm (Myf5) were rarely induced (Fig. 2B), suggesting that neither Activin/Nodal nor TGFβ1 are efficient in driving the differentiation of hESCs into these primary germ layers. Finally, expression of AFP, a marker of visceral endoderm, was not blocked by any of the growth factors, confirming our previous results that Nodal allows extra-embryonic endoderm differentiation in a fraction of the EB cell population (Vallier et al., 2004a). Taken together, these results show that either Activin or Nodal but not TGFβ1 is able to sustain expression of pluripotency markers in a substantial fraction of cells, either in adherent conditions or during the formation of EBs. Intriguingly, the high expression levels of pluripotency markers when combining Activin or Nodal with FGF2 suggest that these two pathways interact to maintain pluripotency.

Activin/Nodal and FGF cooperate to maintain pluripotency-marker expression for prolonged periods

Pluripotency – under any growth conditions – needs to be maintained for a prolonged period of time to convincingly demonstrate its effect. Consequently, H9 hES cells were grown for ten passages (> 40 days in culture) in CDM supplemented with either one growth factor [FGF (40 ng/ml), Activin (10 ng/ml) or Nodal (100 ng/ml)] or a combination of two growth factors [Activin (10 ng) + FGF2 (12 ng/ml), Nodal (100 ng) + FGF2 (12 ng/ml)]. Expression levels of pluripotency markers Tra-1-60, SSEA-4, and SSEA-3 were assessed every five passages by FACS or immunofluorescence analysis (Fig. 2C and data not shown) (Henderson et al., 2002). hESCs grown with FGF or Activin alone began differentiation after three passages and only 3% and 25%, respectively, still expressed markers of pluripotency after five passages (data not shown). This shows that neither FGF nor Activin alone is sufficient to maintain the expression of pluripotency markers in hESCs that are cultured beyond early passages in CDM. However, 50% of cells expressed markers of pluripotency after 10 passages in the presence of a high Nodal dosage (100 ng/ml, data not shown), suggesting that Activin/Nodal signalling has a dose-dependent effect on pluripotency. The best long-term effect was observed with a combination of Activin + FGF2 or Nodal + FGF2. In either of these conditions, expression of Tra-1-60, SSEA-3 and SSEA-4 was seen in 80%, 70% and 90% of the cells, respectively (Fig. 2C and data not shown). Expression of the differentiation marker SSEA-1 was not detected in these culture conditions (data not shown). As a positive control, hESCs grown on feeders for 10 passages were used, and similar results were obtained (Fig. 2C). Consequently, Activin/Nodal and FGF2 signalling together can maintain the expression of pluripotency markers and, in combination, are sufficient to support the culturing of hESCs for prolonged periods of time in the absence of feeders, Matrigel or serum. Importantly, similar results were obtained with hSF-6 cells (supplementary material Fig. S2), which provids evidence that these culture conditions can be extended to another hES cell line.

Alk4/5/7 receptor function is essential for both Activin/Nodal and FGF signalling to maintain the expression of pluripotency markers

To understand the relation between FGF- and Activin/Nodal-signalling in hESCs, we studied the effect of the Alk4/5/7 receptor inhibitor SB431542 (SB) (Inman et al., 2002) and the FGF receptor inhibitor SU5402 (SU) (Mohammadi et al., 1997) on the expression of pluripotency markers in hESCs. This analysis was performed with hESCs grown in CDM, on feeders or on Matrigel in medium conditioned by mouse feeder cells (Fig. 2D). The effect of each combination of growth factors (Activin, Nodal and FGF) and inhibitors (SB and SU) on pluripotency was determined by analysing expression of the pluripotency marker Tra-1-60 by FACS, or by immunodetection of the Oct-4 protein (data not shown). After one week in culture, only 10% of the control cells still expressed Tra-1-60. By contrast, 70% of the cells grown in the presence of Activin plus FGF retained expression of Tra-1-60. The addition of either Activin or FGF resulted in 45% or 30%

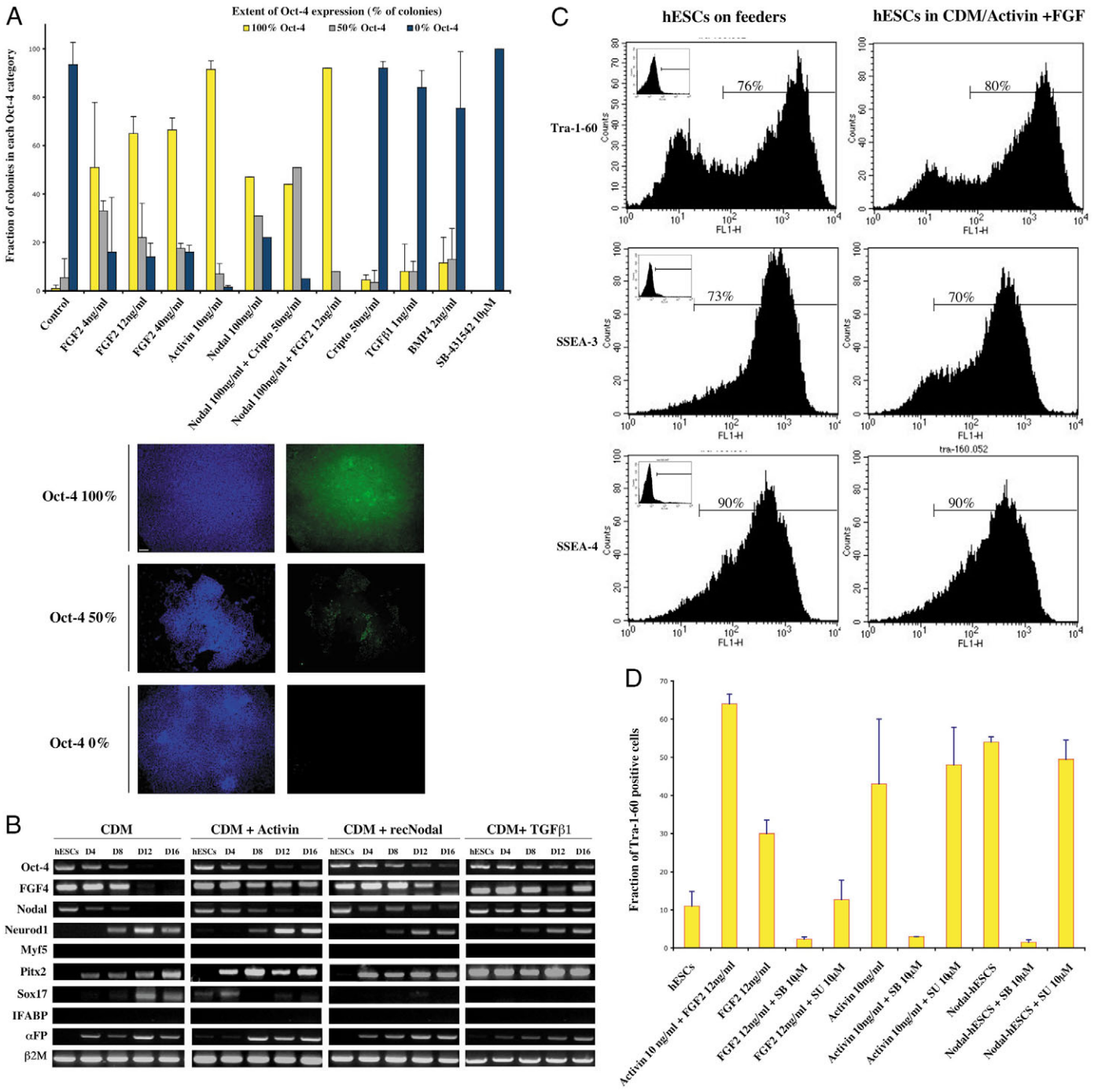

Fig. 2. See next page for legend.

of cells, respectively, expressing Tra-1-60. The positive effect of FGF was totally negated when SU (10 μM) was added; however, this inhibitor did not affect the number of Tra-1-60-positive cells maintained by Activin or Nodal. Moreover, addition of SB (10 μM) induced the disappearance of Tra-1-60-positive cells in the presence of either Activin or FGF2. Therefore, even though FGF is necessary to maintain the expression of pluripotency markers by hESCs, this strictly depends on Activin/Nodal signalling through the Alk4/5/7 receptor pathway. Interestingly, a high dose of Nodal can block differentiation of hESCs grown on feeders in the absence of FGF2 (data not shown), confirming that exogenous FGF is not necessary as long as Activin/Nodal signalling is strongly activated.

Comparable results were obtained using hESCs grown on feeders, or on Matrigel with conditioned medium (Fig. 2E and supplementary material Fig. S3). The effect of the SB and SU inhibitors was also similar on hSF-6 cells grown on feeders (supplementary material Fig. S4), thus demonstrating that these results can be extended to another cell line. Importantly,

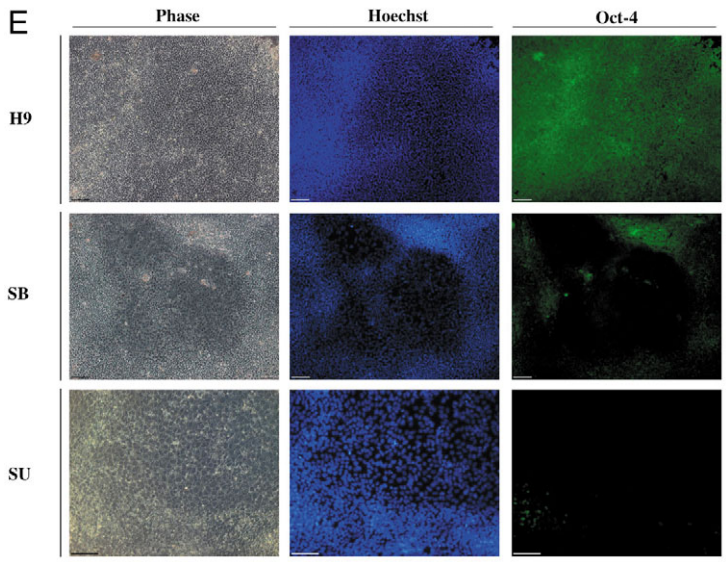

Fig. 2. Effect of Activin and FGF on the pluripotent status of hESCs. (A) Efficiency of FGF and different members of the TGFβ family in maintaining pluripotency marker expression of hESCs in feeder-free conditions. hESCs were grown in CDM for 7 days in the presence of different growth factors and then maintenance-of-pluripotency-marker expression was established by determining the number of colonies expressing Oct-4 using immunofluorescence. Colonies were allocated to three arbitrary categories corresponding to different levels of pluripotency marker expression: colonies containing a preponderance of cells expressing Oct-4 (100% Oct-4, yellow column and green fluorescence, upper right panel), colonies consisting of a relatively even mixture of cells positive and negative for Oct-4 expression (50% Oct-4, grey column and green fluorescence, middle right panel) and colonies containing few or no Oct-4 expressing cells and thus totally differentiated (0% Oct-4, blue column and bottom panel). Nuclei are stained with Hoechst 33258 dye (blue fluorescence). Bar, 100 μM. Values represent the mean and standard deviation of three separate experiments. (B) Expression of pluripotency and differentiation markers during differentiation of hESCs in the absence or presence of Activin (10 ng/ml), recombinant Nodal (recNodal) (50 ng/ml) and TGFβ1 (1 ng/ml). RNAs were extracted every four days for 16 days (D4-D16), then reverse-transcriptase-PCR analysis was performed to detect the expression of the genes denoted. (C) Long term expression of the pluripotency markers Tra-1-60, SSEA-3, and SSEA-4 in hESCs grown either on feeder or in CDM supplemented with Activin (10 ng/ml) and FGF2 (12 ng/ml). hESCs were grown for 10 passages (~ 40 days) in adherent conditions and then the fraction of pluripotent cells was established using FACS to detect expression of Tra-1-60 (upper panels), SSEA-3 (middle panels), and SSEA-4 (bottom panels). hESCs grown on feeder layers were used as positive controls (left panels). (D) Effect of the Activin/Nodal/TGFβ receptor inhibitor SB431542 (SB) and the FGF receptor inhibitor SU5402 (SU) on hESCs grown in feeder-free conditions in CDM. Wild-type hESCs and Nodal-hESCs were grown for 7 days in CDM in the presence or absence of Activin and FGF, and in the presence or absence of the SB and SU inhibitors. FACS was used to determine the fraction of Tra-1-60 expressing cells. Values represent the mean and standard deviation of three separate experiments. (E) Effect of SB431542 (SB) and SU5402 (SU) inhibitors on Oct-4 expression of hESCs grown on Matrigel in feeder cell-conditioned medium. H9 cells were grown for 10 days in the absence (upper row) or in the presence of 20 μm SB (middle row) or 10 μm SU (lower row) inhibitors. The level of differentiation was established by immunofluorescence to determine the expression of Oct-4 (green fluorescence, right panels). Nuclei are shown by Hoechst staining (blue fluorescence). Bar, 100 μM.

higher doses of SB and SU inhibitors were necessary to induce differentiation of cells grown on Matrigel in the presence of conditioned medium, confirming that Matrigel contains compounds that interfere with inhibitor efficiency.

Together, these results demonstrate that both Activin/Nodal and FGF signalling are essential for hESC pluripotency in CDM or any standard hESC culture conditions. However, FGF seems to act as a competence factor for Activin/Nodal signalling because its positive effect on pluripotency strictly depends on Activin/Nodal signalling.

FGF can maintain Cripto expression in hESCs, but cannot be replaced by recombinant Cripto

FGF has previously been found to act as a competence factor for TGFβ signalling in amphibian and fish development (Cornell et al., 1994; Cornell et al., 1995), and it could potentially act through similar means in mammals. Studies in zebrafish have recently revealed that FGF increases the expression of the Nodal co-factor Cripto (Mathieu et al., 2004), suggesting that this is how FGF acts as competence factor for Nodal signalling. hESCs express high levels of Cripto, although this decreases rapidly upon differentiation (Brandenberger et al., 2004; Calhoun et al., 2004; Ginis et al., 2004; Miura et al., 2004). We thus hypothesised that FGF achieves its effects by maintaining Cripto expression, thereby reinforcing the endogenous Nodal activity in hESCs. To examine this hypothesis, Cripto expression in hESCs grown in feeder-free conditions with FGF or Activin was determined using real-time PCR. Expression of Cripto was strongly decreased (14-fold) in control EB cells (Fig. 3A), confirming that Cripto expression marks undifferentiated hESCs. A decrease in Cripto expression (sevenfold) was also observed in Tra-1-60-positive cells, in cultures treated with Activin. By comparison, FGF was more effective than Activin in maintaining Cripto expression, with Cripto transcripts being five times more abundant in the presence of FGF than of Activin alone. However, the level of Cripto expression maintained by FGF signalling in feeder-free conditions was still significantly below the level in hESCs grown on feeders. To confirm that this decrease did not reflect an early step in hESC differentiation, expression of the pluripotent marker Oct-4 was analysed using real-time PCR in similar conditions, showing no significant difference between hESCs grown on feeders and hESCs grown in feeder-free conditions in the presence of Activin or FGF. Therefore, by culturing hESCs in highly defined conditions, we found that FGF was able to restrain the decrease in Cripto expression that accompanies differentiation, although it was less effective than feeders in this regard, suggesting that other factors may also be involved in regulating Cripto expression. Nevertheless, this result appears to support the hypothesis that FGF acts through a Cripto-mediated mechanism.

The hypothesis of Cripto-mediated FGF signalling predicts that Cripto can take the place of FGF when Cripto is added to hESCs cultured in conditions of blocked FGF

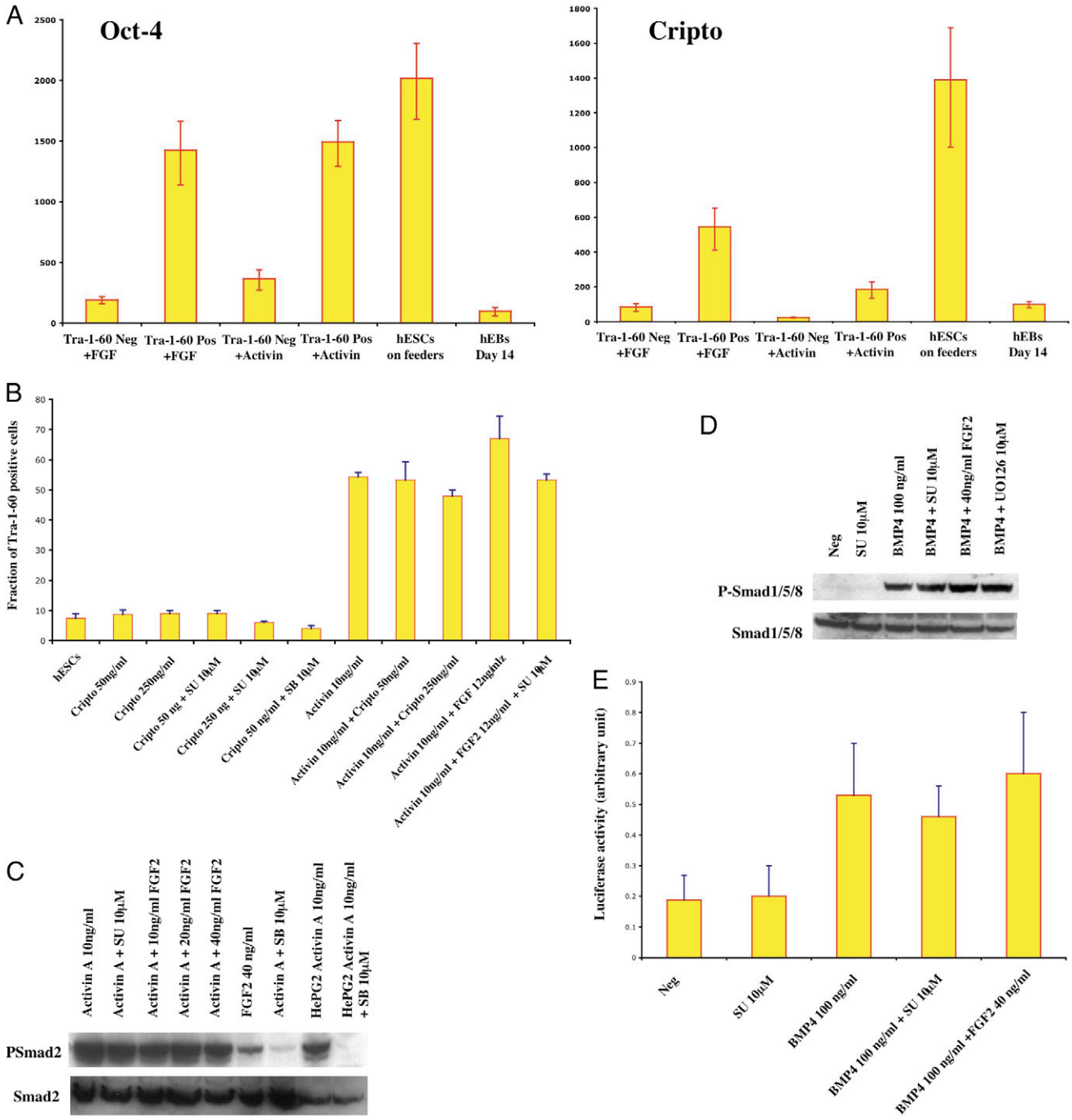

Fig. 3. See next page for legend.

signalling. To further test this hypothesis, hESCs were cultivated in CDM in the presence of Cripto (50 ng/ml or 250 ng/ml), Activin (10 ng/ml), and either SU (10 μM) or SB (10 μM) inhibitors. In control experiments, the efficiency of recombinant Cripto was validated with a reporter system for Nodal signalling (Saijoh et al., 2000) (data not shown). Addition of Cripto did not block differentiation of hESCs in the absence of FGF nor did it rescue the differentiation-inducing effect of SB or SU, showing that Cripto alone was unable to maintain pluripotency (Fig. 3B). Therefore, the increased competence that FGF provides the Activin/Nodal signalling pathway in maintaining expression of pluripotency markers in hESCs appears to act through a mechanism distinct from its effect on regulation of Cripto expression, because the effect cannot be replaced by soluble Cripto.

FGF does not appear to act by controlling P-Smad2/3 or P-Smad1/5/8 nuclear localisation

We thus sought to identify an alternative mechanism by which FGF supports Activin/Nodal signalling. Interestingly, ERK kinase, a downstream target of FGF signalling, can prevent

Fig. 3. Interactions between FGF and Activin signalling pathways in hESCs. (A) Real-time quantitative PCR (QPCR) analysis of Oct-4 and Cripto expression in hESCs grown for four days in CDM supplemented with Activin (10 ng/ml) or FGF (40 ng/ml). Tra-1-60 positive and negative cells were sorted using FACS to separate undifferentiated and differentiated cells. Normalised Oct-4/PBGD (left panel) and Cripto/PBGD (right panel) mRNA levels were measured by QPCR. hESCs grown on feeders and differentiated cells from EBs were used respectively as positive and negative controls. Similar results were obtained in three independent experiments. (B) Effect of Cripto on hESCs grown in feeder-free conditions in CDM. Wild-type hESCs were grown for 7 days in CDM in the presence or absence of Cripto, Activin and FGF and in the presence or absence of the SB and SU inhibitors. FACS was used to determine the fraction of Tra-1-60 expressing cells. Values represent the mean and standard deviation of three separate experiments. (C). Western blot analysis of Smad2/3 phosphorylation in hESCs grown in feeder-free conditions. hESCs were grown for 6 days in CDM supplemented with 10 ng/ml Activin and 12 ng/ml FGF2 without feeders. Then hESCs were grown in different culture conditions for 2 hours. HepG2 cells were used as a positive control. Nuclear proteins were extracted and the expression of the phosphorylated form of the Smad2 protein was analysed using western blot (upper panel). Alternatively, total cellular extracts were used to confirm that expression of the Smad2 protein was the same in all the conditions (lower panel). Similar results were obtained with hESCs grown on feeders (data not shown). (D) Western blot analysis of Smad1/5/8 phosphorylation in hESCs grown in feeder-free conditions in the presence or absence of BMP4 (100 ng/ml). hESCs were grown for 6 days in CDM supplemented with 10 ng/ml Activin and 12 ng/ml FGF2 without feeders. Then hESCs were grown in different culture conditions for 2 hours. Nuclear proteins were extracted and the expression of the phosphorylated form of the Smad1/5/8 protein was analysed using western blot (upper panel). Alternatively total cellular extracts were used to confirm that expression of the Smad2 protein was the same in all the conditions (lower panel). (E) Recombinant BMP4 activates the Tlx2-lux reporter in feeder-free conditions. H9 cells were transiently transfected with the Tlx2-lux vector. After transfection, cells were incubated 48 hours in the absence (Neg) or in the presence of the SU inhibitor (10 μM) or BMP4 (100 ng/ml) alone or combined with FGF2 (40 ng/ml) or SU inhibitor (10 μM). Normalised luciferase activity is expressed as the mean ± SD from three informative experiments.

the nuclear localisation of Phospho-Smad proteins by phosphorylation of their linker region (Grimm and Gurdon, 2002; Kretzschmar et al., 1999). As a result of this mechanism, addition of FGF might limit the activity of Activin/Nodal signalling to prevent induction of differentiation. To test this hypothesis, the amount of Phospho-Smad2 protein localised in the nucleus of hESCs grown in CDM was determined by western blotting (Fig. 3C). Increasing doses of FGF2 (10 ng/ml, 20 ng/ml and 40 ng/ml) did not result in a reduction of nuclear Phospho-Smad2 nor did inhibition of FGF signalling by SU inhibitor result in an increase. Addition of SB inhibitor (or omission of Activin) resulted in a large decrease of nuclear Phospho-Smad2, confirming that Smad2 activation depends on exogenous Activin. Together, these results suggest that the function of FGF in pluripotency does not occur through the modulation of the nuclear localisation of Smad2.

Other recent studies have demonstrated that Serum Replacer contains a BMP-4-like activity capable of inducing differentiation of hESCs into trophectoderm (Xu et al., 2005b). Such differentiation can be blocked either by FGF2 or by Noggin and thus a major function of FGF in maintaining hESC pluripotency could be to inhibit any BMP-like activity (Wang et al., 2005a; Xu et al., 2005b). To test this hypothesis in our culture conditions, we analysed the nuclear localisation of Phospho-Smad1/5/8 by western blotting (Fig. 3D) in hESCs grown in CDM in the presence (Fig. 3D lanes 3-6) or in the absence of recombinant BMP4 (Fig. 3D lanes 1, 2). Nuclear localisation of Phospho-Smad1/5/8 was observed only in the presence of BMP4. Addition of the SU inhibitor in the absence of BMP4 did not induce nuclear localisation of Phospho-Smad1/5/8, thereby excluding any interference from an endogenous source of FGF. These results reaffirm the absence of endogenous BMP activity in hESCs cultures grown in CDM and also suggest that FGF has a function(s) in hESC pluripotency other than the simple control of BMP4 activity. Interestingly, even addition of a high dose of FGF2 (40 ng/ml) did not decrease the quantity of Phospho-Smad1/5/8 localised in the nucleus, nor did addition of the SU inhibitor or the MEK kinase inhibitor UO126 (10 μM) (Duncia et al., 1998) in combination with BMP4 increase it. These negative findings were confirmed using the Tlx2-lux reporter system for Smad1 transcriptional activity (Visser et al., 2001). Only exogenous BMP4 induced activation in this reporter assay (Student's *t*-test, P=0.02), and addition of FGF did not seem to block this induction by BMP4 (Student's *t*-test, P=0.3) (Fig. 3E). Therefore, the function of FGF in pluripotency does not appear to occur through the inhibition of BMP4 activity, because this does not seem to be achieved by controlling the nuclear localisation or function of Phospho-Smad1/5/8.

Discussion

Our findings from a combination of growth factor addition and receptor inhibition experiments provide substantial evidence to support the hypothesis that Activin/Nodal signalling plays the key role in maintaining pluripotency of hESCs. The first indication of such a role emerged from the prevalence of TGFβ signalling pathway components in the transcriptome of hESCs, and their rapid disappearance upon differentiation (Brandenberger et al., 2004). Moreover, exposing hESCs to the TGFβ family member Nodal as recombinant protein upregulated markers of pluripotency, and Nodal transgene expression led to the development of an epiblast/primitive ectoderm-like cell type during EB formation (Vallier et al., 2004a). In addition, Activin and TGFβ1 in combination with other factors has been shown to support hESCs grown on Matrigel or on fibronectin, in medium supplemented with Serum Replacer (Amit et al., 2004; Beattie et al., 2005). TGFβ family members are also less potent than other growth factors in inducing differentiation (Schuldiner et al., 2000). Finally, a recent report (James et al., 2005) describes short-term observations on the effects of the TGFβ inhibitor SB431542 on Oct-4 expression. Our results substantially extend those observations, providing insights into the mechanisms of Activin/Nodal signalling on the status of hESC pluripotency.

There is also ample evidence for a role of the FGF signalling pathway in maintaining pluripotency of hESCs. FGF2 has been included in the media used to derive and maintain existing hES cell lines, suggesting that this pathway has an important function in the mechanisms regulating growth and self-renewal of hESCs (Amit et al., 2000). More importantly, FGF2 has recently been shown to be sufficient to support hESC growth

on Matrigel in the absence of feeders or feeder-conditioned medium (Wang et al., 2005a; Wang et al., 2005b; Xu et al., 2005a; Xu et al., 2005b), further demonstrating the significance of this pathway. Using growth factors and receptor inhibitors to define how FGF achieves its effects, we have demonstrated here its robust action as a competence factor for Activin/Nodal signalling in the absence of other sources of TGFβ family ligands.

Our method of analysis is based on culturing hESCs in medium with decreased complexity, coupled with quantification of the cells that express cell markers of pluripotency (Oct4, SSEA-3, SSEA-4 Tra-1-60, Tra-1-81). This approach expedites analysis of the components of the culture environment that are responsible for TGFβ pathway activation and the extent to which they are capable of deflecting hESCs away from the differentiative pathway. Although not a long-term in vivo assay, the criteria of in-vitro expression of cellular markers for pluripotency has been shown in numerous studies to accurately predict pluripotency as assayed by teratoma formation in immunocompromised mice (Amit et al., 2000; Amit et al., 2004; Reubinoff et al., 2000; Stojkovic et al., 2004; Thomson et al., 1998; Wang et al., 2005b; Xu et al., 2001). Therefore, the persistence of pluripotency markers can be taken as the in-vitro correlate of in vivo pluripotency because differentiation in vitro inevitably results in their imminent disappearance. Accordingly, we find that TGFβ pathway signalling is necessary to maintain pluripotency in a variety of culture conditions, each of which can provide components that either activate the TGFβ pathway (Activin, Nodal, TGFβ1) or serve as competence factors for it (FGF, Cripto).

Activin/Nodal signalling is essential to maintain pluripotency of hESCs

In addition to the existing circumstantial evidence for a role of TGFβ signalling in hESCs offered by profiling studies (Brandenberger et al., 2004), our previous study provided a direct demonstration that Nodal was sufficient to maintain markers of pluripotency both in monolayer cultures and during development of EBs (Vallier et al., 2004a). In that study, we used either recombinant Nodal or expression of a Nodal transgene to determine the effect of this TGFβ ligand, which has been widely identified among vertebrate embryonic systems as an inducer of the mesendoderm pathway of differentiation (Schier, 2003). The effects on pluripotency from sustained exposure to Nodal were twofold: 1) maintenance of pluripotency marker expression in monolayer cultures in the absence of other growth factors and 2) development of an epiblast-like inner layer in Nodal-expressing EBs (Vallier et al., 2004a). The demonstration of the sufficiency of Nodal to induce cell fate decisions in hESCs – resulting in the maintenance of an apparently pluripotent phenotype – provided the impetus for further investigations.

The present study, involving inhibitors of Nodal function, was carried out to determine whether this TGFβ family member, identified as being sufficient to activate the Activin/Nodal signalling pathway, was also necessary to maintain pluripotency. At the outset, we obtained novel insights regarding the ability of Nodal to compensate for the absence of FGF in monolayer cultures and the activity of the Activin/Nodal signalling pathway as indicated by Smad 2/3 phosphorylation and nuclear localisation. In examining whether Nodal itself was necessary for the observed TGFβ pathway activity, we found, however, that expression of either the Lefty or the Cerberus-Short transgenes, both known to be inhibitors of Nodal function (Meno et al., 1999; Piccolo et al., 1999), had no effect on the number of hESC colonies generated in monolayer cultures on feeder layers supplemented with Serum Replacer, nor did it eliminate the expression of the pluripotency marker Tra-1-60. This result was particularly striking in the case of Cerberus, whose shortened form has been identified in *Xenopus* studies to be a specific inhibitor of Nodal (Piccolo et al., 1999). Consequently, although Nodal was sufficient to maintain prolonged expression of pluripotency markers in the absence of other additives, it did not appear necessary for the maintenance of pluripotency in standard culture conditions.

Such evidence for functional sufficiency without evidence for functional necessity implies that alternative and/or redundant factors or pathways are active in this system (Stoffel et al., 2004). Indeed, it is apparent that both Activin and TGFβ1 are transcribed by mouse feeder cells, which are standardly used with hESCs (Beattie et al., 2005), and that even in its growth factor-reduced form Matrigel also provides substantial quantities of TGFβ1 (Kleinman et al., 1982; Vukicevic et al., 1992). Activin would be expected to act through the identical receptors and the Smad 2/3 signalling pathway as Nodal, and TGFβ1 through independent receptors (Alk5 and TβR-II) but through the same Smad 2/3 pathway (Miyazawa et al., 2002). Treatment with the Activin inhibitor follistatin provided evidence for the role of Activin in sustaining the expression of Tra-1-60, by complementing the inhibitory effects of Lefty or Cerberus-Short in hESCs that express either transgene. However, overexpression of Nodal was able to negate the follistatin effect, indicating that both Activin and Nodal sustain the expression of pluripotency markers by similar mechanisms, but independently of each other.

Nevertheless, distinguishing the role of specific TGFβ growth factors in maintaining pluripotency is difficult, particularly when the culture environment includes undefined components such as feeder cell layers, Matrigel or Serum Replacer. Therefore, to define the role of individual growth factors, we utilised the simplified CDM medium devised by Johanssen and Wiles (Johanssen and Wiles, 1995) for growth of mESCs in highly defined conditions. This enabled us to analyse the effects of Nodal and Activin either alone or in combination with FGF, and to distinguish their effects from those of TGFβ1 or BMP4. When hESCs were grown in CDM as monolayers in the presence of FGF2, Activin or Nodal, a substantial majority of the colonies showed extensive or complete retention of Oct4 expression. A striking additive effect was achieved when FGF2 was added to either Activin or Nodal. Consistently with this observation, the combination of Activin and FGF in CDM was sufficient to maintain expression of pluripotency markers for long periods (10 passages, ~40 days) in monolayer cultures in the absence of any other factors hypothesised to be capable of maintaining pluripotency. This contrasted with the modest effects of either TGFβ1 or BMP4 added singly on the maintenance of Oct4 expression, where pluripotency marker expression was largely extinguished

within one week of culture. When hESCs were grown as EBs (which provokes the onset of differentiation in control cultures) in CDM and in the presence of Activin, Nodal or TGFβ1, there was a substantial delay in the loss of gene expression associated with pluripotency (e.g. FGF4). Consequently, Activin, presumptively made by feeders transcribing this gene, and Nodal, synthesised by hESCs themselves, could both actively contribute to the maintenance of pluripotency observed in standard culture conditions containing feeder cell layers, or in feeder-free conditions on Matrigel (Xu et al., 2001; Xu et al., 2005). By contrast, TGFβ1 was far less efficient at maintaining the expression of pluripotency markers on hESCs grown in adherent conditions in CDM. However, TGFβ1 was capable of maintaining the expression of pluripotency markers during differentiation of EBs. The difference between these two results could be explained by an indirect effect of TGFβ through differentiated cells generated during the formation of EBs, or the complementation with other endogenous factor(s). Interestingly, the TGFβ1 pathway seems to be functional in hESCs because addition of TGFβ1 induces activity of the Smad2/3 luciferase reporter gene SBE4-lux (data not shown) (Jonk et al., 1998). Therefore, Activin/Nodal and TGFβ1 can have distinct effects on pluripotency even when they share components of the TGFβ signalling pathway. Further studies are thus needed to fully understand the molecular basis of the TGFβ pathway in maintaining pluripotency, including the identification and role of Smad interacting proteins and their ultimate target genes in hESCs.

Faced with such evidence of a role for Activin/Nodal/TGFβ signalling, it remained important to demonstrate that this function was indeed necessary for hESC self-renewal. This was accomplished through the use of the Alk4/5/7 inhibitor, SB431542, which has been shown to specifically inhibit the receptors responsible for both Activin/Nodal (Alk4, Alk7) and TGFβ1 (Alk5) signalling through Smad2/3 in the majority of cell types studied to date (Inman et al., 2002; James et al., 2005; Piek et al., 1999; ten Dijke and Hill, 2004). This inhibitor not only suppressed Smad2/3 phosphorylation (James et al., 2005) and thus its nuclear translocation but also dramatically reduced the fraction of Tra-1-60-positive cells in a dose-responsive and time-dependent manner. This result confirms the essential role of TGFβ pathway signalling in the maintenance of hESC pluripotency.

FGF is an essential cofactor for Activin/Nodal to maintain pluripotency in hESCs

Although there is now ample evidence for a role of FGF in hESC self-renewal (Amit et al., 2000; Amit et al., 2004; James et al., 2005; Schuldiner et al., 2000; Wang et al., 2005b; Xu et al., 2005a; Xu et al., 2005b), this has all been obtained in complex culture conditions, in which other contributory factors could play supportive roles. Prominent among those studies is the demonstration that high levels of FGF2 maintain long-term expression of pluripotency markers in hESCs grown in feeder-free conditions on Matrigel, by inhibiting a BMP4-like activity contained in Serum Replacer (Xu et al., 2005; Wang et al., 2005). We also found that FGF can maintain a high degree of Oct4 expression in hESCs cultured in CDM but only for a short period of time. Accordingly, it was important to augment such evidence for FGF sufficiency by determining whether it is also necessary for maintenance of pluripotency marker expression. This was accomplished by direct inhibition of FGF-receptor function using the inhibitor SU5402. hESCs treated with SU5402 during monolayer culture in CDM showed a 50% reduction in the fraction of Tra-1-60 cells, although the inhibitor effect was diminished substantially when hESCs were cultured in feeder-free conditions on Matrigel, when the cultures were supplemented with Activin, or when they expressed the Nodal transgene. Consistently with this latter observation, the positive effect of FGF2 on expression of pluripotency markers strictly depended on TGFβ pathway signalling activity, as demonstrated by the observation that FGF could not rescue differentiation induced by inhibiting Alk4/5/7 using SB431542. Therefore, FGF signalling is necessary but not sufficient to maintain pluripotency, and its effect(s) depends on TGFβ pathway signalling. This conclusion contradicts recent studies (Xu et al., 2005; Wang et al., 2005) showing that high doses of FGF permits growth of hESCs in feeder-free conditions by inhibiting BMP4 contained in the Serum Replacer. However, those experiments were performed on Matrigel containing TGFβ1 and in Serum Replacer containing unknown factors which could explain the effects they observed (Kleinman et al., 1982; Vukicevic et al., 1992). Moreover, CDM does not contain any BMP-like activity, yet FGF is still necessary to achieve long-term maintenance of hESCs grown in our culture conditions. Therefore, the FGF function in pluripotency cannot be explained solely by the regulation of BMP activity. In addition, inhibition of TGFβ pathway signalling using the SB inhibitor in high FGF2 culture conditions on Matrigel without conditioned medium (Wang et al., 2005b; Xu et al., 2005a; Xu et al., 2005b) also induced differentiation (data not shown). In view of the modest effect of added TGFβ1 and BMP4 in monolayer cultures of hESCs in CDM, the most obvious candidates for implementing the pluripotency-enhancing effects induced by FGF2 in our experimental conditions are Activin and Nodal.

Interactions between the FGF and TGFβ signalling pathways have been described in *Xenopus* and zebrafish, where FGFs work as a competence factor for Activin/Nodal signalling during mesendoderm differentiation (Cornell and Kimelman, 1994; Cornell et al., 1995). A recent analysis of the role of FGF3 and FGF8 in zebrafish development led to a model in which Nodal-induced transcription of *One eyed pinhead*, a homologue of the Nodal cofactor Cripto, is regulated by FGF (Mathieu et al., 2004). Our results suggest that Cripto transcription is indeed enhanced by FGF2. However, as exogenous soluble Cripto does not take the place of FGF in hESCs treated with SU5402, this mechanism would not account for the positive effects of FGF on the Activin/Nodal pathway unless Cripto activity were limited to its membrane-bound form as in *Xenopus* embryos (Sakuma et al., 2002), rather than being effective in its soluble form as in mESCs (Minchiotti et al., 2001; Parisi et al., 2003). In addition, it is possible that FGFs acts through other mechanisms in hESCs. One such mechanism is the phosphorylation of the linker region of Samd2, thereby preventing its nuclear localisation (Grimm and Gurdon, 2002; Kretzschmar et al., 1999; Lehmann et al., 2000). However, we found no effect of FGF2 on Phospho-Smad2 localisation in hESCs. Another potential mechanism is the modulation of BMP signalling,

preventing the nuclear localisation of activation of Phospho-Smad1/5/8 (Kretzschmar et al., 1997). Once again, we found no evidence of such an effect in hESCs. Recent studies suggesting that FGF2 acts through the PI3K/Akt/PKB pathway in hESCs, provide an alternative route whereby it could act as a competency factor for Activin/Nodal signalling (Kim et al., 2005). However, a direct activation of PI3K/Akt/PKB by FGF receptors was not shown in that study, and all their experiments were performed on feeders, which can alter both the extracellular matrix and growth factor composition of the culture environment. Therefore, additional studies using fully defined conditions are needed to correlate those findings with the present results.

Finally, recent studies in our laboratory suggest that recombinant Wnt protein can rescue the inhibition of FGF activity by the SU inhibitor (our unpublished observation), therefore suggesting that intermediates in the Wnt signalling pathway are a target of FGF signalling in hESCs. These results could explain the positive effect of BIO, a GSK3β inhibitor, on the maintenance of hESCs pluripotency (Sato et al., 2004). Interestingly, it has recently been proposed that GSK3β inhibition was necessary for pluripotency of mouse ESCs with increased stability of cMyc (Cartwright et al., 2005). As a consequence, an alternative mechanism for the function of FGF signalling in hESCs could be to maintain Myc activity in hESCs. Further studies at the molecular level are necessary to examine this among other alternative, viable hypotheses.

The role of TGFβ signalling in vivo in maintaining pluripotency

The preceding observations raise an important question concerning the relevancy of Activin/Nodal signalling to the development of pluripotent cells in vivo. To place the Activin/Nodal effects observed in hESCs in the context of normal mammalian development, it is worthwhile considering their role in early mouse embryogenesis, because human embryos have not yet been studied in molecular detail at the corresponding developmental stages. Although the canonical effect of Nodal signalling in vertebrate development is induction of mesoderm and endoderm differentiation (Schier, 2003), several mutations associated with Nodal signalling lead to decreased proliferation of the epiblast (Conlon et al., 1991; Dunn et al., 2004; Gu et al., 1998; Gu et al., 1999), suggesting a potential function for Activin/Nodal signalling even before the induction of mesendoderm differentiation. Consistently with this, embryos mutant for Nodal failed to express Oct4 in their epiblast cells at stages preceding gastrulation (Brennan et al., 2001; Robertson et al., 2003). Taken together with observations in this study on the importance of Activin/Nodal signalling in hESCs, the in-vivo phenotype of Activin/Nodal signalling deficiency leads to the hypothesis that maintenance of pluripotency during epiblast development in mammalian embryos depends on TGFβ pathway signalling (Vallier and Pedersen, 2005). Indeed, our preceding study showed that Nodal overexpression can block neuroectoderm differentiation, suggesting that an early function of Nodal, before the induction of the primitive streak, is to block epiblast development along a neuroectodermal default pathway (Vallier et al., 2004a; reviewed in Vallier and Pedersen, 2005). Further studies in vivo are needed to examine the predictions of this hypothesis, including the analysis of markers of neuroectoderm differentiation in mouse Nodal mutants and the development of mESCs overexpressing Nodal (Pfendler et al., 2005).

The hypothesis of Nodal-mediated epiblast pluripotency potentially reconciles certain compelling differences between mouse and human ESCs (Ginis et al., 2004). LIF and BMP4 have been shown to maintain pluripotency of mESCs by inhibiting mesendoderm differentiation and neuronal differentiation, respectively (Qi et al., 2004; Ying et al., 2003). However, the mechanism of cooperative lineage-restriction as proposed by Ying et al. (Ying et al., 2003) does not seem to apply to hESCs because the LIF pathway is not active (Daheron et al., 2004; Humphrey et al., 2004) and BMP4 induces differentiation along the trophectoderm pathway (Gerami-Naini et al., 2004; Xu et al., 2002). It seems unlikely that the interplay of FGF and Activin provides the equivalent of LIF and BMP4 by cooperatively inhibiting differentiation in hESCs. First, members of the FGF family including FGF4 and FGF8 are known to be involved in inducing mesendoderm differentiation in numerous systems (Feldman et al., 1995; Sun et al., 1999). Second, high doses of Activin/Nodal are sufficient to block hESC differentiation whereas BMP4 drives differentiation of mESCs in the absence of LIF (Ying et al., 2003). Third, Nodal overexpression results in absence of mesoderm differentiation during the formation of EBs (data not shown). Therefore, Activin/Nodal signalling appears to be the dominant pathway involved in maintaining hESC pluripotency, although it is strongly enhanced by cooperating with other pathways, particularly FGF.

This apparent distinction between mESCs and hESCs could be related to differences in the reproductive strategies of these two species (reviewed in Vallier and Pedersen, 2005). Mouse embryos generated during a postpartum oestrus can undergo development arrest (diapause) just before implantation, until suckling neonates complete their early postnatal development (Mantalenakis and Ketchel, 1966; Yoshinaga and Adams, 1966). The survival of epiblast cells in embryos undergoing diapause depends on LIF signalling (Nichols et al., 2001), suggesting that mESCs arise through in-vitro immortalisation of a cell type equivalent to the early epiblast cells of embryos blocked in a diapause stage. Human embryos, by contrast, do not undergo diapause, and it is therefore not surprising that their pluripotent cells are immortalised through different mechanisms. Moreover, because early human development is relatively protracted when compared with rodent species (Pera and Trounson, 2004), distinct mechanisms may exist to maintain the pluriptotent status of human epiblast cells during a prolonged period. One implication of this hypothesis is that Nodal and Activin have temporally distinct roles in regulating pluripotency. Indeed, Activin is expressed in the mouse blastocyst (Albano et al., 1993) whereas Nodal starts to be expressed in the epiblast after implantation (Conlon et al., 1994; Robertson et al., 2003) suggesting that these two factors could intervene sequentially to maintain pluripotency in vivo. This hypothesis is in agreement with our previous findings showing that Nodal overexpression blocks differentiation of hESCs at an early epiblast-like stage during the formation of EBs (Vallier et al., 2004a). Taken together, our findings suggest that Activin/Nodal signalling, with support from FGFs, have an important function during normal in-vivo development of both mouse and human embryos. Thus, a spectrum of in-vitro

hESC and in-vivo animal modelling studies could provide a powerful approach for understanding molecular mechanisms regulating pluripotency in mammals, including humans.

Moving towards feeder-free conditions for growing hESCs

The potential for clinical application of hESCs is promising and thus the development of chemically defined and animal product-free media is a major focus of human stem cell research. The CDM medium of Johanssen and Wiles (Johanssen and Wiles, 1995) supplemented with Activin and FGF represents a promising system for avoiding the use of other, more complex additives, including animal-derived feeder cells and extracellular matrix. The composition of CDM is precisely known and, to our knowledge, represents the simplest medium supporting the long-term growth of undifferentiated hESCs. In addition, the concentrations of FGF and Activin used in this study are relatively low compared with other feeder-free methods, thus making CDM plus Activin and FGF2 compatible with large-scale culture. However, CDM as used in our experiments still contains animal products (including BSA and FBS-derived matrix components). Therefore, the replacement of BSA by human albumin and the use of a well-defined extracellular matrix are needed, to develop culture conditions that are fully compatible with clinical applications. Once these problems are solved, the obvious remaining step will be to demonstrate that such fully-defined culture conditions can maintain long-term pluripotency of hESCs without inducing genetic or epigenetic alteration of the cells. An understanding of the mechanisms that control in-vitro maintenance of pluripotency is thus the first step towards the development of such culture conditions.

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