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Combinatorial Signals of Activin/Nodal and Bone Morphogenic Protein Regulate the Early Lineage Segregation of Human Embryonic Stem Cells*[S]

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Cell fate commitment of pre-implantation blastocysts, to either the inner cell mass or trophoblast, is the first step in cell lineage segregation of the developing human embryo. However, the intercellular signals that control fate determination of these cells remain obscure. Human embryonic stem cells (hESCs) provide a unique model for studying human early embryonic development. We have previously shown that Activin/Nodal signaling contributes to maintaining pluripotency of hESCs, which are derivatives of the inner cell mass. Here we further demonstrate that the inhibition of Activin/Nodal signaling results in the loss of hESC pluripotency and trophoblast differentiation, similar to BMP4-induced trophoblast differentiation from hESCs. We also show that the trophoblast induction effect of BMP4 correlates with and depends on the inhibition of Activin/Nodal signaling. However, the activation of BMP signaling is still required for trophoblast differentiation when Activin/Nodal signaling is inhibited. These data reveal that the early lineage segregation of hESCs is determined by the combinatorial signals of Activin/Nodal and BMP.

The pre-implantation human blastocyst consists of two cell types: the pluripotent inner cell mass and the trophoblast, or the outer epithelial layer of the blastocyst. Trophoblast formation is the first lineage segregation in mammalian embryos. The inner cell mass forms all three germ layers of the body, and the trophoblast gives rise to the trophoblast lineages, which form the major fetal parts of the placenta. Therefore, the trophoblast is crucial for embryo implantation, as well as promotion of embryo survival and growth in the uterus. Trophoblast developmental disorders result in "missed abortions" (pregnancy loss during first two months of gestation), certain types of intrauterine growth restriction, and pre-eclampsia (1, 2). Moreover, it has become clear that the trophoblast also plays key roles in epiblast signaling to establish axial patterning in the embryo. Prior to gastrulation of the early post-implantation embryo, the extraembryonic ectoderm, a trophoblast derivative, is thought to provide general signals that promote expression of posterior mesoderm-specific genes in the underlying epiblast, such as Brachyury (3). Therefore, correct segregation of the trophoblast from the inner cell mass is essential for body plan establishment and embryo survival.

Mice are used extensively for studying the molecular regulation of early mammalian development, due to the advances of genetic manipulation. In the past few years, there has been significant progress in our understanding of genetic control of trophoblast development, which have mainly stemmed from analyses of targeted mutations in the mouse (1, 2). The current understanding of early human embryonic development is based largely on comparisons to mouse development; however, there are significant differences between murine and primate development that limit the usefulness of the mouse model. The derivation of human embryonic stem cell (hESC)[3] lines from the inner cell mass of the human blastocyst (4, 5), and the manipulation of hESCs *in vitro* (6–15) provide a unique model for studying mechanisms of human embryogenesis.

We, along with others, have previously shown that Activin/Nodal signaling maintains hESC pluripotency (16–18). In the present study, we further demonstrate that inhibition of Activin/Nodal signaling results in the loss of hESC pluripotency and trophoblast differentiation. Both activin and Nodal belongs to the TGF-β superfamily that also includes BMP. The action of specificity of various ligands of this superfamily is controlled at multiple levels. Activin/Nodal as well as TGF-β use one set of receptors (Activin receptor-like kinase 4/5/7) and downstream of signal molecules (SMAD2 and SMAD3), whereas BMPs such as BMP4 utilize a different set of receptors (Activin receptor-like

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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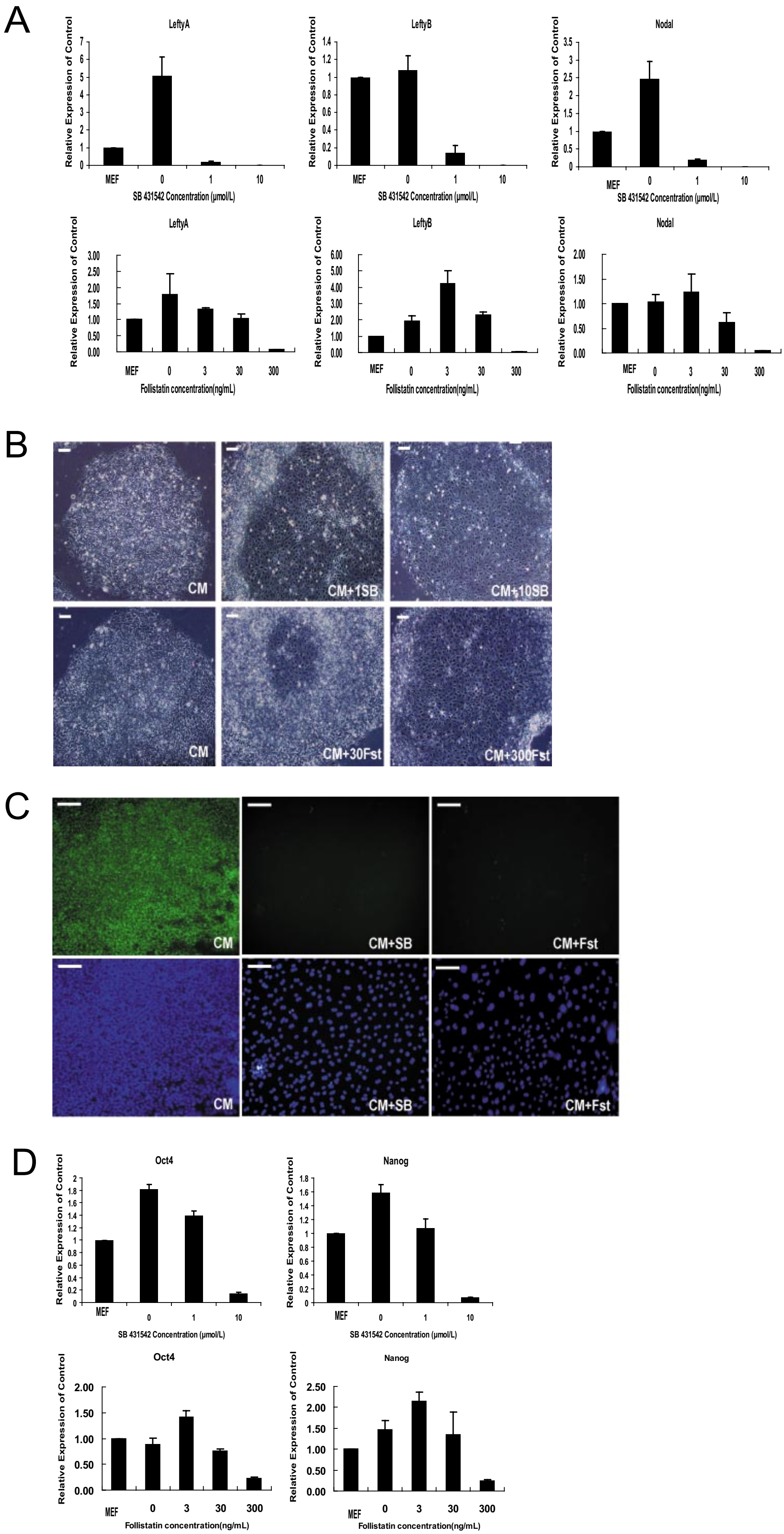
[3] The abbreviations used are: hESC, human embryonic stem cell; CM, conditioned medium; hCG, human chorionic gonadotropin; TGF, transforming growth factor; BMP, bone morphogenic protein; FGF, fibroblast growth factor; ELISA, enzyme-linked immunosorbent assay.

kinase 1/2/3/6) and activates different SMAD transducers (SMAD1/5/8) and other targets (19). Two branches of TGF-β/BMP signaling pathways, one used by BMPs (and Smad1/5/8) and one used by Activin/Nodal/TGF-β (and SMA2/3) naturally antagonize each other, because activated Smad1/5/8 or SMAD2/3 need to compete for the common SMAD4, which is required for the activation of either branch (19).

BMP4 has been reported to induce hESCs to differentiate into trophectoderm (13). We demonstrate here that BMP4 activity depends on inhibition of TGF-β/Activin/Nodal signaling, and this is further supported by results showing that TGF-β/Activin/Nodal signaling is able to reverse the effects of BMP4. We also found that activation of BMP signaling is required for the trophoblast development from hESCs when Activin/Nodal signaling is inhibited. Therefore, we conclude that Activin/Nodal and BMP signaling regulates early hESC lineage segregation. Both inhibition of Activin/Nodal and activation of BMP signaling are required for the trophoblast differentiation from hESCs. In addition, our data suggest that the Activin/Nodal and BMP signals might regulate trophoblast commitment, during human embryonic development *in vivo*.

EXPERIMENTAL PROCEDURES

hESC Culture—The hESC line H1 (WA01) was kindly provided by Dr. Saul Sharkis from Johns Hopkins University, under permission from WiCell Research Institute (5, 16), and HUES-17 was kindly provided by Dr. Douglas Melton, Harvard University (4). All hESC experiments were conducted in accordance with the guidelines for research on human embryonic stem cells, jointly issued by the Ministry of Science and Technology and the Ministry of Health of China (20), and approved by the ethical committee of Shanghai Institutes for Biological Sciences. hESCs were maintained on feeders in hESC medium, which



contained 80% Dulbecco's modified Eagle's medium/Ham's F-12 medium (F12), 20% knock-out serum replacement, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/ml human basic FGF. hESCs cells were passaged approximately once a week by incubation in 1 mg/ml collagenase IV for ~30 min at 37 °C. Protein factors or SB431542 were added directly to the culture in the continued presence of conditioned medium (CM). Recombinant human Activin A, recombinant human BMP-4, and human Follistatin were purchased from R&D Systems Inc. SB431542 was purchased from Tocris Bioscience.

RNA Isolation and Real-time Reverse Transcription-Polymerase Chain Reaction—RNA was extracted using TRIzol reagent for total RNA isolation according to the manufacturer's instructions (Invitrogen). cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed using a Synergy Brand GreenI-based PCR Master mixture (TOYOBO). PCR primers are listed in supplemental Table S1. Each experiment was repeated at least three times. The expression value of each gene was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase cDNA to calculate a relative amount of RNA present in each sample. The expression level of each gene in a single sample was arbitrarily defined as 1 unit. The normalized expression values for all control and treated samples were averaged, and an average -fold change was determined. Analysis of variance was conducted between the normalized relative expression values for control and treated samples to determine statistical significance.

Immunostaining—Immunostaining was performed similarly to previously described protocol (16). The following antibodies were used: anti-SSEA4 (Developmental Studies Hybridoma Bank), anti-hCGα (R&D Systems), and anti-hCGβ (Abcam).

Western Blotting—Cells were lysed with 1× lysis buffer: 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, and complete mini-protease inhibitor mixture (Roche). Total protein (10 μg) was loaded for each lane. Membranes were blocked in Tris-buffered saline with 0.1% Tween and 5% milk. The following antibodies were used: anti-phospho-Smad2/3 (Cell Signaling), anti-Smad2/3 (Cell Signaling), anti-phospho-Smad1 (Santa Cruz), anti-Smad1 (Santa Cruz), anti-Oct4 (Santa Cruz), and β-Actin (Abcam). Primary antibodies were incubated overnight and secondary antibodies for 2 h. Proteins were detected with chemiluminescent (Pierce).

Immunoassays of Placental Hormones in the Culture Medium—H1 cells were cultured in CM with or without SB431542 for 12 days, and the medium was changed every day. The conditioned medium was collected daily from days 2 to 12. The hCG concentration was analyzed using a hCG ELISA kit (RECI), which specifically reacts with CG-β. The concentration of estradiol and progesterone were analyzed with an ELISA kit.

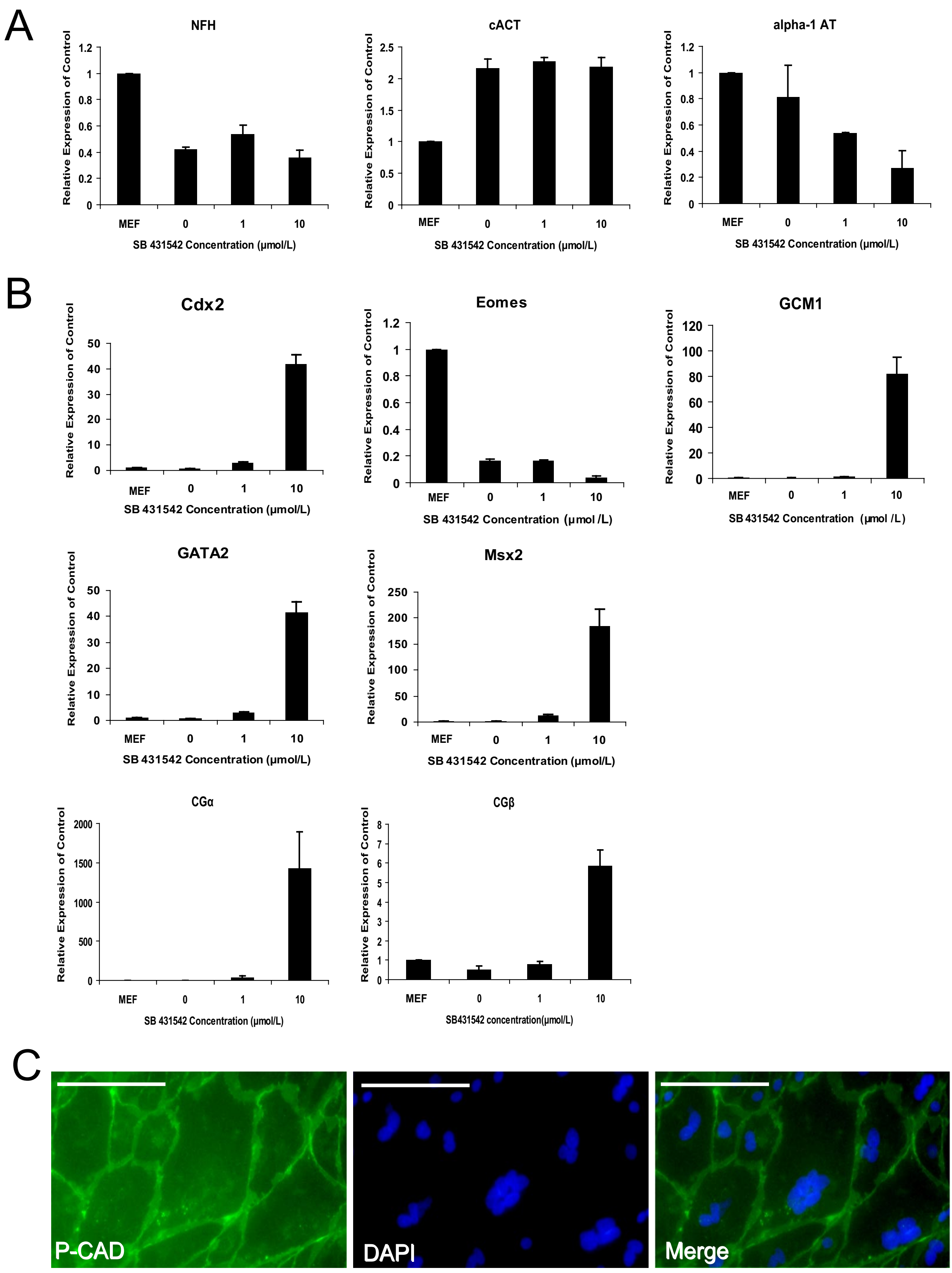
RESULTS

Inhibition of Activin/Nodal Signaling in hESCs Results in Rapid Differentiation—Activin/Nodal signaling has been shown to play a key role in the maintenance of undifferentiated human ES cells (16–18). To further address the function of Activin/Nodal signaling in the developmental fate of hESCs, and to understand the early developmental mechanisms of human embryogenesis, we inhibited Activin/Nodal signaling in hESCs.

Two hESCs lines, H1 and HUES-17, were used in this study, and the results obtained from these two cells lines were very similar. For this reason, only data from the H1 cells have been presented. HESCs were cultured without murine embryonic fibroblast feeder cells in CM, or CM plus different concentrations of Activin/Nodal signaling inhibitors, SB431542 or Follistatin, for 6 days; gene expression was analyzed by real-time PCR. Consistent with previous studies, the conditioned media to the culture system is sufficient for the maintenance of undifferentiated hESCs (21). SB431542 inhibits the function of Activin receptor-like kinase receptors 4/5/7 thereby acting as a selective inhibitor of Activin/Nodal signaling, but not those of BMPs (22, 23). Follistatin is an inhibitor of Activin by directly binding with Activin and preventing the assembly of an active Activin-receptor complex (24). When hESCs were cultured in CM supplied with SB431542, the expression levels of *p*-Smad2, and known downstream targets of Activin/Nodal signaling, namely Nodal, Lefty-A, and Lefty-B, were significantly inhibited (Figs. 1*A* and 5*C*). SB431542 is a very potent inhibitor of Activin/Nodal signaling; in hESCs cultured with CM plus 10 μM SB431542, the expression of Nodal, Lefty-A, and Lefty-B decreased to less than 0.1% of hESCs cultured with CM. We also determined that hESCs underwent differentiation when Activin/Nodal signaling was inhibited, because the treated cells became flattened and enlarged (Fig. 1*B*) and that pluripotency markers, such as Oct4, Nanog, and SSEA4 were significantly down-regulated (Fig. 1, *C* and *D*). Similar results were obtained with Follistatin as an inhibitor of Activin/Nodal signaling in hESCs as observed with SB431542 (Fig. 1). These results demonstrate that and the inhibition of Activin/Nodal signaling promoted differentiation of hESCs.

Inhibition of Activin/Nodal Signaling in hESCs Initiates Trophoblast Differentiation—To determine lineage commitment or differentiation due to inhibition of Activin/Nodal signaling, we analyzed the induction of lineage-specific marker expression. Unlike what we observed with a standard differentiation by embryoid bodies formation, we did not observe a significant up-regulation in expression of ectoderm (neurofilament heavy chain), mesoderm (cardiac actin), or endoderm (α_1-antitrypsin) markers (Fig. 2*A*), indicating that inhibition of Activin/Nodal signaling under the monolayer culture condition did not

FIGURE 1. **Inhibition of Activin/Nodal signaling induces differentiation of hESCs.** The H1 hES cells were cultured under a feeder-free condition and treated with SB431542 or Follistatin for 6 days. Then cells were harvested for analyses. *A*, real-time polymerase chain reaction analysis of the downstream targets of Activin/Nodal signaling. H1 human embryonic stem cells were maintained in CM supplemented with varying concentrations of SB431542 (*upper*) or Follistatin (*lower*) for 6 days. *B*, morphological changes of SB431542-treated or Follistatin-treated H1 cells. *C*, SSEA4 immunofluorescence of H1 cells treated with CM, or CM plus 10 μmol/liter SB431542 or 300 ng/ml Follistatin for 6 days. *D*, real-time polymerase chain reaction analysis of the pluripotent markers, Oct4 and Nanog. The expression level of each gene in H1 hESCs maintained on murine embryonic fibroblast feeder cells is arbitrarily defined as 1 unit. *MEF*, hESCs maintained on murine embryonic fibroblast feeder; *SB*, SB431542; *FST*, Follistatin.


A
NFH
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Relative Expression of Control
SB 431542 Concentration (µmol/L)
MEF
0
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CGα
CGβ
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DAPI
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initiate differentiation of endoderm, mesoderm, or ectoderm in hESCs. However, the trophoblast marker GCM1 was specifically up-regulated (Fig. 2*B*), which suggests that hESCs might have differentiated into trophoblasts when Activin/Nodal signaling was inhibited. The notion of trophoblast differentiation was further supported by the up-regulation of other trophoblast markers, such as Cdx2, GATA2, Msx2, CG-α, and CG-β. CG-α and CG-β are subunits of human chorionic gonadotropin (hCG), which is secreted by giant cells of trophoblast-derived placenta. We also analyzed another key regulator of trophoblast differentiation in mice, eomesodermin (Eomes) (25). Although Eomes plays a key role in mouse trophoblast differentiation, it is a downstream target of Activin/Nodal signaling in mice and *Xenopus* (26, 27). We observed that Eomes showed down-regulation when Activin/Nodal was inhibited. Taken together, these data indicate that the inhibition of Activin/Nodal signaling results in trophoblast differentiation in hESCs. Notably, we also observed a slight up-regulation of neuroectoderm markers, such as Nestin, Sox1, Sox3, and NGN2, when Activin/Nodal signaling was inhibited, which supports a recent article by Smith *et al.* (28) (supplemental Fig. S1).

We attempted to differentiate hESCs that were growing as embryoid bodies or as a monolayer; however, results were similar (supplemental Fig. S1). The differentiation of hESCs as a monolayer produced higher expressions of trophoblast markers and lower expression of other lineage markers, such as Sox3. Therefore, the data presented in this paper pertain to monolayer cultures, unless specifically mentioned.

To understand the kinetics of trophoblast differentiation, we performed time course experiments and analyzed marker expression by real-time PCR. Results showed that inhibition constantly repressed Activin/Nodal signaling (Fig. 3*A*). The expression of pluripotency markers, namely Oct4 and Nanog, decreased in a time-dependent manner (Fig. 3*B*). Cdx2 has been shown to be the key regulator of trophoblast commitment and subsequent self-renewal in mice (29); inhibition of Activin/Nodal signaling in hESCs initiated Cdx2 expression after 2 days, and expression rose to a peak on day 6 and decreased thereafter (Fig. 3*C*). Gcm1 expression was induced by inhibition of Activin/Nodal signaling on day 4 and continued to increase throughout differentiation (Fig. 3*C*). Two additional markers that often associated with BMP activation and trophoblast commitment, GATA2 and Msx2, were also dramatically up-regulated and reached a peak level at day 10 (Fig. 3*C*). Furthermore, CG-α and CG-β expression significantly increased at day 6 and reached a surprisingly high level on day 12 (Fig. 3*C*). Eomes decreased during trophoblast differentiation of hESCs (Fig. 3*C*), which suggests that Eomes might be dispensable in trophoblast differentiation of hESCs. The transient expression of Cdx2 suggests that its function could be to induce Gcm1 and other trophoblast transcriptional factors, and the down-regulation of Cdx2 might allow for further trophoblast maturation.

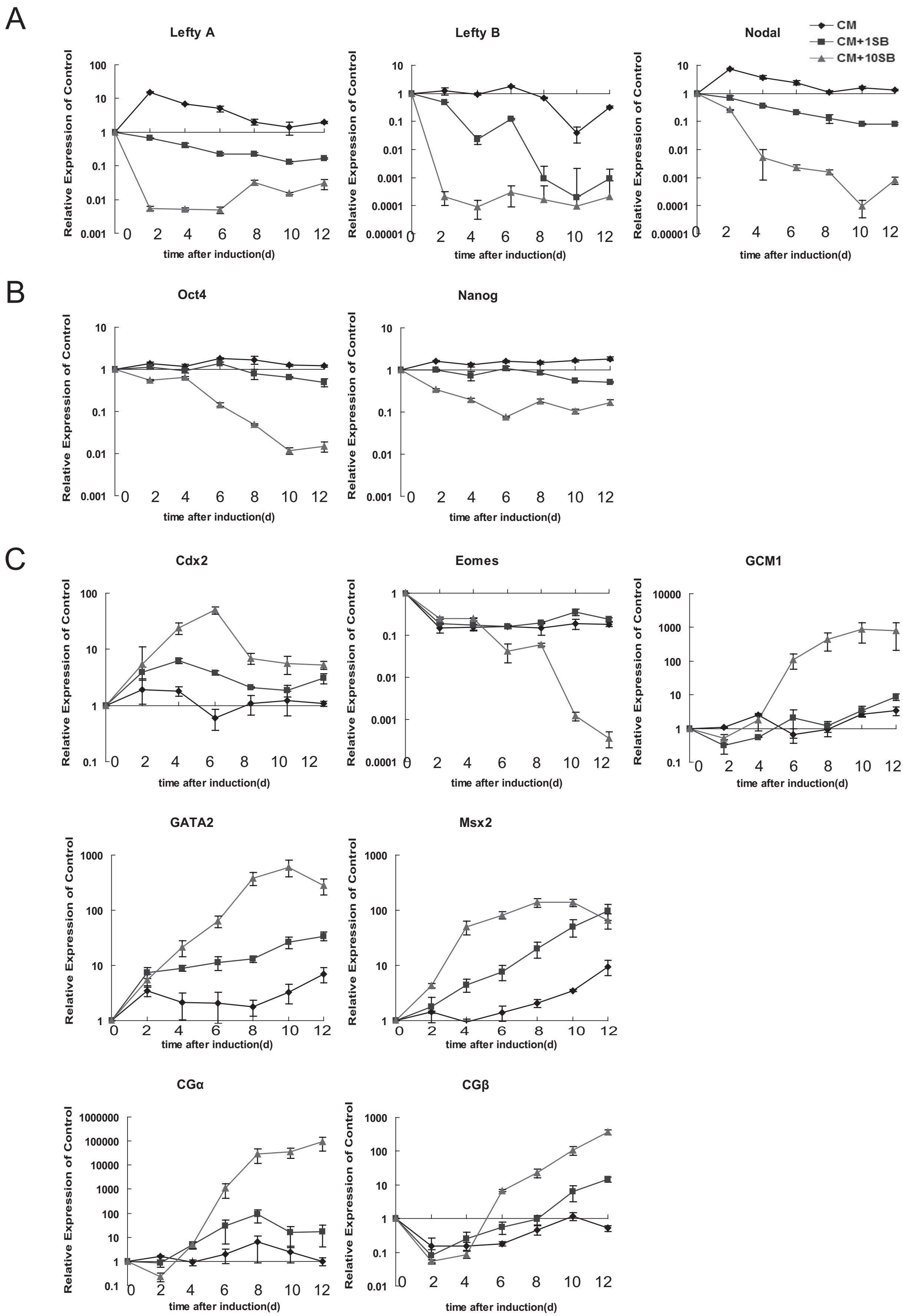
Although the hESC is the only available model thus far for studying human embryonic development, the human ES cell model may not entirely reflect embryonic development *in vivo*. To explore this, we tested the *in vivo* effects of SB431542 in mouse embryos. The 8-cell stage mouse embryos were cultured with 10 μM SB431542 for 3 days. No gross abnormalities were detected at 4.5 days postcoitum; the inner cell mass and trophoblast formed normally (data not shown). These observations are in accordance with previous reports, demonstrating that Activin/Nodal signaling is involved in the propagation of mouse embryonic stem cells, but is not involved in the regulation of pluripotency (18, 30).

hESC-derived Trophoblast Cells Secrete Placental Hormones—Prolonged cultures of hESCs in CM plus SB431542 were performed (12 days); the cells continued to develop, and numerous differentiated cells contained multiple nuclei (Fig. 2*C*). Xu *et al.* (13) reported that syncytial cells were present only among individualized BMP4-treated hESCs plated at low density, whereas BMP4-treated hESC colonies form only mononuclear cells. In contrast, the present study demonstrated that the SB431542- or Follistatin-treated hESC colonies formed syncytial cells (Fig. 2*C*), which suggests that inhibition of Activin/Nodal signaling is more efficient than BMP4 in inducing syncytial cell formation. It was not attempted to induce hESC differentiation in individual cells.

To further confirm trophoblast differentiation from hESCs, the amount of placental hormones in differentiated cells was measured. Both CG-α and CG-β proteins were detected in a large percentage of differentiated hESCs after 12 days treatment with SB431542 or Follistatin (Fig. 4, *A* and *B*). The percentage of the CG-α-expressing cells was 74 $\pm$ 5% ($n = 3$) when Activin/Nodal signaling was inhibited by 10 μM SB431542, and 66 $\pm$ 3% ($n = 3$) when Activin/Nodal signaling was inhibited by Follistatin, respectively. In addition, during hESC differentiation, the placental hormones, hCG (consisting both a and b subunits), estradiol, and progesterone, were secreted in the supernatant in a time- and dose-dependent manner (Fig. 4*C*).

Inhibition of Activin/Nodal Signaling Down-regulates FGF and Wnt Signals, but Up-regulates BMP Signals—FGF signaling has been shown to be important in the maintenance of hESC pluripotency (31, 32), and Wnt signaling has been shown to stimulate the proliferation of hESCs (33–35). Previously, we have reported that Activin/Nodal signaling up-regulates FGF and Wnt signaling in hESCs (16). The present study demonstrates that the expression of FGF2 (Fig. 5*A*), FGF4 (Fig. 5*A*), FGF8 (Fig. 5*A*), and Wnt3 (Fig. 5*A*) was significantly repressed by the inhibition of Activin/Nodal signaling, but *p*-Smad1 and BMP4 expression was significantly up-regulated (Fig. 5, *B* and *C*). These observations further strengthen our previous hypothesis that Activin/Nodal signaling plays a key role in the complex signaling network that maintains the hESC phenotype and function (16).

FIGURE 2. **Inhibition of Activin/Nodal signaling induces trophoblast differentiation.** The H1 hES cells were cultured under a feeder-free condition and treated with SB 431542 for 6 (*A* and *B*) or 12 days (*C*). Then cells were harvested for analyses. *A*, real-time polymerase chain reaction analysis of endoderm (*α1-AT*), mesoderm (*cACT*), and ectoderm (*NFH*) markers. *B*, real-time polymerase chain reaction analysis of multiple trophoblast markers. SB431542 up-regulates trophoblast marker expression in a dose-dependent manner. *C*, differentiated cells form syncytial cells after incubation in CM plus 10 μmol/liter SB 431542 for 12 days. The expression level of each gene in H1 hESCs maintained on murine embryonic fibroblast feeder cells is arbitrarily defined as 1 unit. *α1-AT*, α_1-antitrypsin; *cACT*, cardiac actin; *NFH*, neurofilament heavy chain; *DAPI*, 4′,6-diamidino-2-phenylindole.


A
Lefty A
Lefty B
Nodal
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CM+1SB
CM+10SB
B
Oct4
Nanog
C
Cdx2
Eomes
GCM1
GATA2
Msx2
CGα
CGβ
Relative Expression of Control
time after induction(d)

BMP4-induced Trophoblast Differentiation Correlates with Inhibition of Activin/Nodal Signaling—As reported by Xu *et al.* (16), we also observed that hESCs differentiated into trophoblasts when cultured in CM plus BMP4 (10–50 ng/ml), as evidenced by the down-regulation of pluripotency markers, such as Oct4 and Nanog (Fig. 6*A*), and the up-regulation of Cdx2, Gcm1, GATA2, CG-α, and CG-β (Fig. 6*A*). At the same time, expression of Lefty-A, Lefty-B, and Nodal was largely inhibited in a dose-dependent manner (Fig. 6*B*). Taken together, these results indicate that BMP4 was sufficient to inhibit Activin/Nodal signaling and that BMP4-induced trophoblast differentiation in hESCs correlates to the inhibition of Activin/Nodal signaling.

Inhibition of Activin/Nodal Signaling Is Essential for Trophoblast Differentiation—We further investigated whether inhibition of Activin/Nodal signaling is essential for hESC trophoblast differentiation. hESC differentiation was induced by incubating the cells in CM supplemented with BMP4 and gradients of Activin A. Results showed that Activin A restored the expression of Lefty-A, Lefty-B, and Nodal, indicating release of the BMP inhibition effect on Activin/Nodal signaling (Fig. 6*C*). Activin A also significantly inhibited CG-α and CG-β expression, which was induced by BMP4 (Fig. 6*D*). Immunostaining methods were utilized to detect CG-α and CG-β proteins in hESCs after 6 days of treatment with 10 ng/ml BMP4, or 10 ng/ml BMP4 plus 100 ng/ml Activin A. BMP4 induced the hESCs to produce CG-α and CG-β. However, the number of CG-α- and CG-β-positive cells was reduced dramatically when Activin A was added (Fig. 6*E*). In addition, ELISA analyses demonstrated that Activin A significantly repressed the placental hormones, hCG, estradiol, and progesterone, in a dose-dependent manner (Fig. 6*F*). Therefore, we conclude that inhibition of Activin/Nodal signaling is essential for trophoblast differentiation of hESCs.

BMP Activation Is Required for the Trophoblast Differentiation from hESCs—We showed that inhibition of Activin/Nodal signaling induced the expression of BMP4 (Fig. 5*B*). It is interesting to know if the BMP4 induced by inhibition of Activin/Nodal is required for the trophoblast differentiation. We took advantage of a glycosylphosphatidylinositol-AP deficient hESC line, namely AR1-C1 (37). The BMP signaling depends on a co-receptor, Dragon. Dragon is a glycosylphosphatidylinositol-AP. In AR1-C1 hESCs, the function of Dragon is disrupted due to the lacking of glycosylphosphatidylinositol anchor. Therefore, the extracellular BMP cannot bind with the receptor well and the BMP signaling is blocked. The trophoblast development induced by BMPs in wild type hESCs (G-GFP) is blocked in AR1-C1 hESCs, evidenced by absence of the expression of trophoblast markers like CDX2, CG-a, CG-b (Fig. 7*A*), and Troma-1 in AR1-C1 cells (Fig. 7*B*). The deficiency of BMP signaling can be rescued by transfection of Dragon, which indicates that the deficiency of trophoblast development is caused by deficiency of BMP signaling, not any other signal, in the AR1-C1 cell.[4] We expected that if activation of BMP signaling by BMPs was not required for trophoblast differentiation when Activin/Nodal signaling is repressed, the AR1-C1 cells would differentiate into trophoblast when Activin/Nodal signaling is repressed. If activation of BMP signaling by BMPs is required, the AR1-C1 cells would not differentiate into trophoblast when Activin/Nodal signaling is repressed. When the Ar1-C1 cells were treated with SB431542 to inhibit Activin/Nodal signaling, no evidence of trophoblast differentiation was observed (Fig. 7). Therefore, our data indicated that both inhibition of Activin/Nodal and activation of BMP signaling were required for trophoblast differentiation from hESCs.

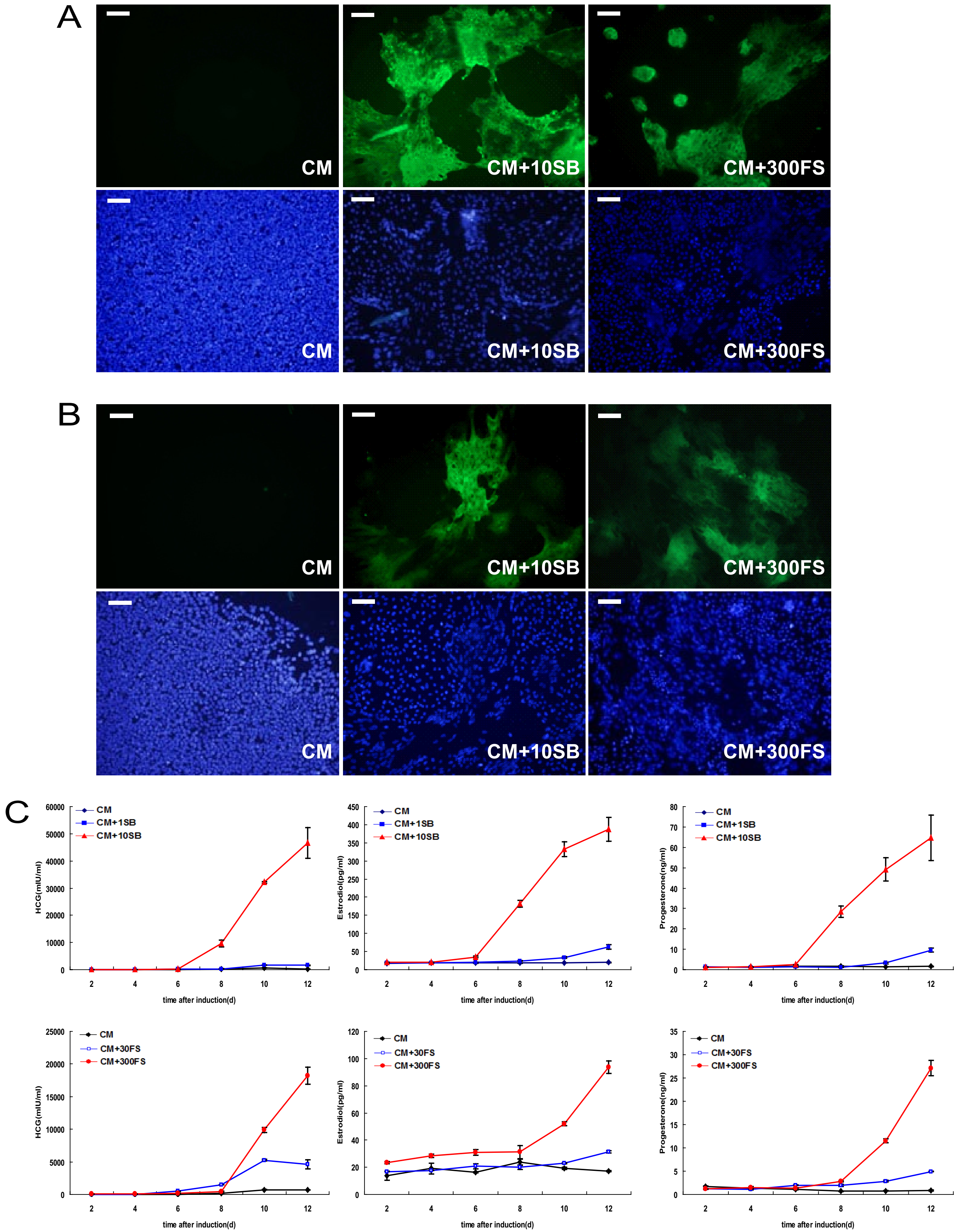
DISCUSSION

The first cell lineage segregation in human embryonic development takes place at the blastocyst stage, when the trophoblast segregates from the inner cell mass. Due to ethical and practical reasons, it has been difficult to determine the key signals in this event (1). We, along with others, have previously shown that Activin/Nodal signaling maintains pluripotency of hESCs (16–18, 36). In the present study, it is demonstrated that hESCs develop into trophoblasts, when Activin/Nodal signaling is inhibited (Fig. 2). Based on these observations, we propose that the segregation of the trophoblast from the inner cell mass is controlled by Activin/Nodal signaling. In the human morula, the cells that receive active Activin/Nodal signals form the inner cell mass; other cells that do not receive sufficient Activin/Nodal signals develop into the trophoblast. This suggests that Activin/Nodal signaling regulates the first differentiation event of human embryonic development.

Xu *et al.* (13) showed that BMP4 is able to initiate trophoblast differentiation. To further address the mechanisms that control cell lineage segregation at the human blastocyst stage, the relation of BMP signal to inhibition of Activin/Nodal signaling was investigated. Results showed that the effect of BMP4 correlates to inhibition of Activin/Nodal signaling. In addition, inhibition of Activin/Nodal signaling induced trophoblast differentiation (Figs. 2 and 3), whereas Activin/Nodal signaling inhibited trophoblast differentiation resulting from BMP4 signals (Fig. 6). Based on these results, we conclude that inhibition of Activin/Nodal signaling is essential for trophoblast differentiation of hESCs.

Our data also showed that when Activin/Nodal was repressed, BMP4 was induced. This raised the possibility that BMP4 induced by Activin/Nodal repression promotes trophoblast differentiation. We used the BMP co-receptor, Dragon, deficient hESCs (37) to investigate if the BMP4 induced by Activin/Nodal repression is required for trophoblast differentiation. We found that trophoblast differentiation was still

FIGURE 3. **Trophoblast differentiation in a dose- and time-dependent manner.** The H1 hES cells were cultured under a feeder-free condition and treated with SB431542 for 12 days. Real-time PCR analyses of the downstream targets of Activin/Nodal signaling (*A*), the pluripotent markers (*B*), and the trophoblast markers (*C*), during differentiation of H1 cells to trophoblast cells following induction by SB431542. Relative expression levels of each gene were analyzed at 0, 2, 4, 6, 8, 10, and 12 days, respectively, after addition of SB431542. The expression level of each gene at day 0 (prior to the addition of SB431542) is arbitrarily defined as 1 unit.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Progesterone(ng/ml)
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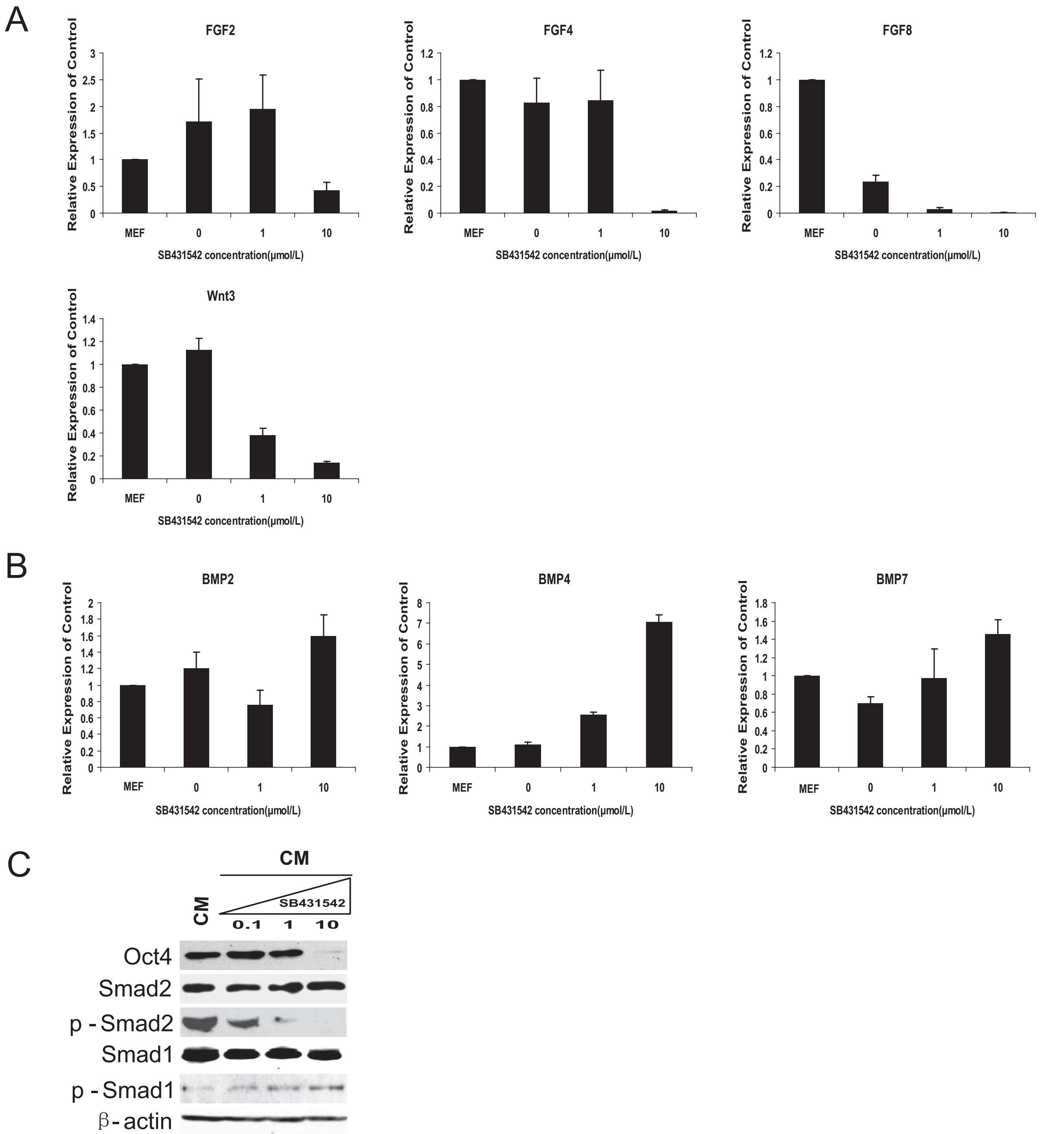
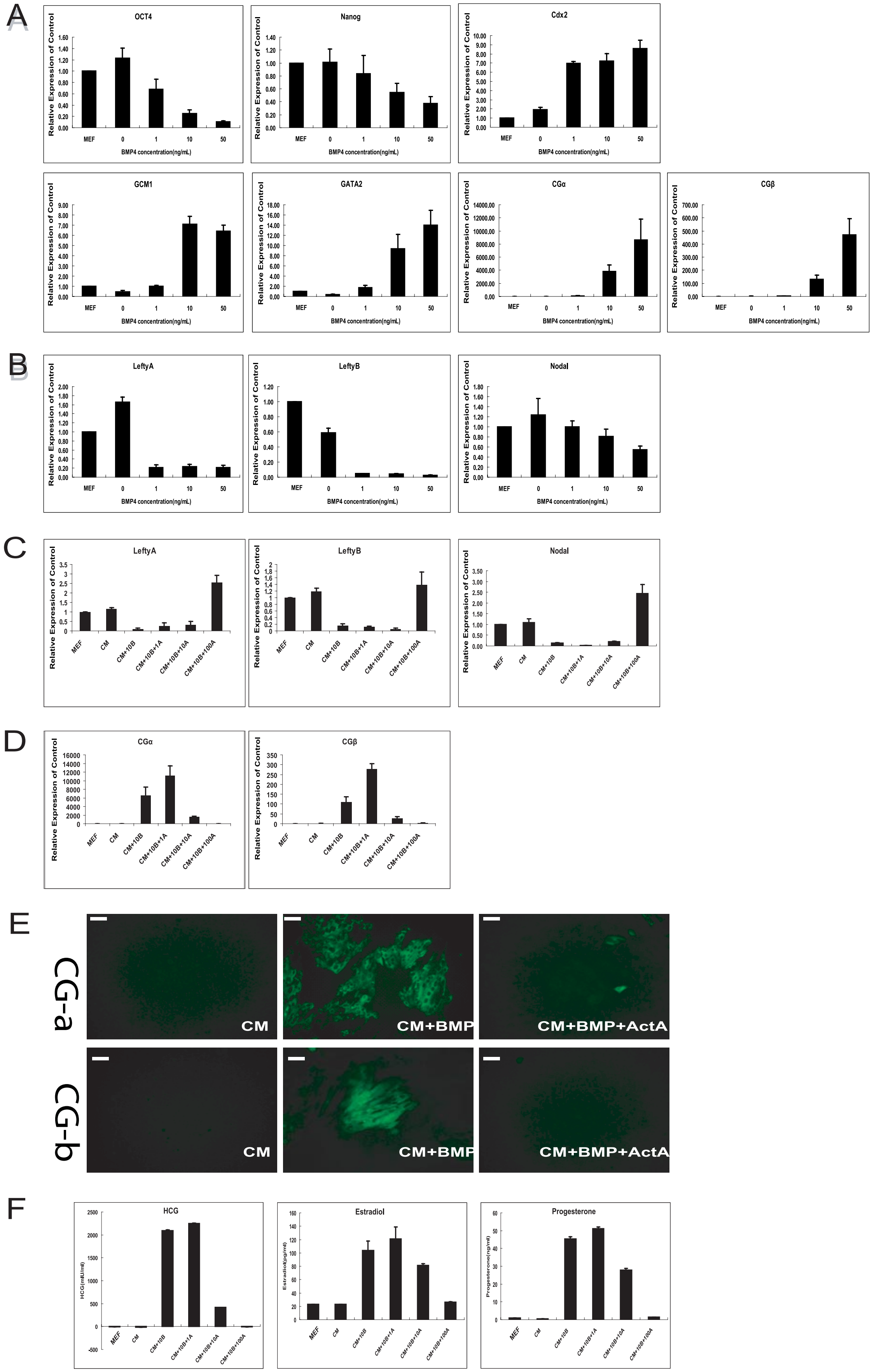


FIGURE 5. **Inhibition of Activin/Nodal signaling down-regulates FGF and Wnt signals, but up-regulates BMP signals.** The H1 hES cells were cultured under a feeder-free condition and treated with SB431542 for 6 days. Then cells were harvested for real-time polymerase chain reaction analysis of ligands of the FGF (*A*), Wnt (*A*), and BMP (*B*) signaling pathways and Western analysis of Oct4, Smad2, *p*-Smad2, Smad1, and *p*-Smad1 (*C*).

blocked when Activin/Nodal was repressed. Our data suggested that BMP signaling is still required for trophoblast development even when Activin/Nodal is repressed. The observation should not be simply interpreted that they are upstream and downstream. Because trophoblast induction of BMP also depends on the inhibition of Activin/Nodal. Activin/Nodal

FIGURE 4. **hESC-derived trophoblast cells secrete placental hormones.** *A* and *B*, Immunofluorescence for CG-α and CG-β. H1 cells were treated with CM, or CM plus 10 μmol/liter SB431542 or 300 ng/ml Follistatin for 12 days. *C*, immunoassays of placental hormones. Conditioned culture medium from H1 cells cultured in CM; CM + 1 SB; CM + 10 SB; CM + 30 FS; or CM + 300 FS were collected at the indicated times and subjected to immunoassays for hCG, estradiol (*E2*), and progesterone (*Prog*). *CM + 1SB*, CM plus 1 μmol/liter SB431542; *CM + 10SB*, CM plus 10 μmol/liter SB431542; *CM + 3FS*, CM plus 3 ng/ml Follistatin; *CM + 30FS*, CM plus 30 ng/ml Follistatin; *CM + 300FS*, CM plus 300 ng/ml Follistatin.


A
OCT4
Nanog
Cdx2
GCM1
GATA2
CGα
CGβ
Relative Expression of Control
MEF
BMP4 concentration(ng/mL)
B
LeftyA
LeftyB
Nodal
C
LeftyA
LeftyB
Nodal
MEF
CM
CM+10B
CM+10B+1A
CM+10B+10A
CM+10B+100A
D
CGα
CGβ
E
CG-a
CG-b
CM
CM+BMP
CM+BMP+ActA
F
HCG
Estradiol
Progesterone

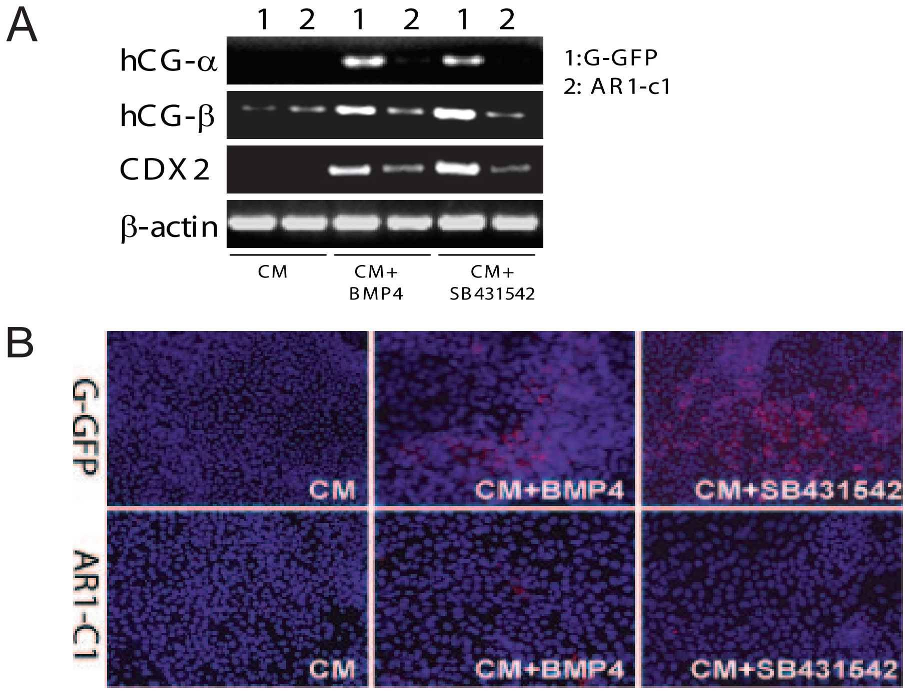


FIGURE 7. **Activation of BMP4 is required for trophoblast differentiation.** The parental (*G-GFP*) and AR1-c1 hES cells were cultured under a feeder-free condition and treated with BMP4 (50 ng/ml) or SB431542 (10 μM) for 10 days. *A*, cells were harvested for real-time PCR analyses. *B*, immunofluorescent staining for trophoectoderm markers TROMA-I (*red*) from the differentiated G-GFP and AR1-c1 cells.

inhibition and BMP activation form a reciprocal feedback loop. Activin/Nodal inhibition induces the expression of BMP and activates BMP signaling; BMP signaling further inhibits Activin/Nodal. Both inhibition of Activin/Nodal and activation of the BMP signal are required for trophoblast differentiation. Our observation reveals that a novel mechanism in which a critical interaction of two related but antagonizing signals by Activin/Nodal and BMP regulates the fate determination of hESCs in culture, and possibly also true for human embryo *in vivo*.

In contrast, Smith *et al.* (28) reported that inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. We did observe a very slight up-regulation of neuroectoderm markers (supplemental Fig. S1); however, we also observed a dramatic up-regulation of trophoblast markers (Figs. 2 and 3 and supplemental Fig. S1). Because Smith *et al.* (28) did not analyze trophoblast marker expression, it is likely they overlooked the dramatic differentiation of trophoblast in their experiments, which led to improper conclusions.

Little is known about normal human development during the early post-implantation period. Although the mouse is the typical model for experimental mammalian embryology, early structures, including the placenta, extraembryonic membranes, and the egg cylinder, all differ substantially from the corresponding human structure. Our results display that although the most key transcriptional factors exhibit similar expression between hESCs and mouse ESCs, some genes, such as Eomes, are completely different. Eomes has been reported to be essential for trophoblast development in mice (25); however, when Activin/Nodal signaling is inhibited, causing hESCs to differentiate into trophoblasts, the expression of Eomes is down-regulated. This suggests that Eomes are not essential for human trophoblast differentiation, which might imply that there are substantial differences between mouse and human early development.

Human and mouse ES cells are both blastocyst-derived; however, they are not equivalent. The mechanisms that human and mouse ES cells use to maintain "stemness" differ greatly (16–18, 30–32, 38–40), as well as their developmental potential, especially the capacity to form cells of the trophoblast lineage (8, 13, 41–43). HESCs and the mouse epiblast stem cell use the same signaling pathways to maintain pluripotency (44), hESCs can differentiate into all embryonic germ layers, as well as trophoblasts (5, 8, 13, 41). In contrast, mouse ES cells are capable of reconstituting all cell types of the body, but do not routinely exhibit a capacity for trophoblast cell differentiation (42, 43, 45). These differences highlight the fact that hESCs are a unique and irreplaceable model for studying early human developmental events. Human ES cells will be particularly valuable for studying development and function of tissues that differ between mice and humans. hESCs give rise to early human cell types that were previously almost unobtainable, which is a major advantage; however, ethical considerations, as well as the practicalities, will make it extremely difficult to validate *in vitro* results with *in vivo* significance. We demonstrate that combinatorial signals of Activin/Nodal and BMP regulate lineage segregation of early human embryo stem cells *in vitro*; however, a

FIGURE 6. **BMP4-induced trophoblast differentiation correlates with and depends on inhibition of Activin/Nodal signaling.** The H1 hES cells were cultured feeder free under the labeled conditions. Real-time polymerase chain reaction analysis of pluripotent markers, trophoblast markers, and downstream targets of Activin/Nodal signaling were performed. *A*, BMP4 promoted the trophoblast differentiation of hESCs; *B*, the trophoblast induction effect of BMP4 correlated with the inhibition of Activin/Nodal signal; *C*, Activin A restores expression of Lefty-A, Lefty-B, and Nodal; *D*, real-time PCR analysis of the expression of CG-α and CG-β; *E*, the H1 hES cells were cultured feeder free with CM plus 10 ng/ml BMP4 and 100 ng/ml Activin A, immunofluorescence of CG-α and CG-β indicates that Activin A inhibit the effect of BMP4; *F*, immunoassays of the placental hormones, hCG, estradiol (*E2*), and progesterone (*Prog*). Cell culture supernatant of hESCs cultured on MEF, in CM, CM + 10B, CM + 10B + 1A, CM + 10B + 10A, or CM + 10B + 100A were collected at the indicated times and subjected to immunoassays for human chorionic gonadotropin, estradiol, and progesterone. Abbreviations: *CM+10B*, CM plus 10 ng/ml BMP4; *CM+10B+1A*, CM plus 10 ng/ml BMP4 and 1 ng/ml Activin A; *CM+10B+10A*, CM plus 10 ng/ml BMP4 and 10 ng/ml Activin A; *CM+10B+100A*, CM plus 10 ng/ml BMP4 and 100 ng/ml Activin A; *ActA*, Activin A.

direct role for Activin/Nodal signaling in early human embryonic lineage segregation has not been demonstrated *in vivo*. Expression profiles, attained by analysis of EST counts at the NCBI database, shows that Activin A, Follistatin, and BMPs are all expressed in the human ovary and/or uterus, which implies their function during early development. The challenge for the future will be to determine whether Activin/Nodal and BMP signals play a role in early lineage segregation of human embryo *in vivo*, and to establish the key transcriptional factor pathways in human embryo trophoblast differentiation using hESCs as a model.

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