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Supplemental Data

NANOG Is a Direct Target of TGFβ/Activin-Mediated SMAD Signaling in Human ESCs

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EXPERIMENTAL PROCEDURES

Microarray

Total cellular RNA was extracted by Trizol Reagent (Invitrogen), and treated with RNase-free DNase according to the manufacturer's instructions. Total RNA was enriched for the polyA fraction using Oligotex mRNA Mini Kit (Qiagen). Enriched mRNA (250 ng) was primed using random hexamers and reverse transcribed using Superscript III (Invitrogen) in the presence of 5-(3-aminoallyl)-dUTP (Ambion, Austin, TX). The purified product was coupled to Cy5-NHS ester (GE Healthcare, Piscataway, NJ). Similarly, sonicated genomic DNA (gDNA) (2 μg) was primed with random octamers and labeled using Klenow in the presence of 5-(3-aminoallyl)-dUTP. The resulting product was coupled to Cy3-NHS ester (GE Healthcare). Arrays containing 60-mer probes each were manufactured by NimbleGen Systems (Madison, WI), which tiled transcripts from approximately 36,000 human locus identifiers. The Cy5-labeled mRNA sample (2 μg) was added onto each array along with the Cy3-labeled gDNA (4.5 μg) that served as a common reference enabling comparison across different arrays. Hybridizations were performed in 3.6X SSC buffer with 35% formamide and 0.07% SDS at 42 °C overnight. Arrays were then washed, dried, and scanned using a GenePix 4000B scanner.

Gene expression raw data were extracted using NimbleScan software v2.1. The signal intensities from both the RNA and gDNA channels in all the arrays were separately normalized with robust multi-array average algorithm (Irizarry et al., 2003). For a given gene, relative RNA level was calculated as (intensity from RNA channel)/(intensity from gDNA channel + median intensity of all genes from the gDNA channel) and displayed as ratio to the level in the control (T1).

Western blotting

Cell lysates were electrophoresized on a 4%–15% linear gradient Polyacrylamide Tris-HCl Precast Gel (BioRad) for western blotting. Rabbit antibodies against human phosphorylated SMAD1/5/8, SMAD1/5/8, phosphorylated SMAD2/3, SMAD2/3, phosphorylated MEK1/2 (Cell Signaling Technology), BMP2/4 (R&D Systems), and β-

Actin (Abcam) were used to detect the corresponding proteins. Horseradish peroxidase-labeled donkey anti-rabbit IgG secondary antibodies (Santa Cruz) and Immuno-star HRP Chemiluminescent solution A and B (BioRad) were used to visualize the detection. NANOG was detected with goat anti-human NANOG (R&D Systems) and horseradish peroxidase-labeled rabbit anti-goat IgG secondary antibodies; βActin as an internal control for NANOG was detected with mouse anti-chicken βActin (Santa Cruz Biotechnology) and goat anti-mouse IgG secondary antibodies (R&D Systems).

TABLES

Table S1. Effect of FGF, TGFβ, and BMP signals on expression of marker genes in hESCs analyzed by microarray

		Relative RNA level (normalized by T1)				
GENE NAME	ACCESSION	T1	T1(+)SU(-)F	T1(+)SB(-)Tb	T1(+)SU(+)SB (-)F(-)Tb	T1(+)BMP4
ES cells						
OCT4	NM_002701	1.00	0.51	0.46	0.07	0.51
NANOG	NM_024865	1.00	0.48	0.12	0.01	0.57
SOX2	NM_003106	1.00	0.97	1.14	0.46	0.04
NODAL	NM_018055	1.00	0.90	0.09	0.05	1.97
Trophectoderm						
EOMES	NM_005442	1.00	1.05	0.69	0.71	25.06
CDX2	NM_001265	1.00	1.39	1.10	2.16	20.88
GCM1	NM_003643	1.00	1.10	0.92	0.99	1.15
CGB5	NM_033043	1.00	0.82	0.85	0.55	0.82
Primitive ectoderm						
FGF5	M37825	1.00	1.00	0.95	0.93	0.91
Neural ectoderm						
SOX1	NM_005986	1.00	1.08	1.77	1.02	1.03
NESTIN	NM_006617	1.00	1.36	1.54	1.55	1.21
NEUROD1	NM_002500	1.00	0.90	1.21	0.98	1.14
Epidermal ectoderm						
CRABP2	NM_001878	1.00	3.70	1.63	4.99	1.99
Mesoderm						
VIMENTIN	AK056766	1.00	1.24	0.61	1.45	2.23
COL2A1	BC007252	1.00	1.38	1.56	6.83	0.30
HAND1	NM_004821	1.00	7.40	1.01	17.54	186.13
MSX1	BC021285	1.00	2.51	0.77	54.59	64.91
BMP4	NM_001202	1.00	2.52	0.74	14.67	53.54

Endoderm						
AFP	NM_001134	1.00	1.17	1.24	2.04	0.78
FABP2	NM_000134	1.00	1.31	0.98	0.96	0.97
Germline						
SYCP3	NM_153694	1.00	1.04	0.97	0.90	1.04
DDX4	NM_024415	1.00	1.28	0.94	0.89	0.94
STELLA	AY317075	1.00	2.05	0.92	0.89	1.14
House-keeping						
beta ACTIN	NM_001101	1.00	0.88	0.83	0.83	0.63
GAPDH	NM_002046	1.00	0.81	0.86	0.94	0.84

Note: H9 cells were cultured in various media for 3 days and followed by microarray for gene expression. T1: T1 medium; SU: 10 μM SU5402; F: 100 ng/ml bFGF; SB: 10 μM SB431542; Tb: 0.6 ng/ml TGFβ1; B4: 100 ng/ml BMP4.

Table S2. Applied Biosystems Taqman® assays for RT-QPCR (Fig. 2B)

Gene Symbol	Gene Name	Assay	RefSeq
B2M	β-2-microglobulin	Hs00187842_m1	NM_004048.2
POU5F1	OCT4	Hs00742896_s1	NM_203289.2
NANOG	NANOG	Hs02387400_g1	NM_024865.1

FIGURES

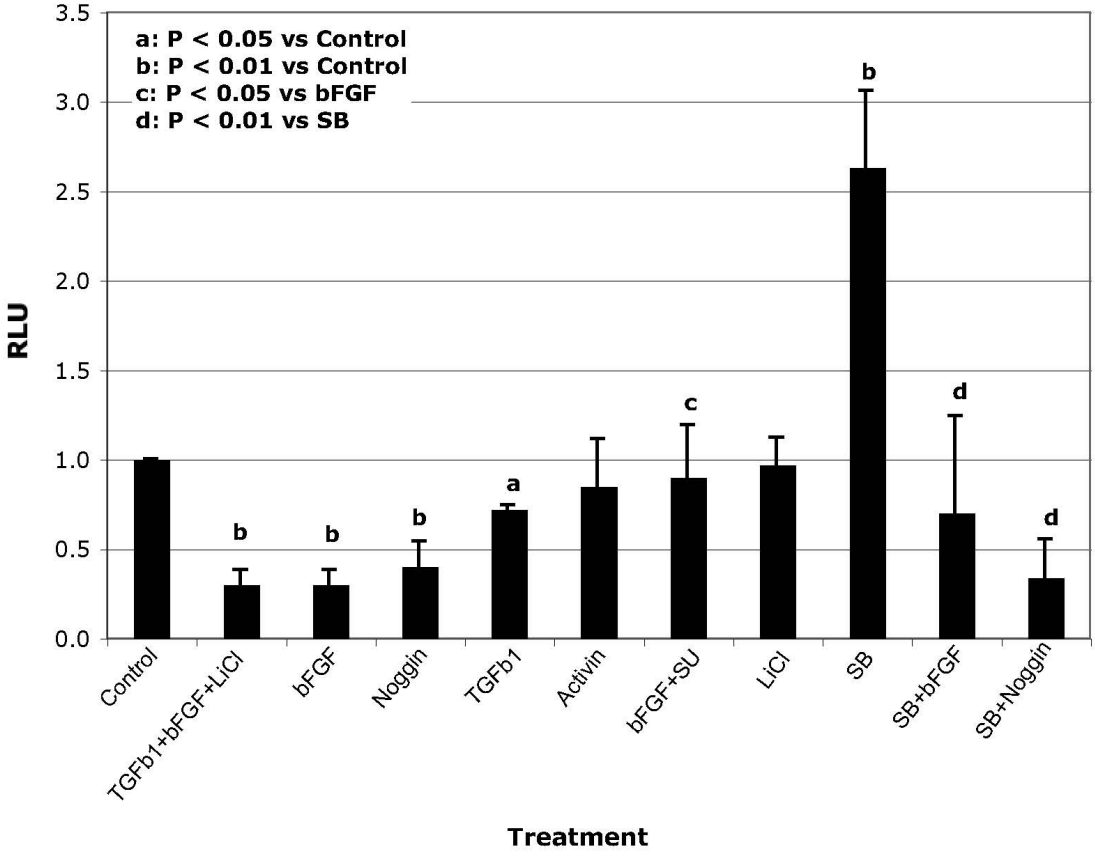
a: P < 0.05 vs Control
b: P < 0.01 vs Control
c: P < 0.05 vs bFGF
d: P < 0.01 vs SB
RLU
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
b
b
a
c
b
d
d
Control
TGFb1+bFGF+LiCl
bFGF
Noggin
TGFb1
Activin
bFGF+SU
LiCl
SB
SB+bFGF
SB+Noggin
Treatment

Fig. S1**. Regulation of BMP signal in human ES cells by ligands or inhibitors of multiple signaling pathways.** H9 cells were transfected with pID120-Luc and a trace amount of pRL-tk on day 1. Treatments of the cells started on day 2 with T1 medium minus 0.6 ng/ml TGFβ1, 100 ng/ml bFGF, and 1 mM LiCl (a stimulator of WNT signal) as a control, or the control medium plus ligands or inhibitors of multiple signaling pathways including the above deleted reagents, 10 ng/ml Activin, 100 ng/ml Noggin, 10 μM SU5402 (SU), and 10 μM SB431542 (SB). The cells were lysed on day 3 for analysis of luciferase activity.

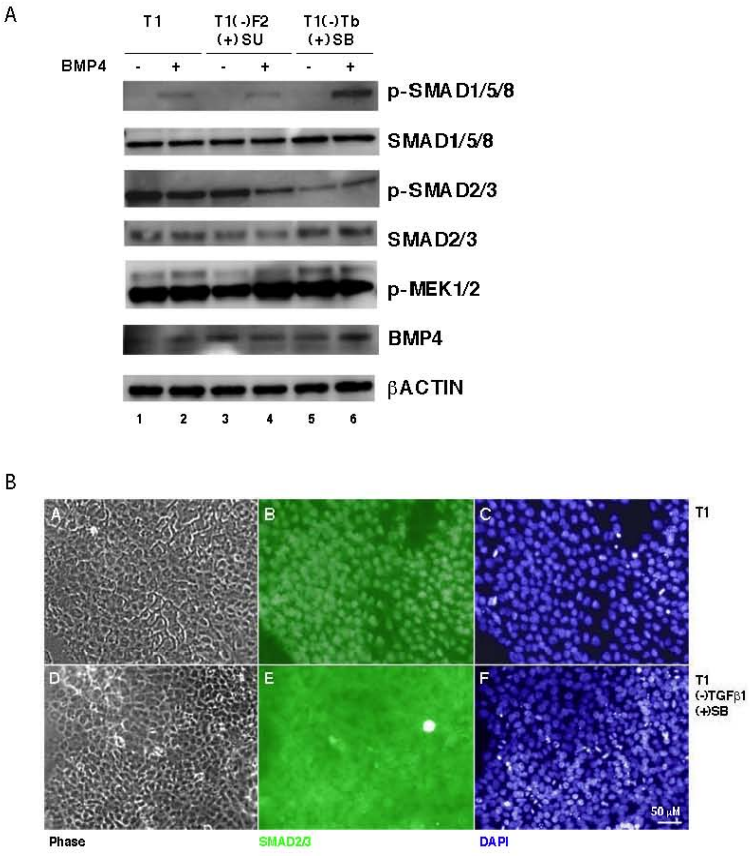

Fig. S2. **Validation of the effects of BMP4 and inhibitors of FGF and TGFβ signaling on human ES cells cultured in T1-based medium.** (**A**) Western blotting was used to test various proteins and their phosphorylated form in H9 cells cultured in T1 medium, T1 minus bFGF plus 10 μM SU5402 [T1(-)F2(+)SU] or T1 minus TGFβ1 plus

10 μM SB431542 [T1(-)Tb(+)SB], each with or without 100 ng/ml BMP4 for 24 hours. βActin was detected as an internal control. (**B**) H9 cells cultured in T1 or T1(-)Tb(+)SB for 24 h were immunostained for SMAD2/3 and the cell nuclei stained by DAPI.

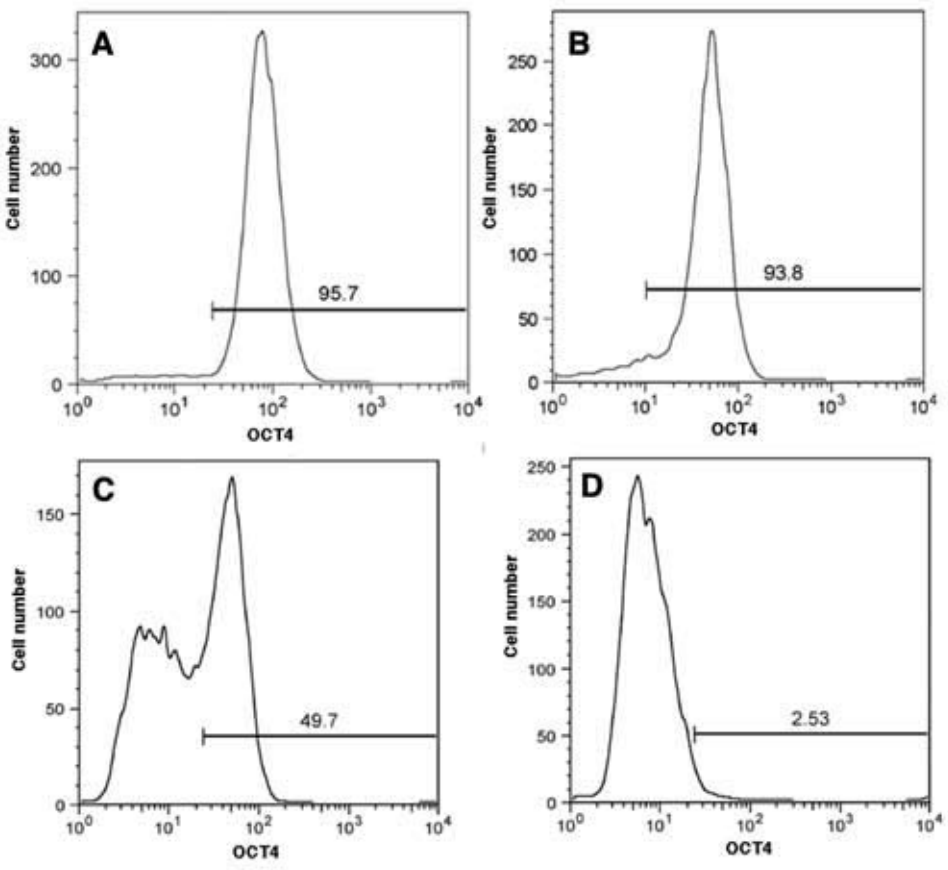

Fig. S3. **Histograms of flow cytometry analysis of H9 cells for OCT4 cultured for 7 days. (A)** T1 (**B**) T1 minus 100 ng/ml bFGF (**C**) T1 plus 10 μM SB431542 (**D**) or T1 minus bFGF plus SB431542.

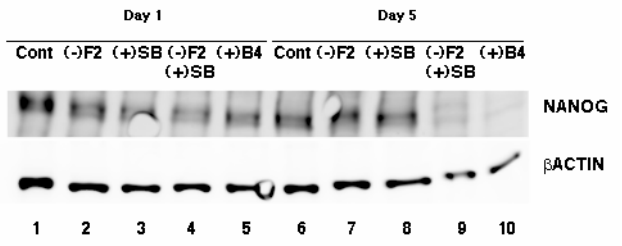

Fig. S4. **Effect of FGF, TGFβ, and BMP signals on the level of NANOG protein in human ES cells.** Western blotting was used to test NANOG in H9 cells cultured in T1 medium (control), T1 minus 100 ng/ml bFGF [T1(-)bFGF], T1 plus 10 μM SB431542 [T1(+)SB], T1(-)bFGF(+)SB, or T1 plus 100 ng/ml BMP4 for 24 hours or 5 days. βACTIN was tested as a loading control.

(GCTGGTTTCAAACTCCTGACTTCAGGTGATCCGCCTGCCACGGCCTCCCAATT
TACTGGGATTACAGGGGTGGGCCACCGCGCCCGGCCTTTTTCTTAATTTTTAAA
AATATTAAAGTTTTATCCCATTC CTGTTGAACCATATTCCTGATTTAAAAGTTGGA
AACGTGGTGAACCTAGAAGTATTTGTTGCTGGGTTTGTCTTCAGGTTCTGTTGC
TCGGTTTTCTAGTTCCCCACCTA GTCTG GGTTactcTGCAGCTAC TTTTGCAT TAC
AATG GCCTTGGTG AGAC TGGT AGAC GGGATTAACTGAGAATTCACAAGGGTGG
GtcagTAGGGGGTGTGCCCGCCAGGAGGGGTGGGTCTAAGGTGATAGAGCCTT
C ATTATAAATCTAG AGAC TCCAGGA)TTTTAACGTTCTGCTGGACTGAGCTGGTT
GCCTCATGTTATTATGCAGGCAACTCACTTTATCCCAATTTCTTGATACTTTTCCT
TCTGGAGGTCCTATTTCTCTAACATCTTCCAGAAAAGTCTTAAAGCTGCCTTAAC
CTTTTTTCCAGTCCACCTCTTAAATTTTTTCCTCCTCTTCCTCTATACTAAC

Fig. S5. **The *NANOG* proximal promoter and putative SMAD binding elements (SBEs).** This region contains 404 bp, from -380 to 24 relative to the transcription start site of *NANOG*. Bold: transcription start site. Underlined: putative SBEs including a consensus sequence [C]AGAC and its complementary form GTCT[G]. Sequences in the parenthesis are the Octamer/Sox elements. Lowercased sequences were chosen for mutation to generate a negative control for the binding site-specific mutations in the *NANOG* proximal promoter in the reporter plasmids.

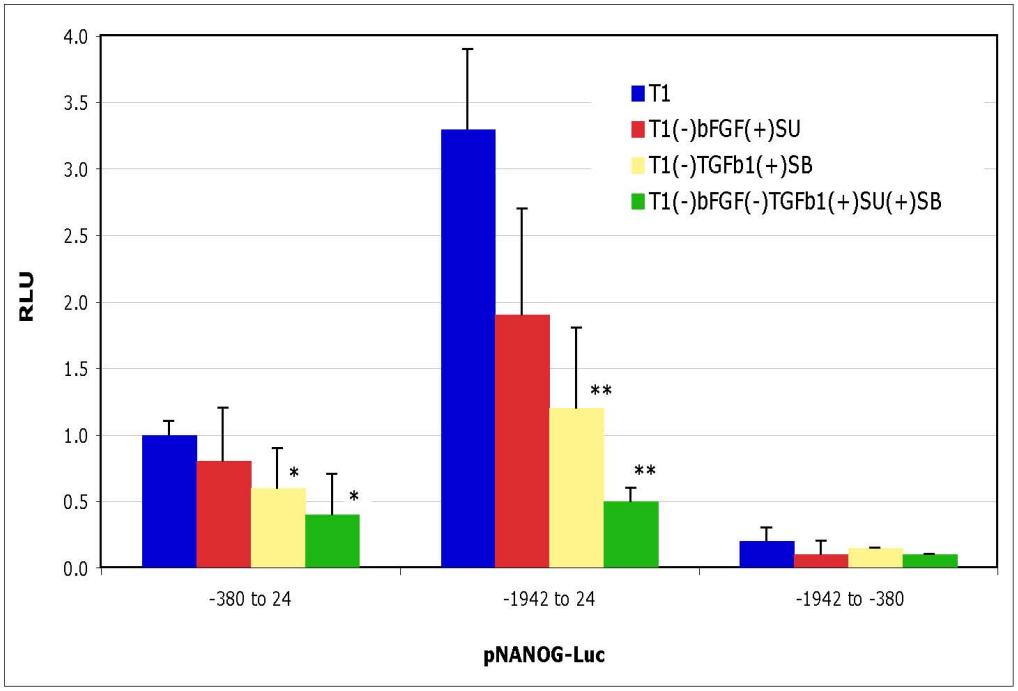
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
RLU
T1
T1(-)bFGF(+)SU
T1(-)TGFb1(+)SB
T1(-)bFGF(-)TGFb1(+)SU(+)SB
*
*
**
**
-380 to 24
-1942 to 24
-1942 to -380
pNANOG-Luc

Fig. S6. **Mapping of the essential role to the proximal region in *NANOG* promoter.** H9 cells were transfected with luciferase reporter plasmids driven by various regions of the *NANOG* promoter and a trace amount of pGL4.70 (as an internal control) on day 1. Treatments of the cells started on day 2 with T1 medium, T1 minus TGFβ1 plus 10 μM SB431542 [T1(-)TGFβ1(+)SB], T1 minus bFGF plus 10 μM SU5402 [T1(-)bFGF(+)SU], or T1(-)TGFβ1(+)SB(-)bFGF(+)SU. After 24 hours of treatment, cells were harvested for analysis of luciferase activity. *$p < 0.05$, **$p < 0.01$ compared to T1.