TGFβ/Activin/Nodal Pathway in Inhibition of Human Embryonic Stem Cell Differentiation by Mechanical Strain

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ABSTRACT Cyclic biaxial mechanical strain has been reported to inhibit human embryonic stem cell differentiation without selecting against survival of differentiated or undifferentiated cells. We show that TGFβ/Activin/Nodal signaling plays a crucial role in repression of human embryonic stem cell (hESC) differentiation under mechanical strain. Strain-induced transcription of TGFβ1, Activin A, and Nodal, and upregulated Similar to Mothers Against Decapentaplegic homolog (Smad)2/3 phosphorylation in undifferentiated hESC. TGFβ/Activin/Nodal receptor inhibitor SB431542 stimulated differentiation of hESCs cultured under biaxial strain. Exogenous addition of TGFβ1, Activin A, or Nodal alone was insufficient to stimulate hESC self-renewal to replicate behavior of hESCs in presence of strain. However, exogenous TGFβ1 and Activin A in combination partially replicated the self-renewing phenotype induced by strain but when combined with strain did not further stimulate self-renewal. In presence of mechanical strain, addition of a neutralizing antibody to TGFβ1 promoted hESC differentiation whereas inhibition of Activin A by Follistatin promoted hESC differentiation to a lesser extent. Together, these findings show that TGFβ superfamily activation of Smad2/3 is required for repression of spontaneous differentiation under strain and suggest that strain may induce autocrine or paracrine signaling through TGFβ superfamily ligands.

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells derived from the totipotent cells of preimplantation embryos and are capable of unlimited self-renewal in vitro when cultured under the appropriate conditions (1–3). ESCs can also differentiate into a large number of somatic cell types (4). Human embryonic stem cell (hESC) self-renewal and differentiation are regulated by extrinsic signals in the cell microenvironment (1,4,5). This microenvironment involves a complex interplay of short-term and long-range signals between stem cells, their differentiating progeny, and neighboring cells. Signals in the microenvironment may be encoded by a range of secreted factors, extracellular matrix (ECM) proteins, or cell–cell interactions mediated by integral membrane proteins (5).

Mechanical forces also play a role in self-renewal and differentiation decisions of hESCs. Cyclic biaxial stretch applied to a deformable substratum at 10% or greater strain, 6–30 cycles/min was found to inhibit the rate of spontaneous hESC differentiation and promote self-renewal, but did not affect cell growth rate or pluripotency (6). Mechanical inhibition of hESC differentiation could not be traced to secretion of chemical factors into the media suggesting that mechanical forces may directly regulate hESC differentiation. Mechanical strain was not sufficient to inhibit differentiation, however; in medium not conditioned by mouse embryonic fibroblasts (MEFs), hESCs exposed to strain differentiated at the same rate as cells cultured in the absence of strain. Thus, although mechanical forces play a role in regulating hESC self-renewal and differentiation, they must act synergistically with chemical signals delivered in the medium or ECM. Despite these observations, the cellular mechanisms by which hESCs sense and respond to mechanical stimuli have yet to be defined.

Models of early vertebrate development have indicated that transformation growth factor β (TGFβ) superfamily signaling plays an important role in the earliest cell fate decisions of embryogenesis in *Xenopus* (7) and in primitive streak and mesoderm formation in the mouse (8). TGFβ and Activin A have been reported to inhibit differentiation of hESC-derived embryoid bodies (EBs) to endodermal and ectodermal cells, but stimulate differentiation toward mesodermal (muscle) cells (9). Several studies have suggested that TGFβ is involved in the maintenance of hESC pluripotent status (10–12). TGFβ has been shown to be one of several growth factors that contribute to maintaining hESCs in an undifferentiated state in feeder-free culture (13). TGFβ/Activin/Nodal signaling in undifferentiated hESCs was required for the maintenance of markers of undifferentiated hESCs (14). Similar to mothers against decapentaplegic homolog (Smad)2/3 activation has also been reported to be necessary for maintenance of the self-renewing pluripotent state in hESCs (15).

TGFβ superfamily ligands bind to receptor complexes composed of TGFβ type I receptors (RI) and type II receptors (RII), also called activin-like kinase (Alk) receptors. These receptors feature two transmembrane glycoproteic domains, which are able to dimerize through cysteine residues after

Submitted August 14, 2007, and accepted for publication December 31, 2007.

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Editor: Jason M. Haugh.

0006-3495/08/05/4123/11 $2.00

doi: 10.1529/biophysj.107.119891

binding of a ligand. In mammals, seven type I receptors, Alk 1–7, and five type II receptors have been identified. Agonist binding induces activation of the serine/threonine kinase of RII domain, which then phosphorylates RI on specific serine and threonine residues present in the juxtamembrane glycine and serine-rich GS domain (16). Phosphorylation-dependent activation of Smads follows and leads to their nuclear translocation. The TGFβ superfamily of ligands signals through two main branches, the Smad1/5 branch and the TGFβ/Activin/Nodal branch. The Smad1/5 branch transduces BMP and GDF ligand signals via the RIs Alk1, Alk2, Alk3, and Alk6. The TGFβ/Activin/Nodal branch activates Smad2/3 via Alk4, Alk5, and Alk7 (17). Nodal and Activin share type I and II receptors and have the same Smad signaling pathway (Smad2/3), whereas TGFβ1 preferentially uses TGFβ1 receptors (Alk5, TβRII) and Smad2/3. After activation of the type I and type II receptor complexes, receptor Smads (Smad5/8 and Smad2/3) are phosphorylated. The common Smad, also known as Smad4, subsequently forms heteromeric complexes with the receptor Smads. This results in transcriptional activation and expression of target genes.

We hypothesized that mechanical strain activates the TGFβ/Activin/Nodal signaling pathway to repress spontaneous differentiation of hESCs. To test this hypothesis, we assessed changes in expression of TGFβ superfamily ligands in the presence of strain, and quantified the effects of these ligands and their inhibitors on cell differentiation in the presence and absence of strain. We found that strain induced TGFβ1, Activin A, and Nodal expression as well as activated Smad2/3 phosphorylation. Also, inhibition of TGFβ/Activin/Nodal signaling by SB435142, an Alk4/5/7 inhibitor, promoted hESC differentiation in the presence of mechanical strain. Addition of exogenous TGFβ1 and Activin A in combination partially repressed spontaneous differentiation, similar to the effects of strain. TGFβ1 neutralization and Activin A inhibition promoted hESC differentiation by mechanical strain. These results suggest that signaling through TGFβ1 ligands plays a crucial mechanistic role in repression of hESC differentiation under mechanical strain.

MATERIALS AND METHODS

Cell culture

hESC lines H1 and H9, passages 33–39, were cultured on MEF feeder layers or Matrigel (Becton Dickinson, Bedford, MA) coated plates as described (18). All media components were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified.

To obtain MEF-conditioned medium (CM), a T75 flask was coated with 10 ml of 0.1% gelatin solution and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. After discarding the gelatin solution, T75 flasks were immediately seeded with 3.8×10^6 irradiated (35 Gy γ-radiation) MEF cells in 15 ml MEF medium (90% DMEM, 10% FBS, 1% MEM nonessential amino acids solution) and incubated overnight at 37°C. The MEF medium was then aspirated and discarded. hESC medium (6) without bFGF (20 ml) was added to the MEF cells and incubated overnight for conditioning. The CM was collected every day for 2 weeks.

Culturing hESCs under strain

To prepare for experiments that tested the effects of mechanical strain on hESC differentiation, cell colonies were plated at a density of $1–4 \times 10^5$ cells per well, and cultured in CM medium supplemented with 4 ng/ml bFGF (CM/F+) on 6-well BioFlex culture plates coated with Matrigel. The Bioflex plates containing the hESCs were exposed to strain using a FX-4000T Flexercell Tension Plus (Flexcell International, Hillsborough, NC), without the loading station. This system provides equibiaxial strain over the center 80% of the membrane surface area (19). Most colonies outside this area detached from the membrane during culture. Strain rate and frequency were maintained constant during an experimental run. Cells cultured on unstrained Bioflex plates were treated in an identical manner and served as controls. Cyclic strain of desired magnitude was applied at 10 cycles/min unless otherwise noted. CM/F+ was exchanged daily with fresh medium.

The effect of TGFβ superfamily signaling on hESC culture was studied by adding 25 ng/ml Activin A (PeproTech, Rocky Hill, NJ), 10 ng/ml TGFβ1 (Pepro Tech), 50 ng/ml Nodal (R&D Systems, Minneapolis, MN), 25 μg/ml monoclonal mouse anti-TGFβ1 antibody (clone 1D11, R&D Systems), 100 ng/ml Follistatin (R&D Systems), or 20 μM SB435142 (Tocris Biosciences, Ellisville, MO).

Flow cytometry

Colonies were detached by adding 1 ml of 1 mg/ml collagenase in DMEM/F12 to each well of the 6-well plate and incubating the plate at 37°C for 10 min. The colonies were scraped off the plate and partially dissociated by gentle pipetting. The detached cells were resuspended in CM/F+ medium and pelleted. Cells were resuspended in 2 ml trypsin/EDTA supplemented with 2% chick serum and incubated in 37°C water bath for 10 min. The cells were diluted by a factor of 2 in CM/F+ and pelleted. Next, the cells were resuspended in FACS buffer (97.9% PBS w/o Ca^{2+}/Mg^{2+}, 2% FBS, 0.1% $NaNH_3$) and filtered through a 40-μm nylon cell strainer (BD Biosciences Discovery Labware, San Jose, CA). For Oct4 analysis, cells were fixed with 0.5% paraformaldehyde for 10 min at 37°C. The cells were then resuspended in 1 ml of ice-cold methanol and incubated on ice for 30 min. Cells were pelleted and resuspended in FACS buffer with 0.2% Triton X-100. The cell concentration was adjusted to 10^6 cells/ml. Cell suspension (100 μl) was treated with a primary anti-Oct4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) and incubated overnight at 4°C. For SSEA-4 analysis, filtered cells were washed twice in FACS buffer and diluted to 10^6 cells/ml. Cell suspension (100 μl) was treated with a primary SSEA-4 antibody (Santa Cruz Biotechnology; 1:50) and incubated for 30 min at 22°C. Cells labeled with anti-Oct4 and anti-SSEA-4 were then washed in FACS buffer containing 0.2% Triton X-100 and labeled with Alexa Fluor 488 $F(ab)_2$ fragment of rabbit anti-mouse IgG or goat anti-rabbit IgG (Molecular Probes, Eugene, OR; 1:1000) and incubated at 22°C for 30 min.

Undifferentiated hESCs served as the positive control for flow cytometry. For a negative control, mouse or rabbit IgG was substituted for the primary anti-Oct4 or anti-SSEA4 antibody. Flow cytometry was carried out using a FACS Calibur and CellQuest acquisition and analysis software (Becton Dickson).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized/blocked with 0.1% Triton X-100 in phosphate-buffered saline at room temperature for 45 min. After blocking, the cells were incubated at room temperature for 1 h with anti-Nanog (R&D Systems; 1:20) antibody. The cells were then washed with phosphate-buffered saline before addition of the secondary antibody fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, MO; 1:100). The cells were washed again before acquisition of fluorescence and phase contrast images. A sample of 1000 cells/well was counted for Nanog expression. Sample

identity was blinded to the researcher counting the fraction of Nanog-expressing cells. The data are reported as a mean ± SD from a total of four wells for each condition.

Western blotting

Cells were lysed with 500 μl/well lysis buffer (100 mM Tris [pH 7.2], 8 M urea). Cell lysates were homogenized with a sonicator (50% power, 10 s) and concentrated by filtering through 3 kD filter at 4750 rpm for 15 min. Quantification of protein concentration in the lysate was carried out using a BCA Protein Assay kit (Pierce Laboratories, Rockford, IL) according to manufacturer's instructions; 20 μg total protein samples were run on each lane. Gels were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked in TBST with 5% milk. Membranes were then stained with rabbit anti-phospho Smad2/3 antibody (Cell Signaling Technology, Beverly, MA) followed by secondary anti-rabbit antibody conjugated to horseradish peroxidase (Molecular Probes). For a loading control, anti-β-actin was used instead of the anti-phospho Smad 2/3 antibody. Membranes were developed for detection of proteins using ECL Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, UK).

ELISA analysis

TGFβ1 protein concentration in culture media was measured by Quantikine ELISA analysis (R&D Systems) according to manufacturer's instructions.

Quantitative polymerase chain reaction

Total RNA was extracted from hESCs using an RNAeasy Minikit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. DNase treatment was carried out on-column using RNase-free DNase Kit (Qiagen). Reverse transcription was carried out using 1 μg of total RNA in a final volume of 20 μl using Omniscript RT kit (Qiagen) according to manufacturer's instructions. Each RNA sample was reverse-transcribed in duplicate, and appropriate negative controls were included in each run. Gene-specific primer pairs were designed and evaluated for an annealing temperature of 60°C using Primer3 software. Primers were designed for the following genes: TGFβ1, Activin A, Nodal, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR samples were prepared using a Quantitect SYBR Green PCR kit (Qiagen) according to manufacturer's instructions. All quantitative polymerase chain reactions (QPCR) were carried out with SYBR Green in an iCycler (Bio-Rad). For QPCR, 1× Jump Start Buffer ×10 (Sigma-Aldrich), 3 mM $MgCl_2$ (Sigma-Aldrich), 0.3 mM dNTP mix (Sigma-Aldrich), 0.4x SYBR Green (Molecular Probes), 0.4 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 0.04 U/μl Jump Start *taq* polymerase (Sigma-Aldrich), and 2 μl cDNA template were used in a final volume of 20 μl. After an initial denaturation/activation step of 3 min at 95°C, 45 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C were carried out. The detection of fluorescent signal was carried out at 72°C in each cycle. C_t (threshold cycle) values were calculated using the iCycler software. All QPCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. QPCR analyses were conducted in two independent experiments with three replicates in each experiment.

RESULTS

Smad2/3 is phosphorylated under mechanical strain

Cyclic biaxial stretch applied to the deformable substratum at 10% strain, 10 cycles/min was found to inhibit hESC spontaneous differentiation and promote self-renewal, as measured by increases in Oct4 and SSEA-4 expression (6). To understand the role of TGFβ/Activin/Nodal signaling in the repression of hESC differentiation under mechanical strain, we first tested whether mechanical strain induces Smad2/3 activation downstream of the TGFβ superfamily receptors Alk4/5/7. hESCs were cultured on Matrigel-coated Bioflex plates and subjected to 10% cyclic biaxial strain at 10 cycles/min in the presence and absence of SB435142. Constant strain magnitude and frequency were maintained throughout each experiment. Cells cultured on Matrigel-coated Bioflex plates in the absence of strain, but otherwise treated in an identical manner, served as controls. All cells were cultured in MEF-conditioned medium supplemented with 4 ng/ml bFGF, changed daily, conditions that normally repress differentiation when cells are regularly passaged. The amount of phospho-Smad2/3 localized in the hESCs cultured for 7 days in presence and absence of strain (10%, 6 cycles/min) and Alk4/5/7 inhibitor SB435142 was determined by Western blotting (Fig. 1 *A*). After 7 days, hESCs cultured in the presence or absence of strain exhibited little spontaneous differentiation, indicated by high levels of Oct4 expression in the flow cytometric analysis (Fig. 1 *C*). In the absence of SB435142, significantly higher Smad2/3 phosphorylation was observed in strained cultures as compared to unstrained culture. SB435142 addition substantially diminished this strain-induced Smad2/3 phosphorylation. Similar results were also observed in strained and unstrained hESCs grown for 12 days in presence and absence of mechanical strain and/or SB435142 (Fig. 1 *B*).

TGFβ1, Activin A, and Nodal expression increases on application of mechanical strain

To address whether strain may induce autocrine or paracrine signaling via TGFβ superfamily ligands, which then would activate Alk4/5/7 receptor activity and induce Smad2/3 phosphorylation, we compared the expression of TGFβ1, Activin A, and Nodal in strained and unstrained hESCs via QPCR. hESCs were harvested and total RNA was extracted from strained and unstrained cultures at day 7 after passaging, at which time significant differences in Oct4 expression were not detected in strained and unstrained hESCs (Fig. 1 *C*). Expression levels of TGFβ1, Activin A, and Nodal in strained and unstrained hESCs were normalized by GAPDH expression in the respective samples to obtain fold increase (Fig. 2). TGFβ1 and Activin A expression were upregulated significantly under strain (fold increase = 8.3 ± 0.2 and 4.0 ± 0.2, respectively; $p < 0.05$ for both TGFβ1 and Activin A expression comparing strained to unstrained cultures). Nodal expression was also upregulated in strained hESC cultures but the magnitude of upregulation was lesser than TGFβ1 and Activin (fold increase = 1.9 ± 0.1; $p < 0.05$). These results suggest that TGFβ1, Activin A, and Nodal ligands might play a role in the activation of the TGFβ1/Activin/Nodal pathway in hESCs under mechanical strain.

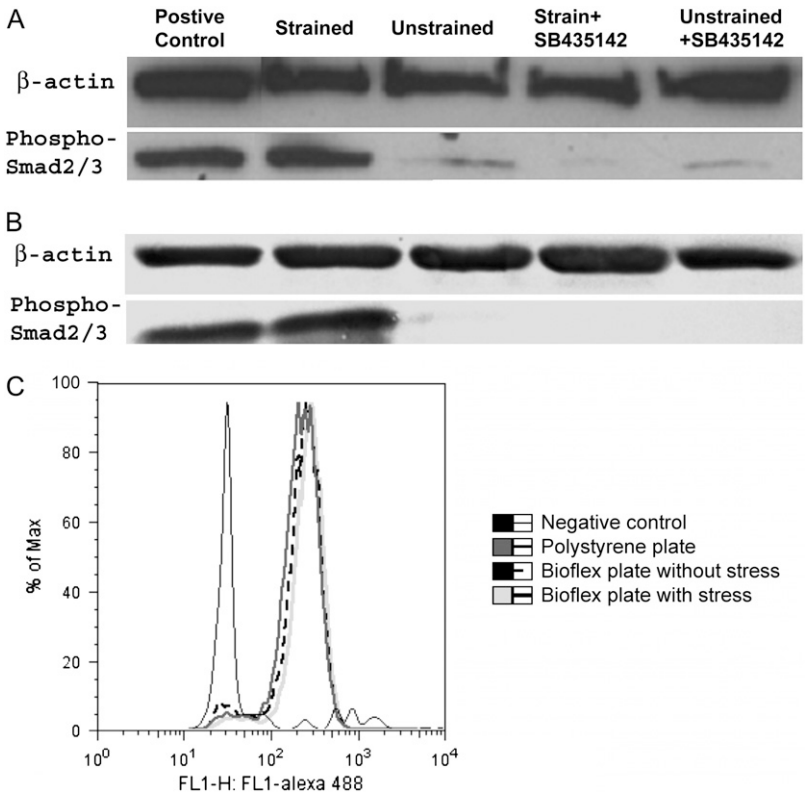

FIGURE 1 Changes in phosphorylated Smad2/3 in the presence or absence of mechanical strain and SB435142. hESCs were cultured for (*A*) 7 days or (*B*) for 12 days. (*Lane 1*) Undifferentiated hESCs grown for 5 days serving as positive control. (*Lane 2*) hESCs grown in presence of mechanical strain (10%, 10 cycles/min). (*Lane 3*) hESCs grown in absence of mechanical strain. (*Lane 4*) hESCs grown in presence of mechanical strain (10%, 10 cycles/min) and SB435142 (20 μM). (*Lane 5*) hESCs grown with SB435142 (20 μM) and in absence of mechanical strain. Phosphorylation of Smad2/3 was evaluated by probing cell lysates with a phospho-specific antibody directed against Smad2/3. (*C*) Flow cytometric analysis of Oct4 levels in cells grown in the presence or absence of 10% average membrane strain, 10 cycles/min for 7 days shows little difference in number of cells expressing Oct4 between strained cells cultured on a Bioflex plate (*solid light gray line*), unstrained cells on a Bioflex plate (*dashed black line*), and positive control cells maintained on Matrigel-coated polystyrene (*solid dark gray line*). The solid black line represents a no primary antibody control staining of hESCs.

Inhibition of TGFβ/Activin/Nodal signaling represses strain-mediated self-renewal in hESCs

To identify time points at which strain does and does not repress spontaneous differentiation, we examined hESC cultures grown in the presence and absence of strain and the presence and absence of SB435142 for expression of Oct4, Nanog, and SSEA-4 7 and 12 days postplating. Oct4 and SSEA-4 expression were quantified by flow cytometry and Nanog expression by immunocytochemistry. Seven days postplating, strained and unstrained cultures exhibited similar levels of hESC marker expression (Fig. 3; $p > 0.05$ for all markers). SB435142 increased spontaneous differentiation in both strained and unstrained populations at day 7, consistent with a prior report by James et al. (14). After 12 days in culture, samples exposed to strain in the absence of SB435142 exhibited substantially higher expression of Oct4, Nanog, and SSEA-4 as compared to unstrained samples (Fig. 4; $p < 0.05$ for all markers). However, strain was unable to repress differentiation in the presence of SB435142.

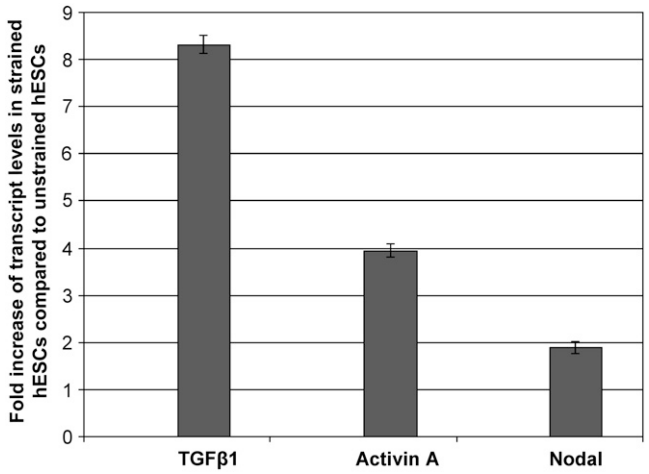

FIGURE 2 Expression of TGFβ1, Activin A, and Nodal in strained and unstrained hESCs. QPCR analysis of marker transcripts (TGFβ1, Activin A, and Nodal) was carried out after 6 days of hESC culture at 10% strain, 10 cycles/min. Assays were carried out in two independent experiments, with three replicates in each experiment, and are shown as the fold increase of strained hESCs over unstrained hESCs $\pm$ SE. Expression of each gene was normalized to GAPDH expression. For each gene tested, $p < 0.05$ comparing strained and unstrained cultures.

Measuring hESC marker expression at 7 and 12 days postplating allowed investigation of the effects of TGFβ pathway signaling at a time point when unstrained cells were largely undifferentiated and where strain did not repress differentiation (7 days) and a time point at which most unstrained cells had spontaneously differentiated but strained cells remained largely undifferentiated (12 days). SB435142 promoted hESC differentiation in both the presence and absence of strain 7–12 days postplating, although effects were much greater at 12 days. The requirement of Alk4/5/7 activity in strain-induced self-renewal is consistent with strain directly mediating self-renewal through this pathway, or through a parallel pathway that requires TGFβ superfamily signaling.

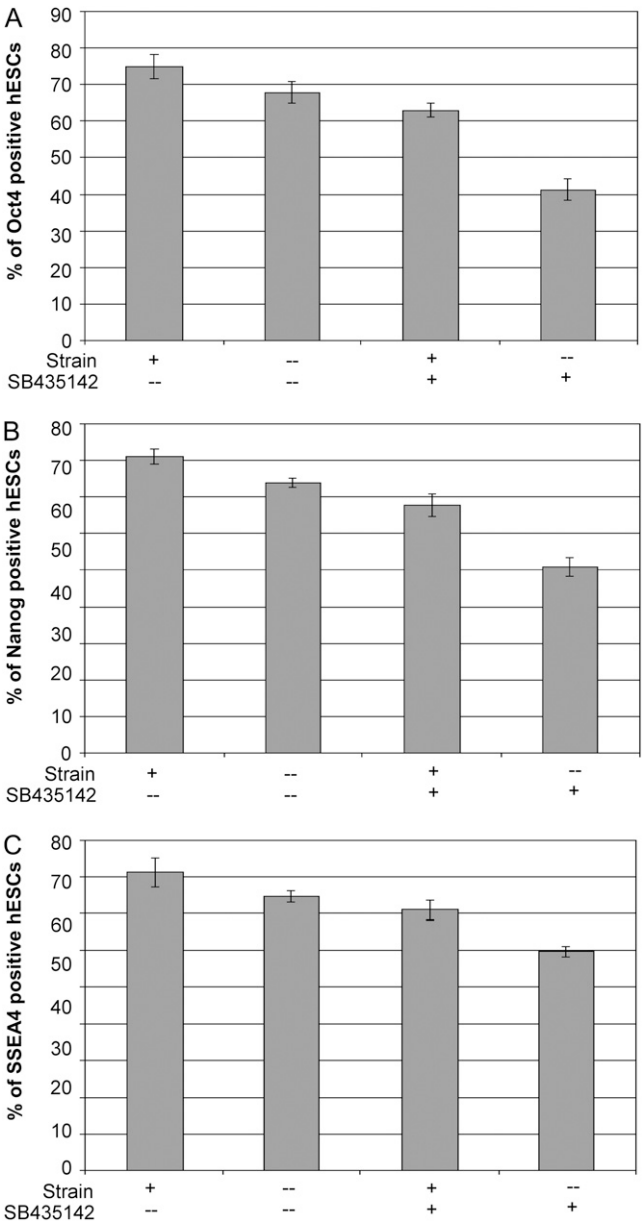

FIGURE 3 Effect of Alk4/5/7 receptor inhibitor SB435142 on Oct4 (*A*), Nanog (*B*), and SSEA-4 (*C*) expression in the presence and absence of mechanical strain after 7 days of culture. hESCs were grown in CM/F+ medium on Matrigel-coated Bioflex plates for 7 days in the presence or absence of mechanical strain (10%, 10 cycles/min) and SB435142 (20 μM). Oct4 and SSEA-4 expression were quantified by flow cytometry. Cells falling in the gated region, determined by hESC staining with nonspecific mouse IgG rather than an anti-Oct4 or anti-SSEA-4 antibody, were considered positive. Flow cytometry assays were carried out in triplicate samples in at least three independent experiments and are shown $\pm$ SE. Nanog expression was quantified from observer-blinded analysis of immunocytochemistry images. At least 1000 cells, taken from four samples from two independent experiments, were analyzed for each condition. Data are shown as the percentage of Nanog-expressing cells $\pm$ SE.

Addition of exogenous TGFβ1, Activin A, or Nodal to unstrained hESCs does not replicate repression of hESC differentiation observed in strained hESCs

Having determined that Alk4/5/7 receptor function and Smad2/3 phosphorylation play roles in repression of hESC differentiation by mechanical strain, we investigated whether addition of exogenous TGFβ1, Activin A, or Nodal to unstrained cultures was sufficient to inhibit hESC differentiation to the same extent as mechanical strain. Colonies were allowed to attach to Matrigel-coated Bioflex plates for 2 days and were then cultured for 10 additional days in MEF–conditioned medium in the presence or absence of 10% strain at 10 cycles/min. At this time point, significant spontaneous differentiation was observed in the absence of strain but not in the presence of strain (Fig. 5), consistent with results shown in Fig. 4. MEF-conditioned medium containing 4 ng/ml bFGF was also supplemented with TGFβ1 (10 ng/ml), Activin A (25 ng/ml), or Nodal (50 ng/ml). Medium was changed daily in each experiment. The effect of the growth factors on spontaneous differentiation was determined by flow cytometric assessment of the fraction of cells expressing Oct4.

Significantly greater spontaneous differentiation was observed in the absence of strain than in the presence of strain (21% vs. 67% of cells expressing Oct4; Fig. 5, *A* and *B*). In the absence of strain, addition of Activin A or TGFβ1 did not result in any significant increase in the fraction of cells expressing Oct4 (Fig. 5, *A* and *B*), suggesting that exogenous TGFβ1 or Activin A alone are not sufficient to prevent differentiation in long term unstrained cultures and cannot solely account for the effects of strain on hESC differentiation. Addition of SB435142 resulted in higher levels of differentiation in strained cultures supplemented with TGFβ1 or Activin A.

In the absence of strain, addition of exogenous Nodal (50 ng/ml) produced a slight repression of hESC differentiation; 31% of cells expressed Oct4 in presence of Nodal as compared to 19% in absence of Nodal (Fig. 5 *C*). This partial repression of hESC differentiation by Nodal was negated by addition of SB435142. As with TGFβ1 and Activin A, Nodal alone is not sufficient to explain the extent of repression of differentiation caused by strain.

Addition of exogenous TGFβ1 and Activin A together partially represses hESC differentiation in the absence of mechanical strain

Next we investigated whether combinations of exogenous TGFβ superfamily ligands were sufficient to repress hESC differentiation of unstrained cultures to levels observed in strained hESCs. hESC colonies were allowed to attach to Matrigel-coated Bioflex plates for 2 days and were then cultured for 10 additional days in MEF-conditioned medium

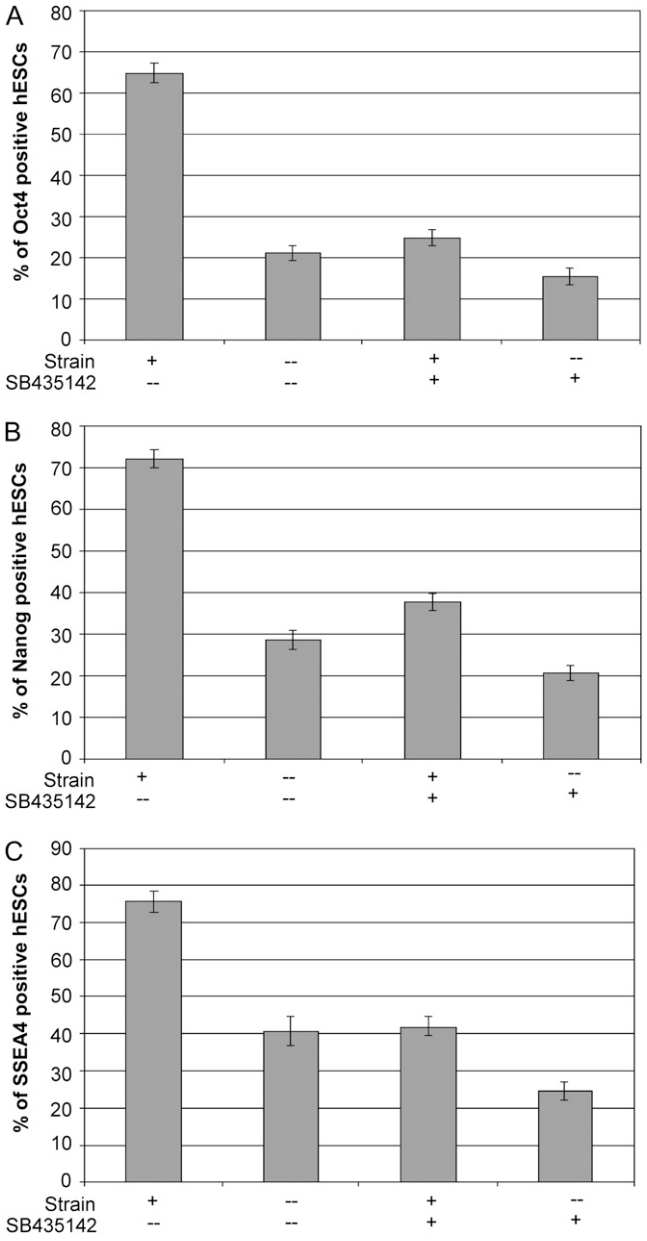

FIGURE 4 Effect of Alk4/5/7 receptor inhibitor SB435142 on Oct4 (*A*), Nanog (*B*), and SSEA-4 (*C*) expression in the presence and absence of mechanical strain after 12 days of culture. hESCs were grown in CM/F+ medium on Matrigel-coated Bioflex plates for 12 days in the presence or absence of mechanical strain (10%, 10 cycles/min) and SB435142 (20 μM). Oct4 and SSEA-4 expression were quantified by flow cytometry. Cells falling in the gated region, determined by hESC staining with nonspecific mouse IgG rather than an anti-Oct4 or anti-SSEA-4 antibody, were considered positive. Flow cytometry assays were carried out in triplicate samples in at least three independent experiments and are shown $\pm$ SE. Nanog expression was quantified from observer-blinded analysis of immunocytochemistry images. At least 1000 cells, taken from four samples from two independent experiments, were analyzed for each condition. Data are shown as the percentage of Nanog-expressing cells $\pm$ SE.

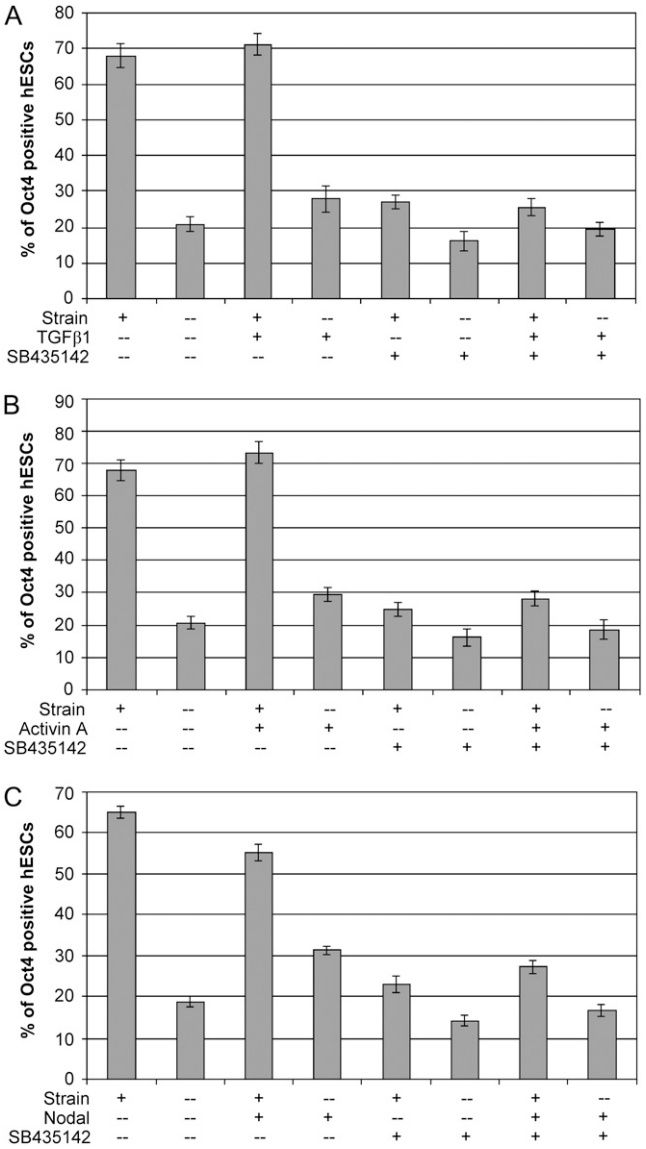

FIGURE 5 Effect of exogenous TGFβ1, Activin A, Nodal on hESC differentiation in the presence and absence of mechanical strain and SB435142. (*A*) Flow cytometric analysis of Oct4 levels in hESCs after 12 days of hESC culture in presence or absence of mechanical strain (10%, 10 cycles/min), TGFβ1 (10 ng/ml), or SB435142 (20 μM). (*B*) Flow cytometric analysis of Oct4 levels after 12 days in presence or absence of mechanical strain (10%, 10 cycles/min), Activin A (25 ng/ml), or SB435142 (20 μM). (*C*) Flow cytometric analysis of Oct-4 levels after 12 days in presence or absence of mechanical strain (10%, 10 cycles/min), Nodal (50 ng/ml), or SB435142 (20 μM). Data shown are representative results from one of at least two independent experiments, each experiment containing at least three replicates, and are shown $\pm$ SE. Cells falling in the gated region, determined by hESCs stained with nonspecific mouse IgG rather than an anti-SSEA-4 antibody, were considered Oct4-positive.

supplemented with TGFβ1 (10 ng/ml) and Activin A (25 ng/ml), in the presence or absence of 10% strain at 10 cycles/min. Seventy percent of strained hESCs expressed Oct4 whereas 22% of unstrained hESCs expressed Oct4 (Fig. 6 *A*). Addition of Activin A and TGFβ1 together to unstrained

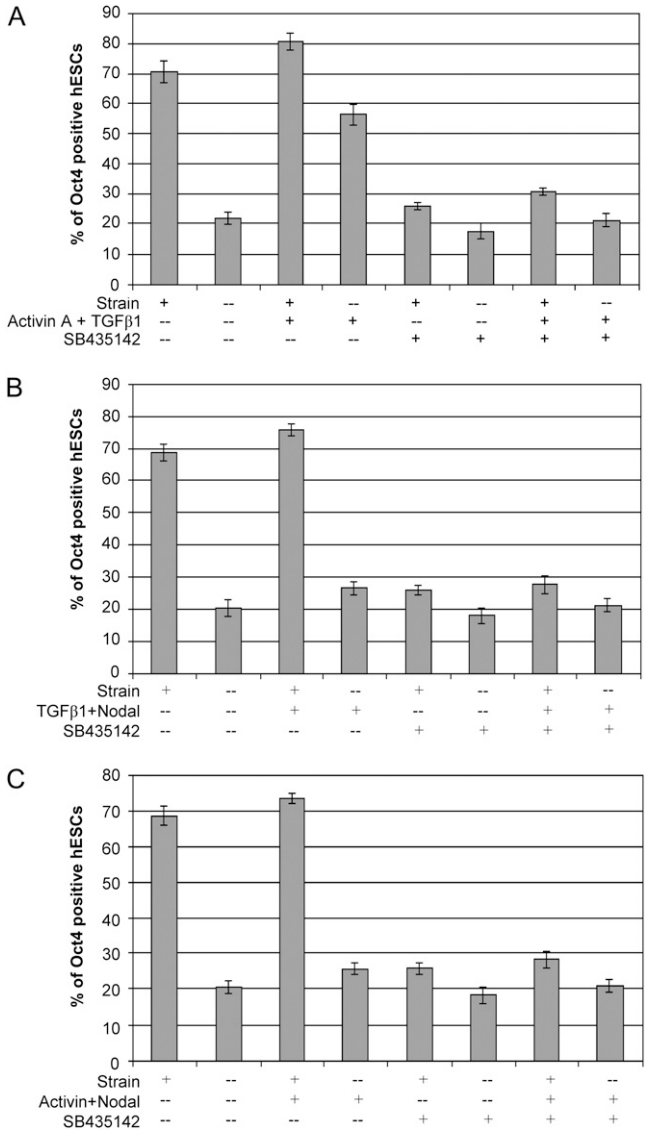

FIGURE 6 (*A*) Effect of TGFβ1 and Activin A in combination on hESC differentiation in the presence and absence of mechanical strain and SB435142. Oct4 expression was evaluated by flow cytometric analysis after 12 days of culture in presence or absence of TGFβ1 (10 ng/ml) and Activin A (25 ng/ml), mechanical strain (10%, 10 cycles/min), or SB435142 (20 μM). (*B*) Effect of Nodal and Activin A in combination on hESC differentiation in the presence and absence of mechanical strain and SB435142. Oct4 expression was evaluated by flow cytometric analysis after 12 days of culture in presence or absence of Nodal (50 ng/ml) and Activin A (25 ng/ml), mechanical strain (10%, 10 cycles/min), or SB435142 (20 μM). (*C*) Effect of TGFβ1 and Nodal in combination on hESC differentiation in the presence and absence of mechanical strain and SB435142. Oct4 expression was evaluated by flow cytometric analysis after 12 days of culture in presence or absence of TGFβ1 (10 ng/ml) and Nodal (50 ng/ml), mechanical strain (10%, 10 cycles/min), or SB435142 (20 μM). Data shown are representative results from one of at least two independent experiments, each experiment containing at least three replicates, and are shown $\pm$ SE. Cells falling in the gated region, determined by hESC staining with nonspecific mouse IgG rather than an anti-SSEA-4 antibody, were considered Oct4-positive.

hESC cultures enhanced Oct4 expression significantly (59%). Addition of TGFβ1 and Activin A did not produce a significant change in hESC differentiation levels under strain as compared to the strained hESC culture grown without TGFβ1 or Activin A ($p = 0.09$). The enhanced self-renewal observed by adding TGFβ1 and Activin A to unstrained cultures was negated by addition of SB435142. This result indicates that Alk4/5/7 inhibition overrides the effects of TGFβ1 and Activin A in presence or absence of mechanical strain, as expected. Higher levels of differentiation observed in strained cultures supplemented with SB435142 both in the presence and absence of TGFβ1 and Activin A suggest potential role of TGFβ1/Activin A pathway signaling through Smad2/3 in mechanical repression of hESC differentiation.

We also investigated whether other combinations of exogenous TGFβ superfamily ligands were sufficient to repress differentiation of unstrained hESCs to levels observed in strained hESCs. Strained cultures exposed to TGFβ1 and Nodal exhibited significantly higher Oct4 expression than unstrained cultures (69% vs. 21%; Fig. 6). Addition of Nodal and Activin A to unstrained cultures did not repress hESC differentiation, however, with 26% of hESCs expressing Oct4. Higher levels of differentiation were observed in strained and unstrained cultures supplemented with SB435142 both in presence and absence of Nodal and Activin A.

Addition of Nodal plus TGFβ1 to unstrained cultures did not increase the fraction of cells expressing Oct4 to levels observed in strained hESC cultures (Fig. 6). In strained cultures, addition of Nodal and TGFβ1 together only marginally increased the fraction of hESCs expressing Oct4. Addition of SB435142 to strained and unstrained cultures resulted in higher levels of differentiation both in the presence and absence of TGFβ1 and Nodal. These results indicate that addition of Nodal and TGFβ1 together does not have a significant effect on hESC differentiation either in the presence or absence of strain.

TGFβ1, but not Activin A, is necessary for strain-mediated repression of hESC differentiation

Having observed that TGFβ superfamily ligand expression was induced by strain, that a combination of Activin A and TGFβ1 repressed spontaneous differentiation of unstrained hESCs to similar levels as observed in strained hESCs, and that signaling through Smad2/3 was required for inhibition of differentiation under strain, we proceeded to investigate whether TGFβ1 and Activin A sequestration affected hESC differentiation under strain. TGFβ1 was blocked with a neutralizing antibody and Activin A with follistatin. hESCs were allowed to attach to Matrigel-coated Bioflex plates for 2 days and were then cultured for either 5 additional days or 10 additional days in MEF–conditioned medium in the presence or absence of 10% strain at 10 cycles/min. After 7 days of culture, anti-TGFβ1 blocking antibody marginally increased

spontaneous differentiation both in presence and absence of strain (Fig. 7 *A*). Significant spontaneous differentiation was observed in the absence of strain but not in the presence of strain after 12 days of culture (Fig. 7 *B*). At 12 days, the anti-TGFβ1 blocking antibody promoted hESC differentiation in presence of strain, reducing Oct4 expression to near the level observed in cells cultured in the absence of strain. In absence of strain, similar levels of differentiation were observed in presence and absence of the anti-TGFβ1 antibody. These results are consistent with a requirement of TGFβ1 signaling during strain-mediated self-renewal.

The fraction of strained cells expressing Oct4 when exposed to Activin inhibitor, follistatin, was similar to the fraction of Oct4-expressing cells cultured under mechanical strain in the absence of follistatin after 7 days in culture (64% vs. 69%, Fig. 7). After 12 days in culture, follistatin slightly reduced the fraction of Oct4 expressing cells exposed to strain (50% vs. 65%). However, follistatin did not diminish Oct4 expression to the level observed in unstrained cells (19%) at 12 days, suggesting Activin A is not the primary mechanism of strain-mediated self-renewal. In the absence of mechanical strain, addition of follistatin had no effect on the fraction of cells expressing Oct4 at either 7 days or 12 days postplating.

Together these results suggest that activation of the TGFβ1/Activin/Nodal pathway by mechanical strain, resulting in inhibition of hESC differentiation is primarily guided through TGFβ1 ligands, whereas Activin A may play a complementary role.

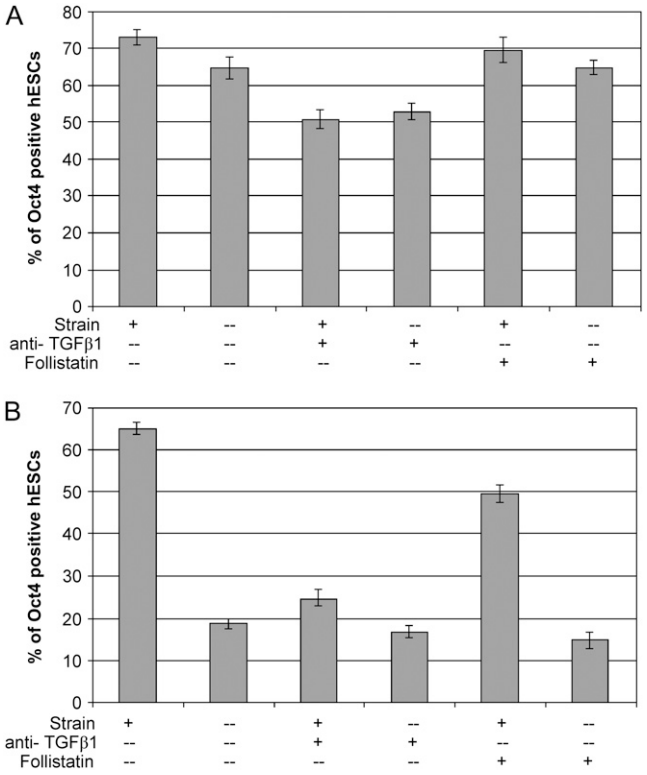

FIGURE 7 Effect of TGFβ1 blocking antibody and Activin inhibitor follistatin on hESC differentiation in the presence and absence of mechanical strain. hESCs were grown in CM/F+ medium on Matrigel-coated Bioflex plates for (*A*) 7 days or (*B*) 12 days. Flow cytometric analysis of Oct4 levels in hESCs grown in the presence or absence of mechanical strain (10%, 10 cycles/min), anti-TGFβ1 antibody (25 μg/ml), or follistatin (100 ng/ml). Data shown are representative results from one of at least two independent experiments, each experiment containing at least three replicates, and are shown as mean $\pm$ SE for all samples. Cells falling in the gated region, determined by hESC staining with nonspecific mouse IgG rather than an anti-Oct4 antibody, were considered Oct4-positive.

DISCUSSION

Mechanical strain has been reported to stimulate self-renewal of hESCs cultured in MEF conditioned medium (6). In this study, we obtained insight into molecular pathways required for mechanical inhibition of hESC differentiation. Mechanical strain induced Smad2/3 phosphorylation in undifferentiated hESCs and a pharmacologic inhibitor of Alk4/5/7 repressed this phosphorylation. These results indicate that TGFβ/Activin/Nodal signaling plays an important role in synergizing mechanical strain with chemical factors during hESC self-renewal and differentiation decisions. Application of strain induced TGFβ1, Activin A, and Nodal expression in hESCs, suggesting a potential autocrine or paracrine signaling mechanism that represses differentiation. In the absence of mechanical strain, TGFβ1 and Activin A in combination significantly repressed hESC spontaneous differentiation to similar levels as observed in strained hESC cultures. Alk4/5/7 inhibition overrode this effect, indicating that Alk4/5/7 receptor function is essential for repression of hESC differentiation by TGFβ1 and Activin A and by strain. However, TGFβ1, Activin A, or Nodal individually did not significantly change the extent of hESC differentiation in the presence and absence of mechanical strain as compared to hESCs cultured without the growth factors. Under mechanical strain, blocking TGFβ1 promoted hESC differentiation, whereas antagonizing Activin A signaling by follistatin promoted hESC differentiation under strain to a lesser extent. These results suggest that activation of the TGFβ1/Activin/Nodal pathway by mechanical strain mimics inhibition of hESC differentiation through TGFβ1 ligands.

Our findings from a combination of growth factor addition and receptor inhibition experiments provide substantial evidence to support the hypothesis that TGFβ/Activin/Nodal signaling through TGFβ1 and Activin A plays a key role in repressing hESC spontaneous differentiation under mechanical strain. Activin and TGFβ1 in combination with other factors have shown to support hESCs grown on Matrigel or on fibronectin, in medium supplemented with Serum Replacer (10,20).

SB435142 has been shown to specifically inhibit the receptors responsible for both Activin/Nodal (Alk4, Alk7) and TGFβ1 (Alk5) signaling through Smad2/3 in most of cell

types studied to date (14,21–23). In vitro, the IC_{50} values against Alk4, 5, and 7 were 1 μM, 0.75 μM, and 2 μM, respectively. SB435142 exhibited no significant effect on the kinase activity of the other known Alk receptors at 10 μM (Alk1, 2, 3, and 6) (14). Likewise, inhibitory activity of SB435142 against a panel of kinases, including JNK1, MAPK2, MKK1, p38 MAPK, MEK1, and others was not detected (14). Alk4/5/7 inhibition has been reported to induce differentiation of hESCs grown on Matrigel in presence of conditioned medium (15). SB435142 suppressed Smad2/3 phosphorylation and also reduced fraction of Tra-1-60-positive hESCs in a dose-responsive and time-dependent manner (14). SB435142 did not affect hESC viability or proliferation and did not significantly affect activation of other pathways (14). Our results indicate that SB435142 suppressed Smad2/3 phosphorylation in strained cells to levels similar to those observed unstrained hESCs (Fig. 1). These results suggest that Alk4/5/7 signaling either mediates mechanotransduction or acts in a parallel, but synergistic pathway.

In this study we have attempted to distinguish the roles of specific TGFβ superfamily ligands (TGFβ1, Activin A, and Nodal) in inhibiting hESC differentiation by mechanical strain. Nodal and Activin A share type I and II receptors and activate the same Smad signaling pathway (Smad2/3), whereas TGFβ1 acts through TGFβ1 receptors (Alk5, TβRII) and also activates same Smad2/3. Although Nodal was sufficient to maintain pluripotency marker expression in hESCs, including Oct4, in monolayer cultures in absence of other growth factors (24), Nodal did not seem necessary for the maintenance of pluripotency in standard culture conditions (15). Activin A and Nodal, but not TGFβ1, have been reported to be able to sustain expression of pluripotency markers in a substantial fraction of hESCs either in adherent conditions or during formation of embryoid bodies (15). In this study, addition of Nodal slightly increased the fraction of Oct4 positive cells. Nodal expression was upregulated in strained hESCs to a smaller extent than TGFβ1 and Activin A expression (Fig. 2) suggesting that Nodal ligands might not be playing an important role in activation of the TGFβ1/Activin/Nodal pathway by mechanical strain.

Such evidence also implies that TGFβ1 and Activin ligands might play an important role in activation of Smad2/3 by mechanical strain. Treatment with follistatin resulted in only a slight increase in hESC differentiation under mechanical strain (Fig. 7) providing evidence that Activin A may not be primarily responsible for Smad2/3 activation by mechanical strain. Addition of Activin A alone did not affect extent of hESC self-renewal in the presence or absence of strain (Fig. 5). However, culture medium enriched with Activin A has been reported to be capable of maintaining hESCs in the undifferentiated state for $>$20 passages on laminin without the need for feeder layers, medium conditioned by MEFs, or STAT3 activation (10). Activin A, which is secreted by MEF feeders (10), was found to be necessary and sufficient to maintain pluripotency of hESCs cultured on Matrigel (25). Activin A alone has also been reported to be sufficient to prevent differentiation for short culture periods (1 week) in the absence of other TGFβ ligands or FGF (15). In these experiments, hESCs were cultured in feeder-free conditions in chemically defined medium (26) without Matrigel or Serum Replacer (15) or in nonconditioned medium supplemented with Activin (25). In our study, hESCs were cultured on Matrigel for 12 days in MEF-conditioned medium containing Serum Replacer and supplemented with bFGF. The difference in culture conditions may account for the higher levels of hESC differentiation observed in our unstrained cultures supplemented with Activin A alone. Strain increased Activin A expression, suggesting possible autocrine or paracrine signaling that then regulates differentiation. Inhibition of Activin A by follistatin increased differentiation in strain cells to a small extent only, suggesting a secondary role of Activin ligands.

TGFβ1 expression was upregulated in strained hESCs to a greater extent than Activin A or Nodal (Fig. 4). Blocking TGFβ1 with a neutralizing antibody resulted in significant increase in hESC differentiation under mechanical strain, consistent with the notion of autocrine or paracrine signaling. We also used ELISA to quantify the TGFβ1 protein concentration in bulk media conditioned by strained and unstrained cultures at multiple time points after application of strain (data not shown). TGFβ1 concentrations were not found to be significantly different in the media conditioned by unstrained and strained cultures at any of the time points tested. This finding is consistent with a previous report that medium conditioned by cells exposed to strain does not promote self-renewal of cells cultured in a static environment (6). This experiment does not preclude the possibility that there might be changes in localized or short-lived TGFβ1 concentration that is not present in the bulk medium, however (i.e., autocrine signaling). Also, it is not entirely clear whether undifferentiated hESCs or a subpopulation of differentiating cells upregulate TGFβ1 superfamily ligand expression under strain. The data in Figs. 1 and 2 suggest that the population of strained and unstrained cells express Oct4 at similar levels as undifferentiated cells at 7 days after plating on Bioflex plates, but it is possible that a subpopulation of Oct4-positive differentiating cells is responsible for TGFβ1 superfamily ligand expression under strain.

The TGFβ/Activin/Nodal signaling pathway seems to be required for inhibition of hESC differentiation by mechanical strain. However, the mechanisms by which cells sense strain and convert strain to chemical signal transduction pathways (e.g., TGFβ/Activin/Nodal) remain unknown. One possible scenario involves reorganization or stimulation of cell–ECM and/or cell–cell adhesions by strain. Mechanical stimulation changes the expression of E-cadherin/catenin complex in salivary adenoid cystic carcinoma in presence of salivary adenoid cystic carcinoma extracellular matrix (27). Integrins trigger signals in response to pulling forces applied to their ECM ligands in fibroblasts (28). Mechanical strain stimulates

conformational activation of $\alpha V\beta 3$ integrin in NIH3T3 cells mediated by phosphoinositol 3-kinase and is followed by an increase in integrin binding to extracellular matrix proteins (29). Alternatively, mechanical strain may stimulate membrane channels or other transmembrane protein receptors. Cyclic mechanical strain induces the upregulation of both the α-1 and α-2 subunits of Na^+,K^+-ATPase in rat aortic smooth muscle cells (30). Mechanical strain generated by subjecting lymphocytes to hypotonic challenge triggered arachidonic acid production and two CYP450 products of arachidonic acid, 5,6-epoxyeicosatrienoic acid and 20-hydroxyeicosatetraenoic acid, induced Ca^{2+} entry into primary B cells (31). Mechanical strain could also cause cytoskeletal reorganization to stimulate cell signal cascades. For example, the structure of the actin stress fibers in osteoblasts is dynamically reorganized under a change in the surrounding mechanical environment (32,33).

Although the blocking experiment (Fig. 7) illustrates that TGFβ1 ligand is necessary for inhibition of differentiation by strain, the TGFβ1 addition results (Fig. 5) show that it is not sufficient to account for the extent of self-renewal conferred by strain. Activin A may contribute to strain repression of differentiation, as discussed earlier. Other intracellular signaling pathways likely synergize with TGFβ/Activin/Nodal pathway to repress hESC differentiation under mechanical strain. bFGF has been reported to act as a competence factor for TGFβ/Activin/Nodal signaling because its positive effect on pluripotency strictly depends on TGFβ/Activin/Nodal signaling (15). High levels of Smad2/3 signaling were observed in hESCs cultured in MEF-conditioned medium supplemented with bFGF (14). However, high levels of bFGF have been shown to 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 (34,11). These contradictory results may be attributable to differences in culture conditions (Matrigel and undefined CM versus a fully-defined culture system). Cross-talk between TGFβ/Activin/Nodal signaling and other pathways including Wnt, Hedgehog, or other tyrosine kinases-linked growth receptor signaling pathways has also been reported (35).

This study indicates that TGFβ superfamily signaling through Smad2/3 plays a crucial role in inhibition of hESC differentiation by mechanical strain. Strain may directly induce Smad2/3 activation through autocrine or paracrine signaling via TGFβ ligands. These results represent a step toward synergizing mechanical and chemical cues to regulate hESC differentiation.

The authors thank James Thomson for providing the hESC lines and the research staff at WiCell for assistance in growing hESCs, and the staff at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Facility for assistance with flow cytometry.

This work was supported by the National Science Foundation (EFRI-0735903) and the National Science Foundation-funded University of Wisconsin Materials Research Science and Engineering Center.

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