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Smad2 Is Essential for Maintenance of the Human and Mouse Primed Pluripotent Stem Cell State*[S]

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Background: TGF-β signaling is required for primed pluripotency, but the roles of Smad2 and Smad3 have not been well defined.
Results: Smad2, but not Smad3, has a role in pluripotency by activating *Nanog* expression and repressing BMP signaling.
Conclusion: Smad2 is essential in the maintenance of pluripotency.
Significance: The roles of Smad2 and Smad3 need to be distinguished in the regulation of pluripotency by TGF-β signaling.

Human embryonic stem cells and mouse epiblast stem cells represent a primed pluripotent stem cell state that requires TGF-β/activin signaling. TGF-β and/or activin are commonly thought to regulate transcription through both Smad2 and Smad3. However, the different contributions of these two Smads to primed pluripotency and the downstream events that they may regulate remain poorly understood. We addressed the individual roles of Smad2 and Smad3 in the maintenance of primed pluripotency. We found that Smad2, but not Smad3, is required to maintain the undifferentiated pluripotent state. We defined a Smad2 regulatory circuit in human embryonic stem cells and mouse epiblast stem cells, in which Smad2 acts through binding to regulatory promoter sequences to activate Nanog expression while in parallel repressing autocrine bone morphogenetic protein signaling. Increased autocrine bone morphogenetic protein signaling caused by *Smad2* down-regulation leads to cell differentiation toward the trophectoderm, mesoderm, and germ cell lineages. Additionally, induction of *Cdx2* expression, as a result of decreased Smad2 expression, leads to repression of *Oct4* expression, which, together with the decreased *Nanog* expression, accelerates the loss of pluripotency. These findings reveal that Smad2 is a unique integrator of transcription and signaling events and is essential for the maintenance of the mouse and human primed pluripotent stem cell state.

Stem cells maintain their identity through signaling pathways that are activated by soluble or extracellular matrix proteins in their microenvironment (1, 2). Embryonic stem cells (ESCs)[4] are derived from the inner cell mass of blastocyst stage embryos and are pluripotent, giving rise to all cell types. Mouse embryos at a later stage also generate pluripotent stem cells, called epiblast stem cells (EpiSCs), that differ in some characteristics from mouse ESCs (mESCs) but share common properties with human ESCs (hESCs) (3, 4). Specifically, although mESCs require leukemia inhibitory factor and bone morphogenetic protein (BMP) signaling to maintain pluripotency, mEpiSCs and hESCs rely on basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) or activin (3–7). Based on differences in their characteristics and signal requirements, it is believed that mESCs show naive or ground pluripotency, whereas mEpiSCs and hESCs represent primed pluripotency (8).

Among the many key transcription factors, Oct4, Sox2, and Nanog play major roles in stem cell pluripotency (9, 10). They either activate their own expression and that of other core pluripotency genes or limit the differentiation programs. For example, Oct4 and Sox2 associate with the *Nanog* promoter and direct its expression (11, 12). Oct4 and the trophectodermal transcription factor Cdx2 regulate lineage segregation between trophectoderm and the inner cell mass of mouse blastocysts (13) by suppressing the expression of one another (14). Disruption of *Nanog* expression in mice results in failure of epiblast generation and peri-implantation lethality (15, 16). Accordingly, elevated Nanog expression in mESCs results in clonal expansion and resistance to differentiation (16) and, in hESCs, promotes cell proliferation (17). However, Nanog expression is heterogeneous in ESC colonies (18, 19) and the inner cell mass of the mouse blastocyst (20) and was shown to be dispensable for mEpiSC pluripotency (21), suggesting more prominent roles of Oct4 and Sox2 in primed pluripotency.

The signaling pathways required for maintaining hESC or mEpiSC pluripotency have been extensively studied. bFGF, an

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[4] The abbreviations used are: ESC, embryonic stem cell; hESC, human ESC; mEpiSC, mouse epiblast stem cell; BMP, bone morphogenetic protein; bFGF, basic fibroblast growth factor; mESC, mouse ESC; qRT, quantitative RT; MEF, mouse embryonic fibroblast; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; PGC, primordial germ cell; esiRNA, endoribonuclease siRNA.

essential factor for hESC and mEpiSC pluripotency, suppresses BMP signaling and neuronal differentiation (22). Although required for mESC pluripotency, BMP induces hESC and mEpiSC differentiation (23, 24). TGF-β signaling, however, suppresses BMP-activated differentiation (25) and neuroectoderm specification (7, 26, 27). Furthermore, bFGF stimulation of hESCs or mouse embryonic fibroblasts (MEFs) results in their release of activin A, TGF-β, and insulin-like growth factor (IGF)-II, promoting hESC and mEpiSC pluripotency (28, 29). Thus, some effects of bFGF might secondarily result from activated TGF-β signaling.

TGF-β, activin, and nodal signal through Smad2 and Smad3, which are activated through phosphorylation by receptor kinases. By forming complexes with the coactivator Smad4 and other DNA-binding transcription factors and coregulators, Smad2 and Smad3 activate or repress gene transcription (30, 31). Although Smad2 and Smad3 have nearly identical transcription activation domains, called MH2 domains, their N-terminal MH1 domains are distinct, with Smad2 unable to directly bind DNA and Smad3 showing DNA binding, indicating functional differences (32–34). Despite their structural and functional differences, the differential roles of Smad2 and Smad3 in ESC pluripotency have not been addressed. TGF-β, activin, and nodal activate both Smad2 and Smad3, and pharmacological inhibition of TGF-β/activin receptor kinases prevents activation of both Smad and non-Smad signaling pathways. Such pharmacological inhibition impairs the pluripotency of hESCs and mEpiSCs, and Smad2 and/or Smad3 were found to directly target *Nanog* expression using an antibody unable to distinguish one from the other (35, 36).

Here, we provide the first evidence for differential roles of Smad2 and Smad3 in the maintenance of pluripotency of hESCs and mEpiSCs. Smad2, but not Smad3, was required for primed pluripotency by directly activating *Nanog* expression in response to TGF-β and by repressing BMP signaling. Enhanced Cdx2 expression, resulting from increased autocrine BMP signaling upon *Smad2* down-regulation, repressed Oct4 expression and accelerated differentiation. These results shed light on specific roles of Smad2 and functional cross-talk of TGF-β with BMP signaling in primed pluripotency.

EXPERIMENTAL PROCEDURES

Cell Culture and in Vitro Differentiation—mEpiSCs isolated from 129SvEv mice were provided by Drs. Paul Tesar (Case Western Reserve University) and Ron McKay (NINDS, National Institutes of Health). hESCs and mEpiSCs were cultured on irradiated MEFs with hESC medium, *i.e.* DMEM/F-12 with 20% knock-out serum replacement, 1× Glutamax, 1× nonessential amino acids, 1× penicillin/streptomycin, 0.1 mM β-mercaptoethanol, 8 ng/ml bFGF. For feeder-free cell cultures, MEF-conditioned medium was prepared by incubating hESC medium overnight with irradiated MEFs at 37 °C, filtered through 0.45-μm pore size nitrocellulose, and used with Matrigel-coated dishes (354234, BD Biosciences). To study BMP responsiveness, cells were cultured overnight with or without 25 ng/ml Noggin and then stimulated with 1 ng/ml BMP4 for 1 h. For *in vitro* differentiation assays, hESCs and mEpiSCs were lifted from feeder cells using Accutase (Chemicon) and seeded onto Aggrewell (Stem Cell Technology) at 300 cells/embryoid body in suspension culture with mTeSR1 (Stem Cell Technology) and 10 μM ROCK inhibitor. Medium was replaced the next day with differentiation medium (DMEM/F-12 containing 20% FBS, 1× Glutamax, 1× nonessential amino acids, 1× penicillin/streptomycin, 0.1 mM β-mercaptoethanol) and changed every other day. Embryoid bodies were transferred to gelatin-coated dishes after 4–7 days in suspension culture and cultured for another 3–5 days with differentiation medium. Endoderm, mesoderm, and neuroectoderm marker gene expression was analyzed by immunofluorescence and quantitative RT-PCR.

Cell Proliferation Assay—At day 5 after shRNA infection, hESCs and mEpiSCs were dissociated with Accutase and seeded onto Matrigel-coated wells with MEF-conditioned medium and 10 μM ROCK inhibitor. For mouse EpiSCs, 1,000 cells were seeded onto a well of a 24-well plate, and cells were counted at day 8, prior to reseeding at the same density (1,000 cells per well of a 24-well plate) and counting at day 16. Cell numbers were averaged from triplicate samples in independent experiments, relative to control cells. For hESCs, cell numbers were counted at days 3, 6, and 10, as in the mEpiSC proliferation assay.

Lentiviral Vector Preparation, Infection, and RNA Interference—Virus preparation and infection were performed as described (37) with the exception of the packaging plasmids (VSV-G, MDL-RRE, and RSVr) used. TRC shRNA vectors (Sigma) or GFP shRNA vectors (from Dr. Louise M. Bilezikjian) (38) were used to stably silence *Smad2, Smad3, Cdx2,* or *Nanog* expression. The shRNA sequences are listed in supplemental Table S1. To transiently silence Smad2, Smad4, or Nanog expression at earlier time points, cells were transfected with corresponding esiRNAs (Smad2 and Nanog) and SMART pool siRNAs (Smad4) (Thermo Scientific). esiRNA to GFP and control siRNA were used to derive control cells. The target sequences of the SMART pool for human Smad4 were 5′-GCAAUUGAAAGUUUGGUAA-3′, 5′-CCCACAACCUUUAGACUGA-3′, 5′-GAAUCCAUAUCACUACGAA-3′, and 5′-GUACAGAGUUACUACUUAG-3′. Transfections of siRNA and esiRNA were performed using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions.

Cell Sorting—To enrich shRNA-GFP-expressing cells, cells were dissociated with Accutase (Chemicon) and resuspended in cell dissociation buffer (Invitrogen) with 10 μM ROCK inhibitor. shRNA-GFP-expressing cells were isolated by cell sorting (FACS Aria; BD Biosciences) and seeded onto irradiated MEFs to expand and examine the morphology of shRNA-expressing cells under undifferentiation conditions.

Quantitative RT-PCR—Total RNA was isolated using RNeasy Mini RNA Isolation kit (Qiagen). cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad). qRT-PCRs were carried out in triplicate using the iQ SYBR Green Mix on CFX96 real time PCR detection system. Primer sequences are listed in supplemental Table S2.

Immunoblotting and Immunofluorescence—Cells were washed with PBS and lysed in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) with protease inhibitor mixture (04693159001, Roche Applied Science). Total cell lysates were analyzed by SDS-PAGE on 4–12% gradient BisTris

gels and immunoblot using antibodies to Smad2/3 (610842, BD Biosciences), Smad3 (C67H9) (9523, Cell Signaling), and SMAD4 (sc-7966x, Santa Cruz Biotechnology). Antibodies to α-tubulin (Sigma) or GAPDH (sc-32233, Santa Cruz Biotechnology) were used to control loading. Anti-Smad1 (ab33902, Abcam) and anti-phospho-Smad1/5 (9516, Cell Signaling) were used to analyze BMP responsiveness. To analyze protein expression in colonies, hESCs and mEpiSCs were fixed on culture plates or glass slide chambers (BD Biosciences) with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then blocked with 2% BSA and stained with antibodies to Oct3/4 (sc-5279, Santa Cruz Biotechnology, 1:500), human NANOG (AF1997, R&D Systems, 1:100), mouse Nanog (SC1000, Millipore, 1:200), human CDX2 (ab76541, Abcam, 1:200), FOXA2 (07633, Upstate Biotechnology, 1:200), SOX17 (AF1924, R&D Systems, 1:200), BRACHYURY (ab20680, Abcam, 1:100), Tuj1 (MMS-435P, Covance, 1:1000), and SMA (M0851, DAKO, 1:10). These antibodies were also used for immunoblotting. Secondary antibodies with Alexa Fluor 633, Alexa Fluor 594, or Alexa Fluor 488 (Invitrogen) were used at 1:500, and cells were incubated with DAPI (Sigma) for 10 min to stain nuclei. Cells were viewed by epifluorescence (Leica, DMI 4000 B) or confocal microscopy (Leica, DM 6000 CS).

Chromatin Immunoprecipitation—To examine binding of SMAD2, SMAD3, or SMAD4 to *NANOG* promoter sequences, hESCs were treated or not with 5 ng/ml TGF-β or 20 ng/ml activin for 1 h after pretreatment with 3 μM SB431542 for 16 h, and ChIP assays were performed as described (39). Briefly, hESCs, lentivirally infected or not to express *SMAD2* or *SMAD3* shRNA for 7 days were cultured until 80% confluence, cross-linked for 45 min at room temperature with 1.5 mM disuccinimidyl glutarate, and then 10 min with 1% formaldehyde. Cross-linking was quenched with 250 mM glycine. Cells were washed twice with PBS, collected using a cell scraper, snap-frozen in liquid nitrogen, and stored at −80 °C prior to use. Cells were resuspended, lysed, and sonicated to shear and solubilize cross-linked DNA. Sonication was performed on 10^7 cells in sonication buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, and 0.5% *N*-lauroylsarcosine) using a Bioruptor. After sonication, samples were centrifuged at 20,000 × *g* for 10 min, and soluble whole cell extracts were divided into two or three tubes depending on the number of antibodies tested to incubate overnight with protein G-agarose beads that had been preincubated with 5 μg of Smad2 antibody (5339, Cell Signaling), Smad3 antibody (ab28379, Abcam), SMAD4 antibody (sc-7966x, Santa Cruz Biotechnology), or control IgG. Incubated beads were washed with 20 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, with 20 mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100; with 10 mM Tris-HCl, pH 8, 250 nM LiCl, 2 mM EDTA, 1% Nonidet P-40; and with 10 mM Tris, pH 7.5, 1 mM EDTA. Bound complexes were eluted from the beads (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5–1% SDS) by heating at 65 °C for 45 min and vortexing every 5 min. Cross-linking was reversed by incubating samples at 65 °C for 6 h. Whole cell extract DNA reserved from the sonication step was treated in the same way to reverse cross-linking. After reversal of cross-linking, samples were treated with RNase and proteinase K prior to cleaning up the DNA using Min Elute Reaction clean-up kit (Qiagen). PCR was performed to detect specific DNA sequences (supplemental Table S2) in the precipitates.

Teratoma Analysis—hESCs and mEpiSCs were dissociated with Accutase at 7–10 days after infection, then pelleted, and resuspended in 100 μl of 30% Matrigel/PBS solution (BD Biosciences) per injection. 10^6 mEpiSCs or 3×10^6 hESCs were injected subcutaneously into the fat pads at both sides of SCID mice near the hind flanks. Tumors were processed after 6–12 weeks for histological analyses at a University of California at San Francisco pathology core facility. Research using mice was carried out following the IACUC guidelines.

Statistics—All quantitative data were analyzed using Prism 6 software (GraphPad) and expressed as means ± S.E. from at least three independent experiments. A two-tailed unpaired Student's *t* test was used to analyze data containing two groups; differences were considered significant at $p < 0.05$.

RESULTS

Smad2, but Not Smad3, Is Required for hESC and mEpiSC Pluripotency—To study the contributions of Smad2 and Smad3 in stem cell pluripotency, we generated hESCs (H9) and mEpiSCs, in which Smad2 or Smad3 expression was selectively decreased using lentiviral shRNA. One vector enabled selection for shRNA expression, based on puromycin resistance (Fig. 1, *A–F*), whereas the other allowed enrichment of shRNA-expressing cells through cell sorting for GFP (Fig. 1, *G* and *H*). In the selected cells, the efficiencies and specificities of gene silencing were evaluated at day 5 after lentiviral infection. As shown in Fig. 1, *A*, *E,* and *G*, Smad2 or Smad3 expression was substantially reduced at day 5 using the selected shRNAs. Assessed by qRT-PCR, *SMAD2* shRNA decreased *SMAD2* mRNA expression with 88 and 91.2% efficiency in S2 KD1 or S2 KD2 cells, respectively, whereas *SMAD3* shRNA decreased *SMAD3* mRNA levels with 91.5 and 89.8% efficiency in S3 KD1 and S3 KD2 cells. *SMAD2* shRNA did not affect *SMAD3* expression, and vice versa, as assessed by immunoblotting (Fig. 1, *A* and *E*) and qRT-PCR (data not shown).

We next evaluated the effects of decreased Smad2 or Smad3 expression at day 5 after lentiviral shRNA vector infection. When grown in Matrigel, hESC and mEpiSC colonies with down-regulated *Smad2* expression no longer had the tight morphology of densely packed cells with close cell-cell contacts, as seen in control cells. Instead, spindle-shaped cells and cells with smaller nuclei were progressively generated (Fig. 1, *B*, *C,* and *H*). Immunoblotting and immunofluorescence showed significant decreases in NANOG and OCT4 expression but not in SOX2 expression in hESCs (Fig. 1, *A* and *C*), and Nanog expression in mEpiSCs (Fig. 1, *E* and *H*). The decrease in Oct4 expression in mEpiSCs followed slower kinetics. Already apparent in hESCs at day 5 after infection (Fig. 1*A*), this decrease was not yet seen in mEpiSCs at that time (Fig. 1*E*), but it was apparent at day 8 (data not shown). When expressed from the GFP-encoding vector, *Smad2* shRNA expression, indicated by GFP, was mutually exclusive with Nanog or Oct4 expression (Fig. 1*H*), indicating that Smad2 is required for stem cell pluripotency. Cell proliferation was decreased upon down-regula-

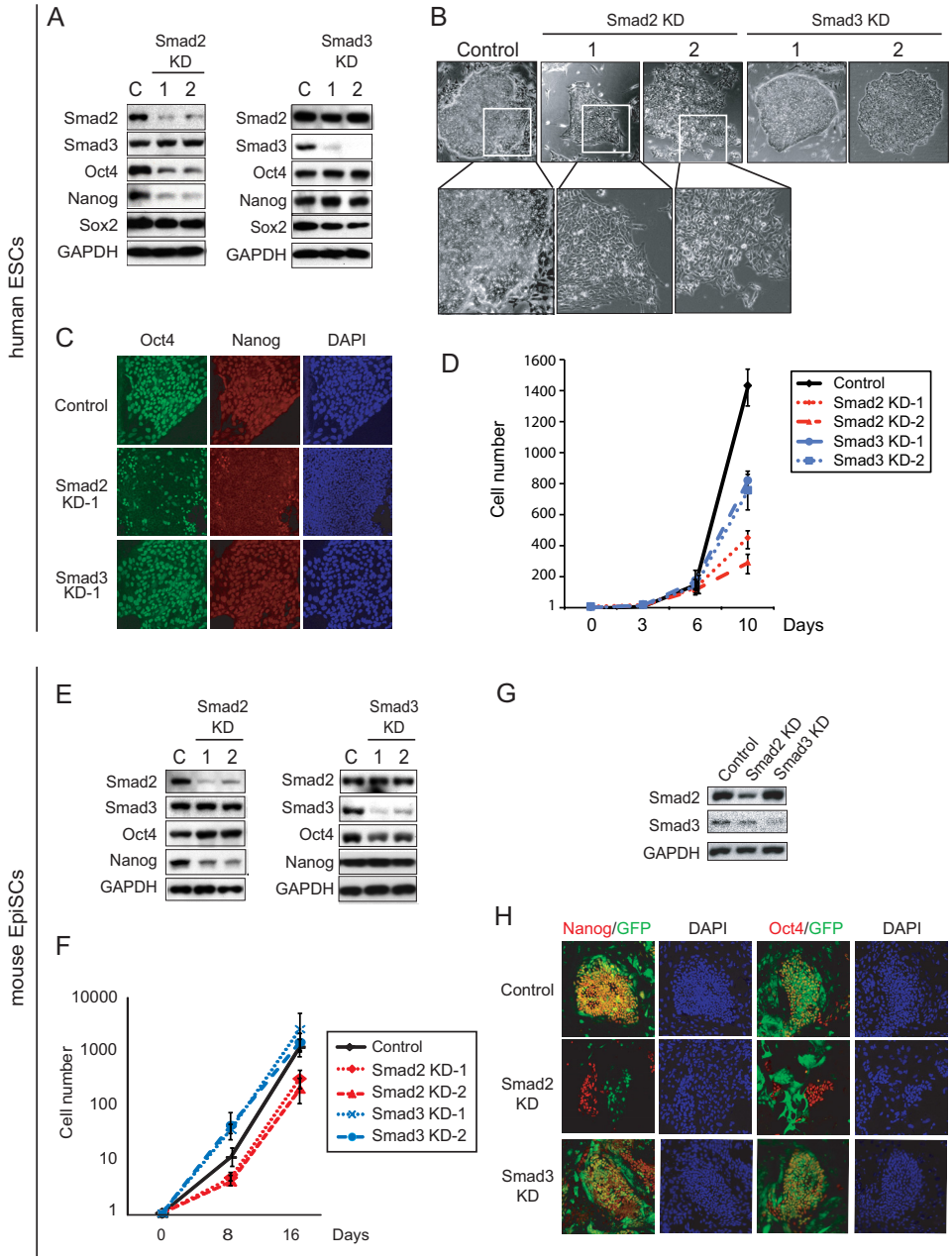

FIGURE 1. **SMAD2, but not SMAD3, is required for pluripotency of hESCs (*A–D*) and mEpiSCs (*E–H*).** *A,* down-regulation of *SMAD2* (*left panel*) but not *SMAD3* (*right panel*) expression for 5 days leads to decreased OCT4 and NANOG but not SOX2 expression shown by immunoblotting. *B,* colony morphology following down-regulation of *SMAD2* or *SMAD3* expression at day 5 after lentiviral shRNA expression. *C,* immunofluorescence shows loss of OCT4 (*green*) or NANOG (*red*) expression, upon down-regulation of *SMAD2* but not *SMAD3* expression for 5 days. DAPI serves as nuclear counterstain. *D,* proliferation of control cells or cells with decreased *SMAD2* (*red lines*) or *SMAD3* (*blue lines*) expression. KD1 and KD2 cells express different shRNAs. *E,* down-regulation of *Smad2* (*left panel*) but not *Smad3* (*right panel*) expression for 5 days leads to decreased Nanog expression, as shown by immunoblotting. *F,* proliferation of control cells or cells with decreased *Smad2* (*red lines*) or *Smad3* (*blue lines*) expression. *G,* immunoblotting shows decreased Smad2 or Smad3 expression using lentiviral shRNA vectors encoding GFP. *H,* immunofluorescence reveals loss of Nanog and Oct4 expression in cells infected to express *Smad2* shRNA and GFP for 10 days. GFP-positive cells lacked Nanog and Oct4 expression. Decreasing *Smad3* expression did not affect Nanog or Oct4 expression in GFP-positive cells. DAPI served as nuclear counter-stain.

tion of Smad2 expression (Fig. 1, *D* and *F*), suggesting cell differentiation and a lower proliferation potential. In contrast to Smad2, decreased Smad3 expression did not affect colony morphology (Fig. 1*B*) or Nanog or Oct4 expression (Fig. 1, *A*, *C*, *E*, and *H*). Although silencing of *SMAD2* expression did not affect *SOX2* expression at day 5, down-regulation of *SMAD3* expression resulted in a reduction of *SOX2* expression in human ES cells (Fig. 1*A*). In cells expressing *Smad3* shRNA from the GFP vector, GFP-positive cells with decreased *Smad3* expression showed Nanog and Oct4 expression similarly to control cells (Fig. 1*H*). Based on these results, Smad2 but not Smad3 appears to be required for the undifferentiated state of both hESCs and mEpiSCs.

Decreased Smad2 Expression Enhances Neuroectoderm, Mesoderm, and Trophectoderm Differentiation—We next examined if the decrease in the undifferentiated state, due to decreased Smad2 levels, correlated with increased cell differentiation under conditions normally used to maintain pluripo-

tency. Assessed by qRT-PCR at day 5 after lentiviral infection, decreased *SMAD2* expression resulted in increased expression of the trophoblast transcription factor *CDX2* and decreased expression of the endodermal marker *SOX17* in hESCs (Fig. 2*A*). The effects of decreased SMAD2 expression on gene expression were stronger at earlier time points in *SMAD2* KD1 cells, when compared with *SMAD2* KD2 cells, but *SMAD2* KD2 cells were more effective at later time points at retaining their effects on differentiation gene expression (data not shown). Decreased SOX17 and increased CDX2 expression were also apparent by immunoblotting (Fig. 2*B*). These striking changes were not seen upon down-regulation of *SMAD3* expression (Fig. 2*A*). Decreased *SMAD2* expression also resulted in reduced expression of other endodermal genes, *FOXA2, GATA4*, and *CER* (Fig. 2*C*), and increased expression of the trophectodermal genes *DLX3, HAND1,* and *MSX2* (Fig. 2*D*). The cells also showed a moderate increase in mRNA levels of the mesodermal gene *BRACHYURY* and neuroectodermal gene *TUJ1* (Fig. 2*A*), yet these increases were more striking when protein levels were assessed by immunoblotting (Fig. 2*B*). The effects of decreased Smad2 expression on trophectodermal genes were similar in mEpiSCs, although endodermal genes such as *Sox17* were not down-regulated (Fig. 2*E*). Despite differences in responses, including the timing of *Oct4* down-regulation after decreasing *Smad2* expression, *Cdx2* and *Brachyury* expressions were also increased in mEpiSCs (Fig. 2*E*), as in hESCs. Along with the loss of pluripotency gene expression (Fig. 1), these results indicate that Smad2 is required to maintain pluripotency and to prevent differentiation, most notably toward the trophectoderm lineage. Decreased Smad3 expression did not have these immediate effects on pluripotency and differentiation.

We also examined the spontaneous differentiation of cells, grown as spheroids in suspension. Under these conditions, hESCs and mEpiSCs form embryoid bodies with differentiation along the three lineages. Five days after infection, cells were dissociated, seeded to low attachment plates, cultured for 4–7 days in suspension, and transferred for culture for 3–5 days. Under these conditions, hESCs with down-regulated *SMAD2* expression formed much smaller embryoid bodies than control cells or cells with decreased *SMAD3* expression (data not shown). qRT-PCR analyses revealed strongly decreased expression of endodermal *CER*, *FOXA2*, and *SOX17* genes and increased expression of neuroectodermal *SOX1, NESTIN,* and *TUJ1* genes (Fig. 2*F*). Immunostaining for endoderm (FOXA2), mesoderm (SMA), and neuroectoderm (TUJ1) markers confirmed that embryoid bodies of hESCs with decreased *SMAD2* expression had increased neuroectodermal and lower endodermal differentiation, as compared with control cells (Fig. 2*G*). This finding suggested decreased pluripotency and endodermal differentiation capacity of cells after decreasing Smad2 expression.

We also evaluated the effects of decreasing *Smad2* or *Smad3* expression on pluripotency and differentiation capacity of hESCs and mEpiSCs in teratoma formation. Control cells or cells with decreased *Smad2* or *Smad3* expression were injected into fat pads of the hind limb of 8–12-week-old female SCID mice. Tumors were collected at 6–12 weeks after injection, when teratomas of control cells reached 1 cm in the longest diameter. Consistent with their poor proliferation and differentiation capacity in embryoid body assays, cells with decreased *Smad2* expression generated fewer and much smaller teratomas, compared with control cells (Table 1), preventing a meaningful analysis of their differentiation. In contrast, cells with decreased *Smad3* expression formed teratomas with all three germ layers, albeit with less mesodermal and more neuroectodermal and endodermal cells (data not shown).

Smad2, but Not Smad3, Directly Targets Nanog Expression—Because decreased expression of Smad2, but not Smad3, strongly repressed Nanog expression in hESCs and mEpiSCs (Fig. 1, *A*, *C, E,* and *H*), we hypothesized that Smad2 directly targets Nanog expression. The Nanog promoter was shown to bind Smad2 and/or Smad3, but the antibodies used did not distinguish between these two Smads (35, 36).

We first evaluated whether decreased *SMAD2* or *SMAD3* expression affected activation of *NANOG* expression in hESCs (Fig. 3*A*). Without treatment, cells maintained *NANOG* expression through autocrine signaling and/or in continued response to ligands secreted by MEFs in the conditioned medium. We therefore used SB431542, which blocks the TGF-β and activin type I receptor kinases (40), to suppress the basal TGF-β/activin signaling and Smad2/3 activation. Subsequently added TGF-β or activin activated *NANOG* mRNA expression in control cells and cells with decreased *SMAD3* expression. In contrast, decreasing *SMAD2* expression attenuated the induction of *NANOG* mRNA expression in response to TGF-β or activin, with *SMAD2* KD2 cells being less effective than KD1 cells in repressing *NANOG* at day 7 after lentiviral shRNA infection (Fig. 3*A*). These results implicate Smad2 but not Smad3 in the control of *Nanog* expression by TGF-β or activin.

Using chromatin immunoprecipitation (ChIP) with antibodies specific for Smad2 or Smad3, we found low level binding of SMAD2 and SMAD3 to a proximal promoter region of *NANOG* in the presence of SB431542 (Fig. 3*B*). Treatment of cells with activin or TGF-β rapidly increased SMAD2 but not SMAD3 binding to this region (Fig. 3*B*), indicating that Smad2, but not Smad3, targets the Nanog promoter in response to activin or TGF-β. Consistent with the basal *NANOG* expression, untreated samples showed SMAD2 interaction with the *NANOG* promoter. Surprisingly, we also found SMAD3 association with the *NANOG* promoter in untreated cells, suggesting steady state association of SMAD2-SMAD3 complexes. In control experiments, TGF-β and activin induced association of both SMAD2 and SMAD3 to regulatory sequences of the *SMAD7* gene (Fig. 3*C*) with known sites for binding of Smad2-Smad3-Smad4 complexes (41). Unlike SMAD2, ligand-induced SMAD4 association with the *NANOG* promoter was only minimal, if at all, which was in contrast to the association of SMAD4 with the *SMAD7* promoter (Fig. 3*D*). This negative result may be due to impaired antibody accessibility of SMAD4 at the *NANOG* promoter or other experimental reasons. However, silencing *SMAD4* expression did not confer down-regulation of pluripotency gene expression (Fig. 3*E*) or induce differentiation marker gene expression (data not shown), consistent with previous observations (42), raising the possibility that Smad2 recruitment to the *NANOG* promoter may not involve Smad4.

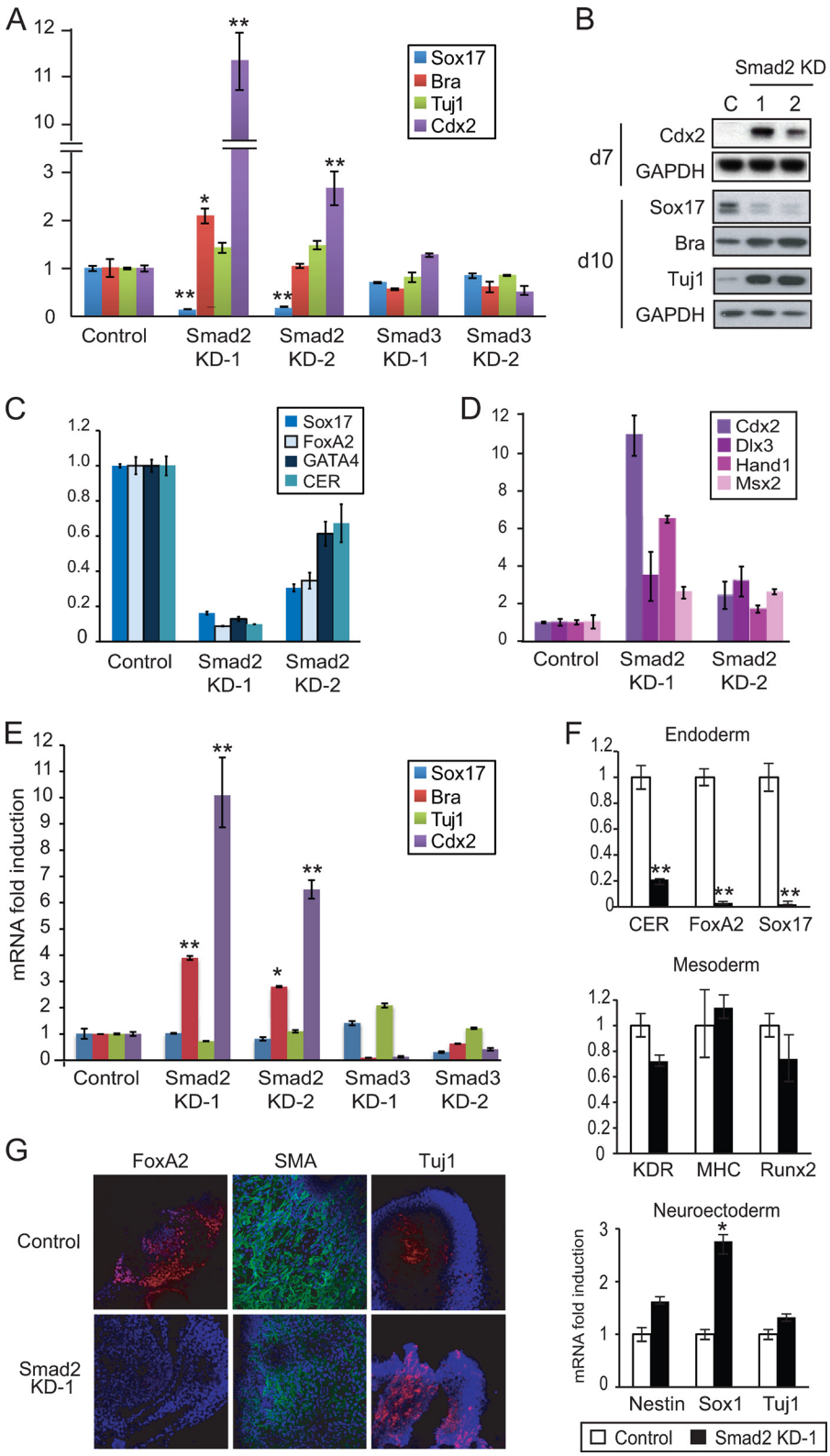

FIGURE 2. **Decreasing Smad2 expression affects stem cell differentiation.** *A, SOX17, BRACHYURY, TUJ1,* and *CDX2* mRNA expression in control hESCs and hESCs with decreased *SMAD2* or *SMAD3* expression for 5 days, as quantified by qRT-PCR. *B,* immunoblots of CDX2, SOX17, BRACHYURY, and TUJ1 expression in control hESCs and hESCs with decreased *SMAD2* expression at days 7 or 10 after infection with lentiviral shRNA vector. *Lane C*, control. *C* and *D, SOX17, FOXA2, GATA4,* and *CER* (endodermal markers) mRNA levels (*C*) and *CDX2, DLX3, HAND1,* and *MSX2* (trophectodermal markers) mRNA levels (*D*) in control hESCs and hESCs with decreased *SMAD2* expression at day 5 after infection with lentiviral shRNA vector. *E, Sox17, Brachyury, Tuj1,* and *Cdx2* mRNA expression in control mEpiSCs and mEpiSCs with decreased Smad2 or Smad3 expression for 5 days, as quantified by qRT-PCR. *F,* mRNA levels of *CER, FOXA2,* and *SOX17* (endodermal markers), *KDR, MHC,* and *RUNX2* (mesodermal markers), and *NESTIN, SOX1,* and *TUJ1* (neuroectoderm markers) in embryoid bodies from control hESCs or hESCs with decreased *SMAD2* expression. *G,* immunofluorescence of FOXA2, SMA, and TUJ1 expression during spontaneous differentiation of embryoid bodies of control hESCs and hESCs with decreased *SMAD2* expression. $n = 3$ assays. *, $p < 0.05$; **, $p < 0.01$, as compared with control cells by Student's *t* tests.

TABLE 1

Decreased efficiency of teratoma formation of hESCs and mEpiSCs with decreased Smad2 expression, as compared with control hESCs and mEpiSCs, in SCID mice

Sample	Cell number injected per site	Total injection sites	No. of teratomas generated		
			Large[a]	Small[a]	None
Epi/control	3 million	6	5/6 (83.3)	1/6 (16.7)	0/6 (0.0)
Epi/*Smad2* KD1	3 million	6	1/6 (16.7)	2/6 (33.3)	3/6 (50)
Epi/*Smad2* KD2	3 million	6	0/6 (0.0)	1/6 (16.7)	5/6 (83.3)
H9/control	1 million	10	7/10 (70.0)	2/10 (20.0)	1/10 (10.0)
H9/*SMAD2* KD1	1 million	6	0/6 (0.0)	1/6 (16.7)	5/6 (83.3)
H9/*SMAD2* KD2	1 million	10	0/10 (0.0)	2/10 (20.0)	8/10 (80.0)

[a] Large represents size of teratomas with 0.5 cm or longer diameter, and small represents size of teratomas shorter than 0.5 cm in diameter.

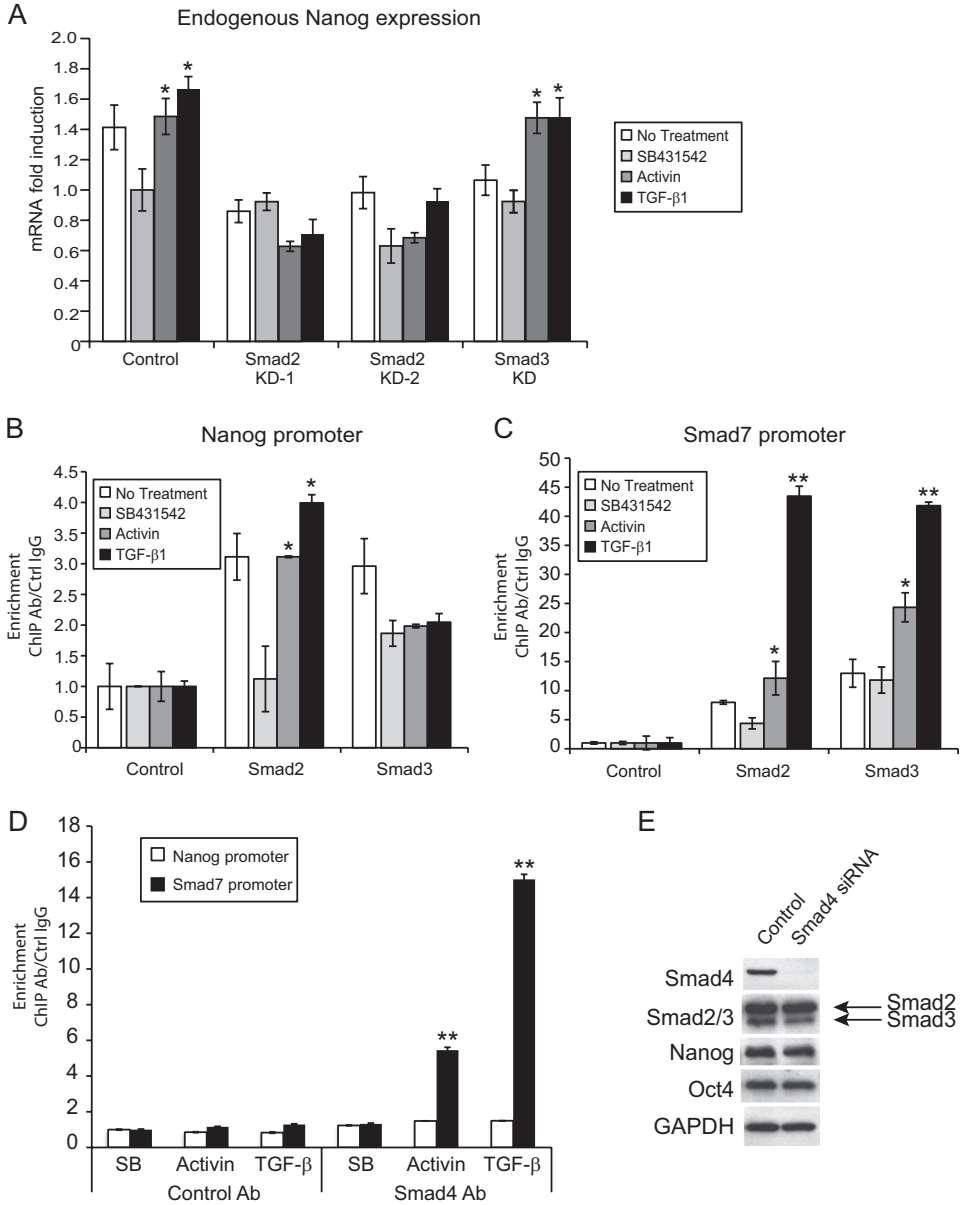

FIGURE 3. **Smad2, but not Smad3, regulates Nanog expression through direct binding to the Nanog promoter.** *A,* effects of activin, TGF-β, SB431542, or basal TGF-β/activin signaling (*No Treatment*) on endogenous *NANOG* mRNA expression in control hESCs and hESCs with decreased *SMAD2* or *SMAD3* expression at day 7 after lentiviral shRNA vector infection. *B,* binding of endogenous SMAD2 or SMAD3 to the *NANOG* promoter, assessed by chromatin immunoprecipitation, in cells with or without ligand stimulation and after SB431542 treatment, at day 7 after lentiviral shRNA vector infection. *C,* binding of endogenous SMAD2 and SMAD3 to the *SMAD7* promoter, assessed by chromatin immunoprecipitation, in cells with or without ligand stimulation and after SB431542 treatment. $n = 3$ assays. *, $p < 0.05$; **, $p < 0.01$, compared with SB431542-treated cells (*A–C*) by Student's t tests. *D,* association of endogenous SMAD4 with the *SMAD7* promoter but not the *NANOG* promoter, as assessed by chromatin immunoprecipitation, in cells with or without ligand stimulation after SB431542 (*SB*) treatment. $n = 3$ assays. **, $p < 0.01$, compared with SMAD4 ChIP with SB431542 treatment by Student's *t* tests. *E,* immunoblots of SMAD4, SMAD2/3, NANOG, OCT4, and GAPDH in control hESCs and hESCs with down-regulated *SMAD4* expression at 96 h after siRNA transfection.

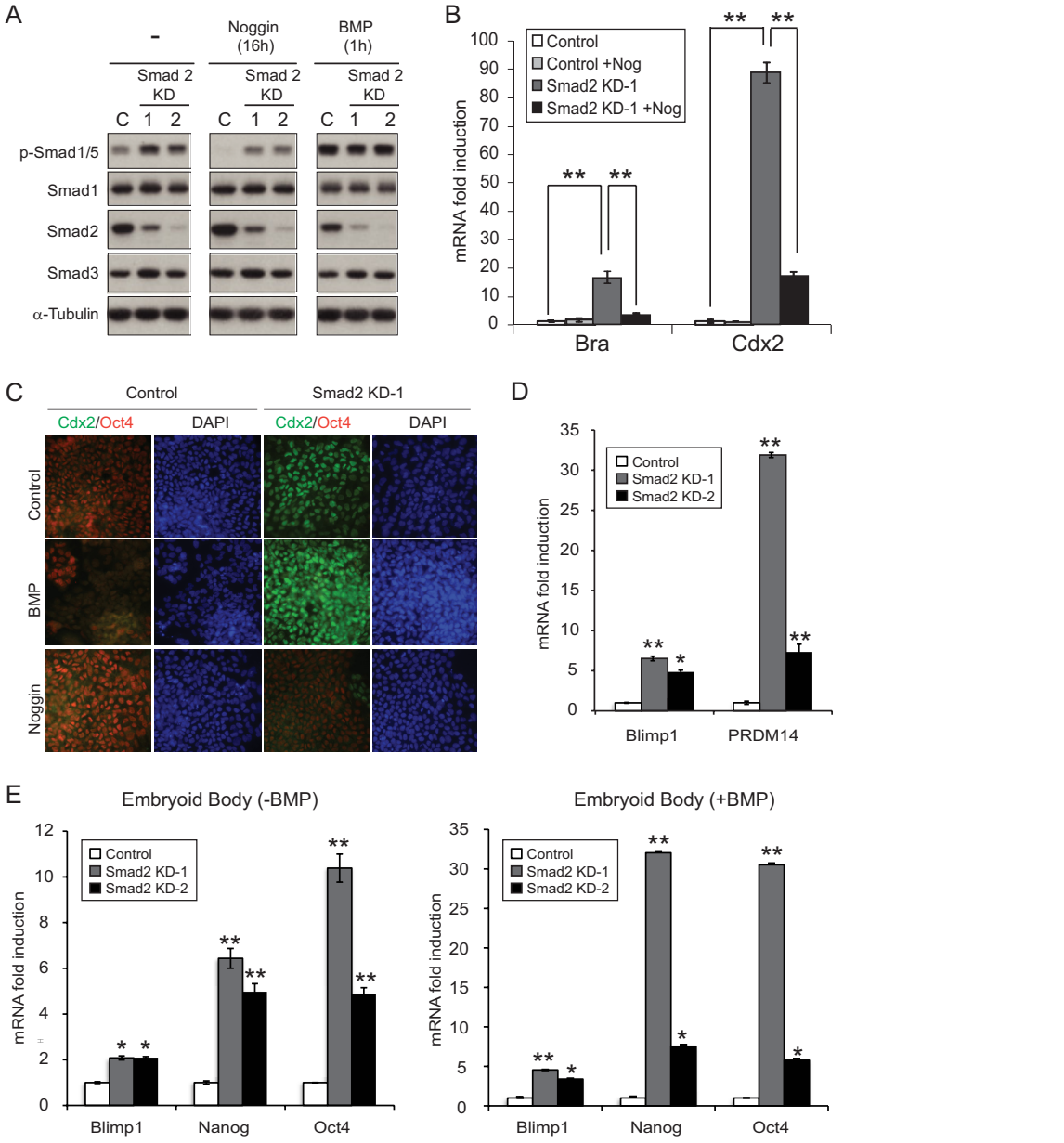

FIGURE 4. **Increased BMP responsiveness in cells with decreased Smad2 expression leads to increased BMP target gene expression.** *A,* decreased *SMAD2* expression in hESCs results in higher levels of phosphorylated SMAD1/5 at day 6 after lentiviral shRNA infection. Noggin or BMP were added at 16 or 1 h before analysis. *Lane C*, control. *B, BRACHYURY* (*Bra*) and *CDX2* mRNA expression in control hESCs and hESCs with decreased *SMAD2* expression in the presence or absence of Noggin from day 3 to day 8 after infection. *C,* immunofluorescence for CDX2 and OCT4 in control hESCs and hESCs with decreased *SMAD2* expression at day 8 after infection. Noggin or BMP were added from day 3 to 8. DAPI served as nuclear counterstain. *D,* mRNA expression of primodial germ cell markers, *BLIMP1* and *PRDM14*, in control mEpiSCs and mEpiSCs with decreased Smad2 expression at day 6 after infection. *E, Blimp1, Oct4,* and *Nanog* mRNA expression in embryoid bodies from control mEpiSCs or mEpiSCs with decreased *Smad2* expression. Cells were dissociated at day 6 after infection and subjected to suspension culture for 4 days. Embryoid bodies were transferred to gelatin-coated plastic plates for two-dimensional culture for 2 more days. BMP4 or vehicle were added to the two dimensional culture for 2 days. $n = 3$ assays. *, $p < 0.05$; **, $p < 0.01$, compared with control cells by Student's *t* tests.

These results indicate that SMAD2 and SMAD3 differ in the control of *NANOG* expression and hESC pluripotency and that *SMAD2* targets *NANOG* expression through regulated binding to its proximal promoter. Because Nanog may be dispensable for maintaining EpiSC pluripotency (21), we sought additional critical events that affect pluripotency in these stem cells upon *Smad2* down-regulation.

Smad2 Down-regulation Enhances Autocrine BMP Signaling Leading to BMP Target Gene Expression and Oct4 Suppression—Because decreased *SMAD2* expression enhanced trophectoderm gene expression (Fig. 2, *A*, *B*, *D*, and *E*), we examined whether this might be due to increased BMP signaling. Indeed, BMP is known to induce trophectodermal and/or mesodermal differentiation of hESCs and mEpiSCs, by increasing *Cdx2* and Brachyury expression (23, 43). Without adding BMP, hESCs with decreased *SMAD2* expression showed higher autocrine SMAD1/5 activation, assessed by C-terminal Smad phosphorylation, as compared with control cells (Fig. 4*A*). The increase in SMAD1/5 activation was reduced by Noggin but not fully inhibited, consistent with the autocrine nature of endogenous BMP signaling and competitive inhibition by Noggin. Upon stimulation with 1 ng/ml BMP4, the SMAD1/5 activation levels

were similar in control hESC and hESCs with decreased *SMAD2* expression.

The increased autocrine BMP stimulation in cells with decreased Smad2 expression may result from various changes in the complex BMP signaling system with its many ligands and secreted inhibitors and the different type I/type II receptor combinations (44). Additionally, the levels or activation states of accessory or intracellular components of this system may have changed, and the levels, activities, and presentation of many BMP signaling mediators are post-translationally controlled. As an initial step, we compared the expression of several BMP signaling mediators in hESCs with decreased *SMAD2* expression and control hESCs. Among the many BMPs, we detected 2-fold increases in *BMP4* and *BMP8b* mRNA levels (data not shown). Among the common BMP receptors, *i.e.* BMPRII and BMPRIA and BMPRIB, we found a 20–30% increase in *BMPRIB* mRNA (data not shown). Additionally, the mRNA levels of two BMP inhibitors, NOGGIN and CHORDIN, were enhanced, whereas GREMLIN expression was unaffected (data not shown). Although these analyses suggest increased ligand as a cause of enhanced autocrine BMP signaling, the complexity of this signaling system does not warrant a simple conclusion on the basis of increased autocrine BMP signaling.

Because BMP signaling stimulates trophectoderm and/or mesoderm gene expression (23, 43), we examined whether increased autocrine BMP signaling enabled *Cdx2* and *Brachyury* expression. As shown in Fig. 4*B*, *BRACHYURY* and *CDX2* mRNA levels were substantially increased in cells with decreased *SMAD2* expression, when compared with control cells; and Noggin, added to decrease autocrine BMP signaling, attenuated this induction. Immunofluorescence confirmed the correlation of BMP signaling with *CDX2* expression (Fig. 4*C*). Adding BMP enhanced both the CDX2 signal intensity and number of cells with visible CDX2 expression, although Noggin suppressed CDX2 expression in broad areas of *SMAD2*-silenced cells with, however, patches of strongly CDX2-positive cells remaining. Increased CDX2 expression correlated with decreased OCT4 expression, when compared with control cells. This result is consistent with the decreased OCT4 expression in cells with decreased *SMAD2* expression, as shown in Fig. 1*A*. Conversely, the decrease in CDX2 levels upon Noggin treatment of these cells correlated with the moderate recovery of OCT4 expression (Fig. 4*C*).

Decreased Smad2 Expression Enhances Primordial Germ Cell Specification—In mouse embryos, primordial germ cell (PGC) specification at the epiblast stage is regulated by BMP signaling, and mouse epiblasts with a *Smad2* null mutation show elevated PGC specification marked by *Blimp1* expression (45). As in hESCs, mEpiSCs showed increased *Cdx2* and *Brachyury* expression, when Smad2 expression was down-regulated (Fig. 2*E*). Consistent with their increased BMP responsiveness, mEpiSCs at day 6 after infection with the Smad2 shRNA vector showed increased *Blimp1* and *Prdm14* mRNA expression, as compared with control cells (Fig. 4*D*). When allowed to differentiate in embryoid bodies, mEpiSCs with decreased Smad2 expression showed increased expression of *Blimp1*, *Nanog*, and *Oct4*, markers of PGCs (Fig. 4*E*, *left panel*), consistent with the decrease in pluripotency in undifferentiated culture conditions and suggesting bias toward PGC specification. The induction of *Blimp1*, *Nanog*, and *Oct4* expression was further enhanced by BMP treatment (Fig. 4*E*, *right panel*). Other PGC markers, such as *Dazl*, were also up-regulated without BMP treatment in embryoid bodies of cells with down-regulated *Smad2* expression (data not shown), further suggesting that mEpiSCs with decreased Smad2 levels differentiated toward PGCs. Together with our results using hESCs, these observations suggest that increased autocrine BMP signaling arises with decreased Smad2 expression, leading to differentiation toward trophectoderm, mesoderm, and germ cell lineages.

Suppressing Cdx2 Expression Rescues Decreased Oct4, but Not Nanog, Expression in Cells with Decreased Smad2 Expression—In mouse blastocysts, *Cdx2* expression is mutually exclusive with *Oct4* and *Nanog* expression. Furthermore, Cdx2 binds *Oct4* and *Nanog* gene promoter sequences, and Oct4 and Nanog can bind the *Cdx2* promotor, conferring reciprocal repression (14, 46). Therefore, decreased OCT4 expression in hESCs with decreased *SMAD2* expression (Fig. 1*A*) may result from increased CDX2 expression. Consistent with this hypothesis, Noggin not only decreased CDX2 expression, but also restored OCT4 expression in cells with decreased *SMAD2* expression (Fig. 4*C*). To address the role of CDX2 in the repression of *OCT4* expression caused by *SMAD2* down-regulation, we expressed *CDX2* or control shRNA in hESCs with decreased SMAD2 expression. Reducing *CDX2* mRNA expression with 82 or 76% using separate shRNAs (Fig. 5*A*) restored OCT4 expression to a level comparable with control hESCs (Fig. 5*B*).

We then addressed the role of CDX2 in regulating *NANOG* expression in hESCs with decreased *SMAD2* expression. In contrast to *OCT4* expression, Noggin did not affect *NANOG* expression in control hESCs or hESCs with decreased SMAD2 expression (Fig. 5, *C* and *D*), suggesting that suppression of CDX2 expression by Noggin is insufficient to recover *NANOG* expression. In addition, silencing *CDX2* expression using shRNA also did not rescue the decreased Nanog levels in hESCs with decreased *SMAD2* expression (Fig. 5*B*). These results indicate that increased *CDX2* expression, resulting from enhanced BMP signaling, does not repress *NANOG* expression in cells with decreased *SMAD2* expression.

Decreased Nanog Expression and Increased BMP Responsiveness Cooperatively Result in the Loss of Pluripotency when Smad2 Expression Is Down-regulated—We next evaluated whether decreased *NANOG* expression may contribute to increased CDX2 expression in cells with decreased *SMAD2* expression. To address the direct effects of decreased *NANOG* expression on activation of BMP signaling, we analyzed hESCs at day 3 after siRNA transfection, taking advantage of the transient but efficient effects of transfected siRNA at this early time point. Decreasing *NANOG* expression did not enhance and slightly decreased *CDX2* expression in hESCs (Fig. 6*A*). Furthermore, suppressing NANOG expression did not enhance BMP-induced autocrine SMAD1/5 activation to the extent seen after down-regulating *SMAD2* expression, even though a slight increase in phospho-SMAD1/5 was apparent (Fig. 6*B*). Furthermore, at day 3 after *SMAD2* siRNA transfection, the down-regulation of *SMAD2* expression had not yet resulted in

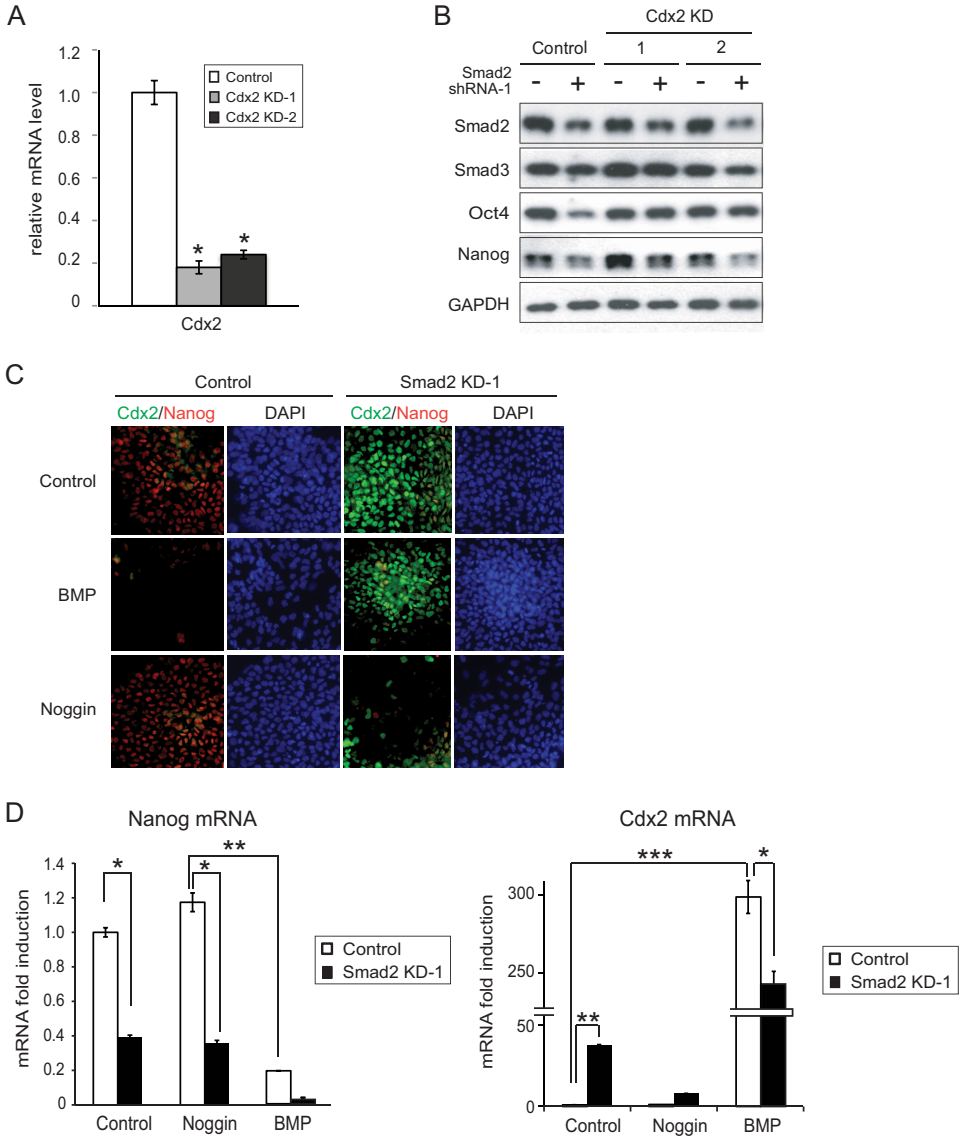

FIGURE 5. **Decreased Oct4, but not Nanog expression, is rescued by silencing *Cdx2* expression.** *A, CDX2* mRNA expression in control hESCs and hESCs with *CDX2* shRNAs. *B,* decreasing CDX2 expression in hESCs using *CDX2* shRNA for 4 days restores expression of OCT4, but not NANOG, as assessed by immunoblots. *C,* immunofluorescence of CDX2 or NANOG expression in control hESCs and hESCs with decreased *SMAD2* expression at day 8 after shRNA infection. Noggin or BMP4 was added from day 3 to day 8. DAPI served as a nuclear counter-stain. *D, NANOG* and *CDX2* mRNA expression in control hESCs and hESCs with decreased *SMAD2* expression, in the presence or absence of Noggin or BMP4 between day 3 and day 8 after infection. $n = 3$ assays. *, $p < 0.05$; **, $p < 0.01$, compared with control cells by Student's *t* tests; ***, $p < 0.005$.

substantial reduction of NANOG expression (Fig. 6*B*) as seen at day 5 after lentiviral shRNA infection (Figs. 1*A* and 5*B*). Because at that time SMAD1/5 activation was already strongly increased (Fig. 6*B*), these data, together with those in Fig. 6*A*, suggest that down-regulation of Nanog expression and increased BMP responsiveness occur independently. Accordingly, decreasing *NANOG* expression neither enhanced *CDX2* or *BRACHYURY* mRNA expression in control cells (no BMP added) nor suppressed their expression in the presence of Noggin (Fig. 6*C*). In response to BMP4, however, down-regulation of *NANOG* contributed to the expression of these BMP target genes (Fig. 6*C*), possibly resulting from lack of inhibition by NANOG bound to SMAD1 (47). Finally, down-regulation of *Nanog* expression did not induce *Cdx2* expression in mouse EpiSCs (Fig. 6*D*), suggesting a similar cooperation of *Nanog* reduction and *Cdx2* induction in hESCs and mEpiSCs, after decreasing *Smad2* expression.

These data suggest that increased BMP responsiveness in cells with decreased *Smad2* expression does not result from direct control of *Nanog* expression by Smad2. Additionally, the increased *Cdx2* expression, resulting from increased autocrine BMP responsiveness, decreases *Oct4* expression in these cells. Thus, decreased *Nanog* expression and enhanced BMP signaling, which lead to increased *Cdx2* expression and then cause *Oct4* down-regulation, cooperate in the loss of pluripotency when TGF-β/activin-activated Smad2 signaling is blocked (Fig. 7).

DISCUSSION

That signaling by TGF-β, activin, or nodal plays key roles in primed pluripotency has been established. Accordingly, their

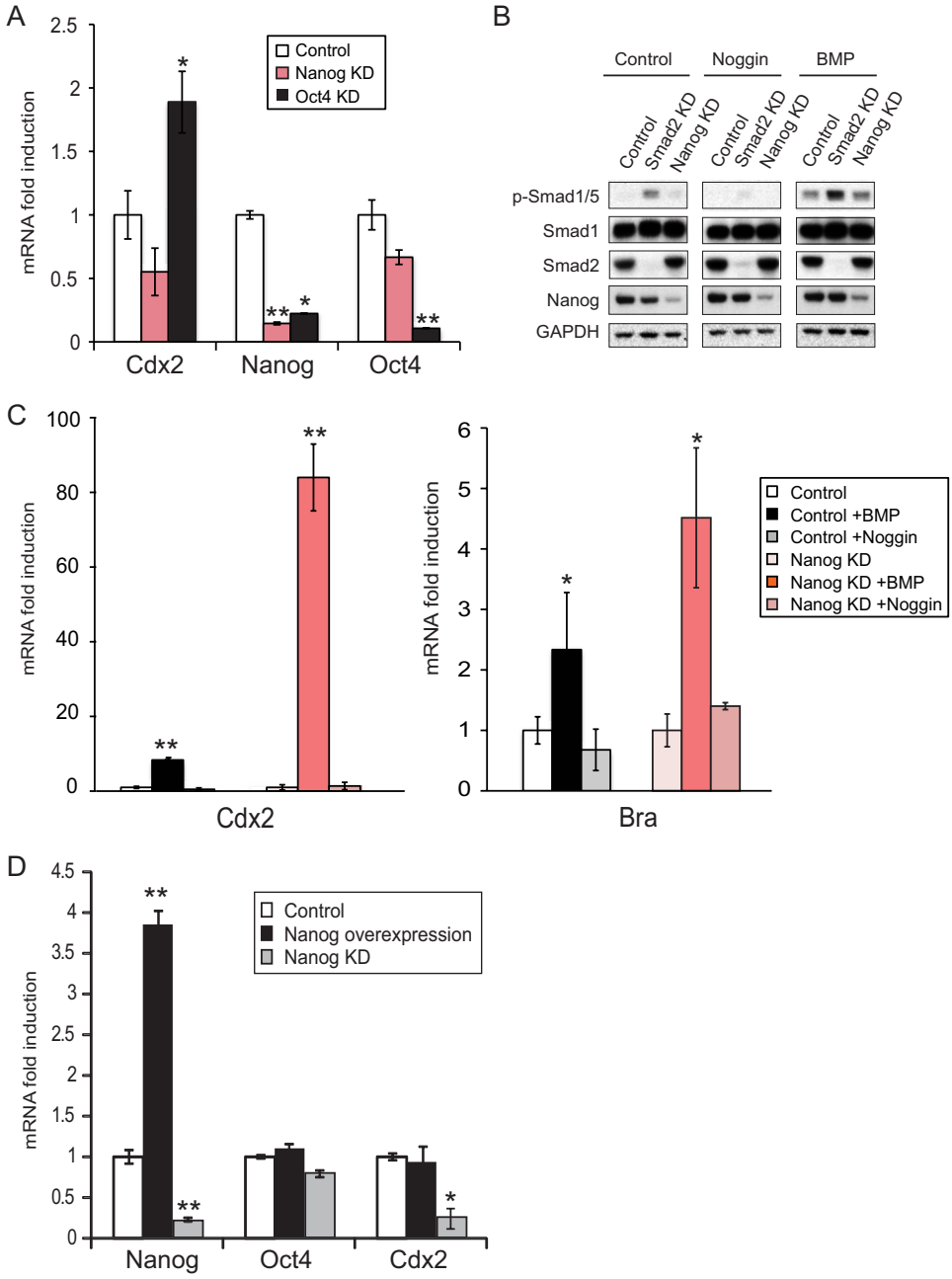

FIGURE 6. ***NANOG* down-regulation does not induce *CDX2* expression or increased BMP responsiveness in undifferentiated hESCs.** *A, CDX2, NANOG*, and *OCT4* mRNA in control hESCs or cells with down-regulated *NANOG* or *OCT4* expression. Reduction of *NANOG* expression does not result in enhanced *CDX2* expression. *B,* down-regulation of Nanog expression does not increase BMP responsiveness, as assessed by immunoblotting for phosphorylated SMAD1/5, as high as in cells with down-regulated *SMAD2* expression. Noggin (25 ng/ml) or BMP (1 ng/ml) was added at 48 or 1 h before analysis. *C, CDX2* and *BRACHYURY* mRNA in control hESC or hESCs with down-regulated *NANOG* expression, with or without BMP stimulation. Noggin (25 ng/ml) or BMP (10 ng/ml) was added for 2 days. *D,* decreased *Nanog* expression does not induce Cdx2 expression in mEpiSCs. qRT-PCR analysis of *Nanog, Oct4,* and *Cdx2* mRNAs in mEpiSCs at day 5 after lentiviral infection with control, Nanog-expressing, or *Nanog* shRNA-expressing vectors. $n = 3$ assays. *, $p < 0.05$; **, $p < 0.01$, compared with control cells by Student's *t* tests.

effectors, Smad2 and Smad3, are seen as transcription regulators for maintaining pluripotency. However, these Smads are functionally distinct, with differences in DNA binding and interactions with transcription factors (33, 34). This study starts delineating distinct roles of Smad2 and Smad3 in maintaining primed pluripotency. We selectively silenced the expression of either Smad, without affecting TGF-β-induced non-Smad signaling. Studying hESCs and mEpiSCs, we found that Smad2, but not Smad3, has a key role in maintaining pluripotency through a dual mechanism. Smad2 targets directly the *Nanog* promoter and directs *Nanog* expression in response to signal activation. Additionally, Smad2 suppresses autocrine BMP signaling, thus controlling the expression of *Cdx2* and other BMP target genes. These two functions of Smad2 are conserved in mEpiSCs and hESCs and act independently from each other, yet cooperate in controlling stem cell pluripotency.

Smad2 Controls Pluripotency and Nanog Expression—In hESCs and mEpiSCs, decreased Smad2 expression resulted in

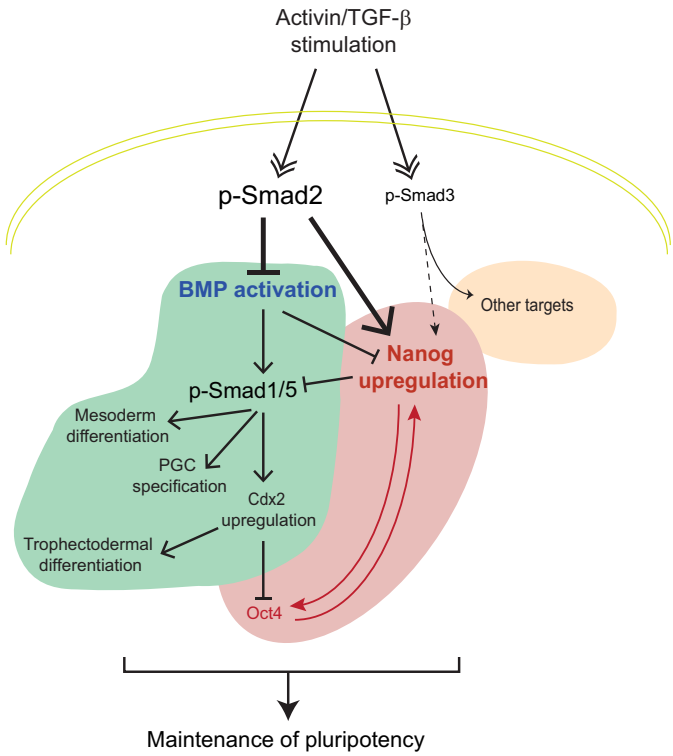

FIGURE 7. **Schematic model of the dual functions of Smad2 in the maintenance of hESC and mEpiSC pluripotency.** Autocrine BMP activity and differentiation upon activation of the BMP pathway (*green area*) need to be suppressed by Smad2. BMP signaling induces the expression of master regulators of differentiation, including Cdx2, and drives differentiation toward mesoderm, PGC, and trophectoderm. Smad2 directly induces *Nanog* expression and maintains expression of other pluripotency regulators such as *Oct4* through enhanced Nanog expression (*pink area*). Suppression of Cdx2 expression by antagonizing BMP signaling is essential to maintain Oct4 expression.

loss of pluripotency. Upon decreasing *Smad2* expression, the cells started differentiating, with increased differentiation along the neuroectoderm, mesoderm, and trophectoderm lineages and decreased endodermal differentiation. In contrast, decreasing *Smad3* expression did not affect *Nanog* or *Oct4* expression, cell proliferation, and colony morphology at day 5, when cells with *Smad2* down-regulation exhibited obvious phenotypes, and it allowed the generation of teratomas composed of three germ layers. These were significantly smaller, possibly due to decreased proliferation (Fig. 1*D*), with less mesodermal differentiation, when compared with teratomas derived from control cells (data not shown). It remains possible that SMAD3 has a distinct role in the maintenance of human ES cell pluripotency. Recently, activin signaling was shown to have opposing effects, depending on the activation of other signaling pathways. At higher Akt activation, activin signaling through Smad2/3 promotes pluripotency, although at lower Akt activation, Smad3 promotes mesodermal gene expression in cooperation with Wnt/β-catenin signaling (48). Thus, differences between Smad2 and Smad3 may be further regulated by specific signaling contexts.

Using antibodies specific for Smad2 or Smad3, we found that TGF-β or activin enriched SMAD2 at a proximal sequence of the *NANOG* gene that has four potential Smad-binding sites. Because Smad2 cannot bind DNA, Smad2 binding at that sequence is likely mediated by Smad4 and/or other DNA-binding transcription factors. However, we did not find evidence for the recruitment of SMAD4 into the promoter-bound SMAD2 complexes. SMAD3 may also associate with this promoter, but SMAD3 binding was not enhanced in response to activin or TGF-β. Additionally, because decreased *SMAD2* but not *SMAD3* expression prevented induction of *NANOG* expression, we conclude that Smad2 directly activates *NANOG* expression upon ligand stimulation. Our results extend the reported Smad2/3 binding to the *Nanog* promoter and the finding that mutation of Smad-binding sites renders this promoter segment unresponsive to TGF-β (35). Overexpression of wild-type or dominant negative Smad3 was also shown to enhance or decrease *Nanog* expression (36). However, overexpressed Smad3 can displace Smad2-Smad4 complexes from Smad-binding sequences, and dominant negative Smad mutants participate in trimeric Smad complexes, interfering with Smad interactions with CBP/p300 coactivators (49–52). Because Smads act through cooperation with high affinity DNA binding transcription factors (53, 54), further identification of Smad2 partners at the *Nanog* promoter will help define the detailed molecular mechanism that confers activation of *Nanog* expression by TGF-β/Smad2.

Suppression of BMP-induced Differentiation by Smad2—While promoting pluripotency in mouse and human ES cells, Nanog was shown to be dispensable for maintaining EpiSC pluripotency (21). We thus surmised that Smad2 might have additional critical roles in maintaining pluripotency. Indeed, by complementing the direct control of *Nanog* expression, Smad2 repressed BMP signaling and BMP-induced differentiation toward the mesoderm, trophectoderm, and germ cell lineages (23, 55, 56). That Smad2 represses BMP responsiveness in hESCs and mEpiSCs was apparent from increased phospho-Smad1/5 levels and activation of BMP target genes encoding *Cdx2, Brachyury, Blimp1*, and *Prdm14* (23, 43, 45), when *Smad2* expression was decreased. Because Smad1/5 activation occurred upon Smad2 down-regulation without adding BMP, even in the presence of Noggin, this responsiveness likely results from autocrine BMP signaling, although we cannot exclude hyper-responsiveness to trace BMP levels in the medium. Competitive titration of Smad4 that is shared with TGF-β/activin and BMP signaling (57) may contribute to increased BMP target gene expression, when Smad2 expression is decreased. However, Smad1/5 phosphorylation occurs independently from the cooperation with Smad4. Considering the complexity of BMP signaling, with its many ligands, inhibitors, coreceptors, and intracellular modulators (44), it is not feasible at this time to delineate the exact basis for increased autocrine BMP signaling. With the increase in *BMP4* and *BMP8b* mRNA levels when SMAD2 signaling is decreased (data not shown), it is tempting to speculate that increased autocrine BMP signaling results from increased ligand expression.

Our results also extend a previous finding that SB431542 enhances BMP responsiveness in mESCs (58). As recently shown, a potent Smad2/3 corepressor SnoN predominantly associates with Smad2 at promoters of primitive streak and early definitive endoderm marker genes. This specific inhibition of definitive endoderm formation was shown to contribute

to the maintenance of hESC pluripotency (59). Intriguingly, ectopic expression of SnoN diverts hESCs toward an extraembryonic fate without repressing pluripotency genes. Thus, increased extraembryonic gene expression, observed in *Smad2* knockdown cells, could be partly due to increased free SnoN levels and increased access to extraembryonic gene promoter sequences.

In embryonic development, TGF-β/activin signaling often contrasts with BMP signaling and can result in functional antagonism. In mice, the site of distal visceral endoderm formation is defined by antagonism between Smad2 and Smad1 (60). Similarly, antagonism of Smad5 with nodal/Smad2 signaling in mouse amnion prevents ectopic primitive streak formation (61). Antagonism between Smad2 and Smad1/5 was also shown to be critical in *Xenopus* dorsal-ventral mesoderm polarity (62) and in *Drosophila* wing development (63). However, a direct link between decreased Smad2 expression and increased Smad1/5 activation has only been shown in a *Drosophila* model (63). Therefore, our results provide the first evidence for a conserved Smad2 function in suppressing BMP-induced Smad activation. Additionally, competition between BMP-activated Smads and TGF-β/activin Smads may control *Nanog* expression (35). This antagonism may be explained by competition for Smad4 (57) or heteromeric Smad combinations in transcription regulation (64). How Smad2 represses BMP signaling in the control of stem cell pluripotency remains to be further defined.

Activation of Nanog Expression and Suppression of BMP Signaling by Smad2 Are Independent Events in Stem Cell Pluripotency—As a core transcription factor in ESC pluripotency, Nanog participates with Oct4 and Sox2 in an autoregulatory circuit to maintain pluripotency (9, 65, 66). Furthermore, Oct4, Sox2, and Nanog control, often in binary combinations, the expression of target genes that define pluripotency (11, 67, 68). Nanog also suppresses neuroectoderm differentiation (36). Thus, reduction of Nanog expression upon decreasing Smad2 expression may eventually be detrimental by leading to misregulation of other core transcription factor and target genes. Conversely, reducing *Nanog* expression did not cause immediate down-regulation of Oct4 expression nor up-regulation of Cdx2 expression in our study. Thus, enhanced BMP signaling may decisively contribute to the loss of pluripotency upon immediate Smad2 down-regulation.

Cdx2 expression is directly repressed by Oct4 or Nanog, and repression of *Nanog* or *Oct4* expression was shown to enhance *Cdx2* expression (14, 46). In our study, decreased *SMAD2* expression in hESCs resulted in repression of NANOG and OCT4 expression and enhanced *CDX2* expression through increased BMP signaling. In hESCs, suppression of *CDX2* expression restored only *OCT4* but not *NANOG* expression (Fig. 5*B*). When kinetically evaluating the effects of *SMAD2* down-regulation, *CDX2* mRNA expression was already enhanced at day 4 after lentiviral infection, without any effect on Oct4 expression, although the *NANOG* mRNA level was mildly decreased (data not shown). In mEpiSCs, a decrease in *Oct4* expression similarly occurred more slowly than that of *Nanog* expression upon down-regulation of *Smad2* expression (Fig. 1*E*). These results argue for direct control of *Oct4* expression by Smad2 and epistatically position the link between Cdx2 and Oct4 under control of BMP signaling. Consistent with our findings, targeted disruption of the *Smad2* gene impairs mouse development between E8.5 and 12.5 with attenuated expression of Oct4 (69).

In contrast, *Nanog* expression was directly controlled by Smad2, and decreased *Cdx2* expression did not restore *Nanog* expression, although *Nanog* down-regulation did not result in increased Cdx2 expression. Furthermore, Noggin did not rescue the decreased *Nanog* expression in *Smad2* down-regulated cells. Importantly, up-regulation of *Cdx2* expression in these cells did not result from decreased *Nanog* expression but from activated BMP signaling. That we did not see cross-regulation between Nanog and Cdx2 expression differs from observations in mESCs, in which the role of *Nanog* is controlled by Oct4 (70, 71). Additionally, the effect of Nanog down-regulation on lineage specification upon BMP stimulation still remains unclear. One report shows that BMP-induced differentiation occurs irrespective of the Nanog expression levels (36), although the other shows that changes in Nanog expression affect the outcome of BMP-mediated differentiation (72). Our results do not conflict with these reports because we found that Smad2 activates *Nanog* expression and suppresses BMP signaling independently from each other.

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