Supplementary Data

Supplementary Materials and Methods

Cell culture

The human ES lines H1 and H9 [1] were obtained from WiCell and maintained under feeder-free conditions in an MEF-conditioned medium (CM) [2] on Matrigel-coated plates and passaged using the colony precutting and dispase (2 mg/mL; Gibco, Invitrogen) method. The CM was prepared in the presence of 4 ng/mL FGF2 (Peprotech), and before feeding the cells with CM, 4 ng/mL of FGF2 was again freshly supplemented. All the experiments (Fig. 1A, Supplementary, Table S1) were carried out in defined conditions, using an N2B27 medium [3]. For maintenance of human embryonic stem cells (hESCs) in an undifferentiated state under feeder-free defined conditions, 20 ng/mL of FGF2 was supplemented. The hESCs were passaged and maintained in the presence of CM till they reached a confluence of ~50%. Then, they were rinsed once with prewarmed PBS without $Ca^{2+}Mg^{2+}$ (Gibco, Invitrogen), followed by various treatments.

The recombinant proteins and reagents used are the folowing: BMP4 (R&D 314-BP-010): 10 ng/mL; SB-431542 (Sigma S4317): 20 μM; SU5402 (Calbiochem 572630): 20 μM; FGF2 (Peprotech): 4–20 ng/mL.

Quantitative reverse–transcription PCR

Cells were lyzed directly on the culture dishes with an RLT lysis buffer after shortly rinsing with PBS, and RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions, including the on-column DNase digestion step. The quantity and quality of RNA and cRNA were determined using a spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis. For reverse transcription, M-MLV reverse transcriptase (Promega), RNA (2 μg), and 0.5 μL of Oligo-dT primer (1 μg/μL; Invitek, Berlin, Germany) were brought to a final volume of 10 μL with distilled water. The mixture was spun briefly, heated to 70°C for 5 min, and immediately cooled on ice to prevent the secondary structure from reforming. An reverse transcription (RT) master mix of 15 μL was prepared using the following components for one reaction: 9.4 μL of distilled water, 5 μL of 5× reaction buffer (Promega), 0.5 μL of 25 mM dNTP, and 0.1 μL of M-MLV reverse transcriptase (200 U/μL; Promega). After thorough mixing and a short centrifugation, reverse transcription was carried out at 42°C for 1 h, and then the reaction was stopped by incubating at 65°C for 10 min.

For RT-qPCR, around 30 ng total RNA equivalent of cDNA was used for a 10-μL reaction mixture, including 5× SYBRGreen PCR Master Mix (Applied Biosystems) and primers (5 pmol; MWG). The reactions were performed in 96-Well Optical Reaction Plates (Applied Biosystems) in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), using the following program: stage 1: 50°C for 2 min; stage 2: 95°C for 10 min; stage 3: 95°C for 15 s and 60°C for 1 min, for 40 cycles; stage 4: 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. The last heating step in stage 4 was performed with a ramp rate of 2% to enable the generation of a dissociation curve of the product. Triplicate amplifications were carried out per gene. *GAPDH* or *ACTB* was amplified along with the target genes as endogenous controls for normalization. The output data generated by SDS 2.1 software (Applied Biosystems) were transferred to MS Excel for analysis. The differential mRNA expression of each gene was normalized against GAPDH or ACTB mRNA expression in the respective samples, and calculated using the ΔΔCt method and to obtain the fold change in comparison to the undifferentiated hESCs (control or untreated):

$$\delta CT = CT_{gene} - CT_{GAPDH\ or\ ACTB}$$
$$\delta\delta CT = \delta CT_{treated} - \delta CT_{untreated}$$
$$\text{Fold change} = 2 - \delta\delta CT$$

Supplementary Table S1. Experimental Setup: Details of Treatments, Replicates, and Time Points

Treatments	*Abbreviation used*	*Replicates*	*No. of replicates*	*Time points (hours/days)*
Control (Undifferentiated hESCs: with FGF2)	UD	Biological	2	5 D
BMP4	B	Biological	2	3 H, 5 D
BMP4 + SB431542	B/SB	Biological	2	3 H, 1 D, 3 D, 5 D
BMP4 + SB431542 + SU5402	B/SB/SU	Biological	2	3 H, 1 D, 3 D, 5 D
BMP4 + SB431542 + FGF2	B/SB/F	Biological	2	3 H, 1 D, 3 D, 5 D
SU5402	SU	Biological	2	1 D, 3 D, 5 D
BMP4 + SU5402	B/SU	Biological	2	5 D
SB431542 + SU5402	SB/SU	Biological	2	5 D
Placenta	Placenta	Technical	2	
Control (BMP4 + DMSO)		Biological	2	5 D
Control (DMSO)		Biological	2	5 D

BMP4, 10 ng/mL; SB431542, 20 μM; SU5402, 20 μM; FGF2, 20 ng/mL; H, hours; D, days; BMP, bone morphogenetic protein; FGF2, fibroblast growth factor 2; hESCs, human embryonic stem cells.

Supplementary Table S2. Primers Used for This Study

Gene name	*Product size (bp)*	*Forward primer (5′-3′)*	*Reverse primer (5′-3′)*
ACTB	87	TCAAGATCATTGCTCCTCCTGAG	ACATCTGCTGGAAGGTGGACA
CDH1	107	TTGAGGCCAAGCAGCAGTACA	ATCCAGCACATCCACGGTGA
CDX2	78	TCACTACAGTCGCTACATCACCATC	TTAACCTGCCTCTCAGAGAGCC
EOMES	76	CGGCCTCTGTGGCTCAAA	AAGGAAACATGCGCCTGC
ERVWE1	100	GAAAAGGCCCAAGAGGTAATAAAGG	CCTGGAAAGCAGGGCTATTGTC
FST	100	ACCGAGGAGGACGTGAATGAC	CCACGTTCTCACACGTTTCTTTAC
GAPDH	81	CTGGTAAAGTGGATATTGTTGCCAT	TGGAATCATATTGGAACATGTAAACC
GATA3	117	ACTCCAGCCACATGCTGACC	AGCATCGAGCAGGGCTCTAAC
GATA6	83	TGTGCGTTCATGGAGAAGATCA	TTTGATAAGAGACCTCATGAACCGACT
GCM1	101	TCTCTTCACCTACGCCTCTCATC	GATCCAAACCCAAGTATGTCATTTC
GREM2	126	CTGCCAGCTGAAGACAGAGTATTC	AGTGTGTCCCTGGTGCTAACG
HAND1	114	TCCCTTTTCCGCTTGCTCTC	CATCGCCTACCTGATGGACG
HCG (CGB)	112	GTGCATCACCGTCAACACCA	CACATCGCGGTAGTTGCACA
HERV-FRD	100	GGACCCTAATCTGAAAGGACTGATG	TCCGAAAATGGGAGGTTTCTG
HLA-G	82	GCACAGACTGACAGAATGAACCTG	TCCACTGGAGGGTGTGAGAACT
HOPX	102	GTGGCTTCACTGGAAAAATGG	CTGGGAGGTGATGGTCAAAAG
ID2	85	AATCCTGCAGCACGTCATCG	CTGGTGATGCAGGCTGACAA
IPL (PHLDA2)	113	GGCGCCTTAAGTTATTGGAC	TGCAATGGGCACAGTGATG
KRT7	74	AGATCGCCACCTACCGCAAG	ATTCACGGCTCCCACTCCAT
NANOG	78	CCTGTGATTTGTGGGCCTG	GACAGTCTCCGTGTGAGGCAT
NCAM1	112	TCATGTGCATTGCGGTCAAC	ACGATGGGCTCCTTGGACTC
OCT4	119	GTGGAGGAAGCTGACAACAA	ATTCTCCAGGTTGCCTCTCA
PAX6	84	CCAGGGCAATCGGTGGTAGT	ACGGGCACTCCCGCTTATAC
SNAI2	83	ATCTGCCAGACGCGAACTCA	CAACAATGGCAACCAGACAACC
SOX1	84	TTGGCATCTAGGTCTTGGCTCA	CGGGCGCACTAACTCAGCTT
SOX17	86	ACGTGTACTACGGCGCGATG	CTGGTGCTGGTGCTGGTGTT
SOX2	78	GTATCAGGAGTTGTCAAGGCAGAG	TCCTAGTCTTAAAGAGGCAGCAAAC
SOX7	110	TGCCCACTTCATGCAACTCC	AGGTACCCTGGGTCTTTGGTCA
T	79	CCTTGCTCACACCTGCAGTAGC	GGCCAACTGCATCATCTCCA
TP53	102	CAGGGCAGCTACGGTTTCC	CAGTTGGCAAAACATCTTGTTGAG
VIM	117	GGAGCTGCAGGAGCTGAATG	GACTTGCCTTGGCCCTTGAG

For better representation, the data have been presented in Log2 scale.

Microarray experiment: sample preparation and Illumina bead chip hybridization

Biotin-labeled cRNA was generated by the means of a linear amplification kit (Ambion) using 500 ng of quality-checked DNase-free total RNA as input. Chip hybridization, washing, Cy3-streptavidin staining, and scanning were performed on an Illumina Bead Station 500 platform (Illumina) using reagents and following protocols supplied by the manufacturer. RNA quality assessment was done using a spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis, both before (total RNA) and after cRNA preparation. The quality-checked cRNA samples were hybridized on human-8 Bead Chips.

Data analysis and interpretation

The raw data were normalized using the rank invariant algorithm of Bead Studio 1.0 (Illumina) software. Data analysis, including statistical tests, comparisons and filtrations such as significance tests, principal component analysis, clustering, Venn diagram, and data filtering and compilations were performed using TIGR-MEV software [4], R statistical software (Bioconductor), VENNY interactive tool [5], MS Excel, and MS Access. R version 2.4.0 was used for implementing the packages of Bioconductor and also for plotting scatter plots.

One-way analysis of variance was performed on the normalized data for obtaining the significant differentially regulated genes between the samples. For all the analyses, the genes that were detected (detection P value < 0.01), significant (P value ≤ 0.05) and regulated (at least 2-fold) in at least one of the samples being considered were only taken into consideration. The regulated genes included those that were at least 2-fold up or downregulated when compared to the undifferentiated hESCs (control or untreated). Database for annotation, visualization, and integrated discovery [6,7] was used for pathway analyses. Groups of genes associated with specific pathways, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), were analyzed together to assess pathway regulation during various treatments of hESCs.

Immunoblotting and immunocytochemistry

For protein extraction, the monolayer culture of cells was rinsed with prewarmed PBS (37°C), followed by addition of a lysis buffer (25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM HEPES), supplemented with a protease inhibitor (Roche), after which the cells were scraped out. To ensure effective cell lysis, the lysates were vortexed for ~1 min, and mechanical lysis was carried out by passing the lysates through a 1-mL syringe and injection needle (BD microlance 3; Becton Dickinson) several times, while placing the samples on ice. Then, the samples were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant was collected

Supplementary Table S3. Pearson's Correlation Coefficient Between Samples

	Placenta_1	Placenta_2	hESCs_1	hESCs_2	B/SB/SU 3H_1	B/SB/SU 3H_2	B/SB/SU 1D_1	B/SB/SU 1D_2	B/SB/SU 3D_1	B/SB/SU 3D_2	B/SB/SU 5D_1	B/SB/SU 5D_2	B/SB/F 3H_1	B/SB/F 3H_2	B/SB/F 1D_1	B/SB/F 1D_2	B/SB/F 3D_1	B/SB/F 3D_2	B/SB/F 5D_1	B/SB/F 5D_2	B 3H_1	B 3H_2	B 5D_1	B 5D_2	B/SB 3H_1	B/SB 3H_2	B/SB 1D_1	B/SB 1D_2	B/SB 3D_1	B/SB 3D_2	B/SB 5D_1	B/SB 5D_2
Placenta_1	1.00	1.00	0.56	0.56	0.56	0.56	0.57	0.57	0.57	0.57	0.60	0.60	0.56	0.56	0.57	0.57	0.57	0.58	0.58	0.58	0.57	0.57	0.58	0.57	0.57	0.56	0.56	0.57	0.57	0.56	0.58	0.58
Placenta_2	1.00	1.00	0.56	0.57	0.56	0.56	0.57	0.57	0.58	0.58	0.60	0.60	0.56	0.56	0.57	0.57	0.58	0.58	0.58	0.59	0.57	0.57	0.58	0.58	0.57	0.57	0.56	0.57	0.57	0.56	0.58	0.58
hESCs_1	0.56	0.56	1.00	0.99	0.99	0.98	0.95	0.96	0.94	0.94	0.92	0.92	0.97	0.97	0.99	0.99	0.99	0.98	0.97	0.97	0.98	0.98	0.99	0.98	0.99	0.98	0.99	0.99	0.98	0.98	0.95	0.95
hESCs_2	0.56	0.57	0.99	1.00	0.98	0.98	0.95	0.95	0.93	0.93	0.91	0.92	0.97	0.97	0.99	0.98	0.98	0.98	0.96	0.96	0.98	0.98	0.98	0.98	0.99	0.98	0.98	0.99	0.98	0.98	0.95	0.95
B/SB/SU 3H_1	0.56	0.56	0.99	0.98	1.00	1.00	0.95	0.97	0.94	0.94	0.93	0.93	0.98	0.98	0.99	0.99	0.98	0.98	0.96	0.96	0.99	0.99	0.98	0.98	0.99	0.99	0.99	0.99	0.98	0.98	0.95	0.95
B/SB/SU 3H_2	0.56	0.56	0.98	0.98	1.00	1.00	0.95	0.97	0.94	0.94	0.93	0.93	0.98	0.98	0.99	0.99	0.98	0.98	0.96	0.96	0.99	0.99	0.98	0.98	0.99	0.99	0.99	0.99	0.98	0.98	0.95	0.95
B/SB/SU 1D_1	0.57	0.57	0.95	0.95	0.95	0.95	1.00	0.98	0.96	0.96	0.95	0.95	0.96	0.96	0.96	0.96	0.96	0.96	0.95	0.95	0.96	0.96	0.96	0.96	0.96	0.95	0.96	0.96	0.96	0.96	0.95	0.95
B/SB/SU 1D_2	0.57	0.57	0.96	0.95	0.97	0.97	0.98	1.00	0.97	0.97	0.96	0.96	0.97	0.97	0.97	0.97	0.97	0.97	0.96	0.96	0.97	0.96	0.97	0.96	0.97	0.96	0.97	0.97	0.98	0.97	0.96	0.96
B/SB/SU 3D_1	0.57	0.58	0.94	0.93	0.94	0.94	0.96	0.97	1.00	1.00	0.98	0.97	0.95	0.95	0.95	0.95	0.96	0.96	0.97	0.97	0.94	0.94	0.96	0.95	0.95	0.94	0.95	0.95	0.97	0.97	0.97	0.97
B/SB/SU 3D_2	0.57	0.58	0.94	0.93	0.94	0.94	0.96	0.97	1.00	1.00	0.98	0.98	0.95	0.95	0.95	0.95	0.96	0.96	0.97	0.97	0.94	0.94	0.96	0.95	0.95	0.94	0.95	0.95	0.97	0.97	0.97	0.97
B/SB/SU 5D_1	0.60	0.60	0.92	0.91	0.93	0.93	0.95	0.96	0.98	0.98	1.00	1.00	0.94	0.94	0.93	0.93	0.94	0.94	0.96	0.95	0.93	0.93	0.95	0.94	0.93	0.93	0.93	0.94	0.94	0.94	0.96	0.96
B/SB/SU 5D_2	0.60	0.60	0.92	0.92	0.93	0.93	0.95	0.96	0.97	0.98	1.00	1.00	0.94	0.94	0.93	0.93	0.94	0.94	0.95	0.95	0.93	0.93	0.95	0.94	0.93	0.93	0.94	0.94	0.94	0.94	0.96	0.96
B/SB/F 3H_1	0.56	0.56	0.97	0.97	0.98	0.98	0.96	0.97	0.95	0.95	0.94	0.94	1.00	1.00	0.98	0.98	0.97	0.97	0.95	0.95	0.99	0.99	0.97	0.97	0.99	0.98	0.98	0.98	0.97	0.97	0.94	0.94
B/SB/F 3H_2	0.56	0.56	0.97	0.97	0.98	0.98	0.96	0.97	0.95	0.95	0.94	0.94	1.00	1.00	0.98	0.98	0.97	0.97	0.95	0.95	0.99	0.99	0.97	0.97	0.98	0.99	0.98	0.98	0.97	0.97	0.94	0.94
B/SB/F 1D_1	0.57	0.57	0.99	0.99	0.99	0.99	0.96	0.97	0.95	0.95	0.93	0.93	0.98	0.98	1.00	1.00	0.99	0.99	0.97	0.97	0.99	0.99	0.99	0.98	0.99	0.98	0.99	1.00	0.99	0.99	0.96	0.96
B/SB/F 1D_2	0.57	0.57	0.99	0.98	0.99	0.99	0.96	0.97	0.95	0.95	0.93	0.93	0.98	0.98	1.00	1.00	0.99	0.99	0.97	0.97	0.99	0.99	0.98	0.98	0.99	0.98	0.99	1.00	0.99	0.99	0.96	0.96
B/SB/F 3D_1	0.57	0.58	0.99	0.98	0.98	0.98	0.96	0.97	0.96	0.96	0.94	0.94	0.97	0.97	0.99	0.99	1.00	1.00	0.99	0.99	0.98	0.98	0.99	0.98	0.99	0.97	0.99	0.99	0.99	0.99	0.97	0.97
B/SB/F 3D_2	0.58	0.58	0.98	0.98	0.98	0.98	0.96	0.97	0.96	0.96	0.94	0.94	0.97	0.97	0.99	0.99	1.00	1.00	0.99	0.99	0.98	0.98	0.99	0.98	0.99	0.98	0.99	0.99	0.99	0.99	0.97	0.97
B/SB/F 5D_1	0.58	0.58	0.97	0.96	0.96	0.96	0.95	0.96	0.97	0.97	0.96	0.95	0.95	0.95	0.97	0.97	0.99	0.99	1.00	1.00	0.96	0.96	0.98	0.98	0.97	0.96	0.97	0.97	0.98	0.98	0.99	0.99
B/SB/F 5D_2	0.58	0.59	0.97	0.96	0.96	0.96	0.95	0.96	0.97	0.97	0.95	0.95	0.95	0.95	0.97	0.97	0.99	0.99	1.00	1.00	0.96	0.96	0.98	0.97	0.97	0.96	0.97	0.97	0.98	0.98	0.99	0.99
B 3H_1	0.57	0.57	0.98	0.98	0.99	0.99	0.96	0.97	0.94	0.94	0.93	0.93	0.99	0.99	0.99	0.99	0.98	0.98	0.96	0.96	1.00	1.00	0.98	0.98	1.00	0.99	0.99	0.99	0.98	0.98	0.95	0.95
B 3H_2	0.57	0.57	0.98	0.98	0.99	0.99	0.96	0.96	0.94	0.94	0.93	0.93	0.99	0.99	0.99	0.99	0.98	0.98	0.96	0.96	1.00	1.00	0.98	0.98	0.99	0.99	0.99	0.99	0.97	0.97	0.95	0.95
B 5D_1	0.58	0.58	0.99	0.98	0.98	0.98	0.96	0.97	0.96	0.96	0.95	0.95	0.97	0.97	0.99	0.98	0.99	0.99	0.98	0.98	0.98	0.98	1.00	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.98	0.98
B 5D_2	0.57	0.58	0.98	0.98	0.98	0.98	0.96	0.96	0.95	0.95	0.94	0.94	0.97	0.97	0.98	0.98	0.98	0.98	0.98	0.97	0.98	0.98	0.99	1.00	0.98	0.99	0.98	0.98	0.98	0.98	0.97	0.97
B/SB 3H_1	0.57	0.57	0.99	0.99	0.99	0.99	0.96	0.97	0.95	0.95	0.93	0.93	0.99	0.98	0.99	0.99	0.99	0.99	0.97	0.97	1.00	0.99	0.99	0.98	1.00	0.99	0.99	0.99	0.98	0.98	0.95	0.96
B/SB 3H_2	0.56	0.57	0.98	0.98	0.99	0.99	0.95	0.96	0.94	0.94	0.93	0.93	0.98	0.99	0.98	0.98	0.97	0.98	0.96	0.96	0.99	0.99	0.98	0.99	0.99	1.00	0.99	0.98	0.97	0.97	0.95	0.95
B/SB 1D_1	0.56	0.56	0.99	0.98	0.99	0.99	0.96	0.97	0.95	0.95	0.93	0.94	0.98	0.98	0.99	0.99	0.99	0.99	0.97	0.97	0.99	0.99	0.99	0.98	0.99	0.99	1.00	1.00	0.99	0.99	0.96	0.96
B/SB 1D_2	0.57	0.57	0.99	0.99	0.99	0.99	0.96	0.97	0.95	0.95	0.94	0.94	0.98	0.98	1.00	1.00	0.99	0.99	0.97	0.97	0.99	0.99	0.99	0.98	0.99	0.98	1.00	1.00	0.99	0.99	0.96	0.96
B/SB 3D_1	0.57	0.57	0.98	0.98	0.98	0.98	0.96	0.98	0.97	0.97	0.94	0.94	0.97	0.97	0.99	0.99	0.99	0.99	0.98	0.98	0.98	0.97	0.99	0.98	0.98	0.97	0.99	0.99	1.00	1.00	0.98	0.98
B/SB 3D_2	0.56	0.56	0.98	0.98	0.98	0.98	0.96	0.97	0.97	0.97	0.94	0.94	0.97	0.97	0.99	0.99	0.99	0.99	0.98	0.98	0.98	0.97	0.99	0.98	0.98	0.97	0.99	0.99	1.00	1.00	0.98	0.98
B/SB 5D_1	0.58	0.58	0.95	0.95	0.95	0.95	0.95	0.96	0.97	0.97	0.96	0.96	0.94	0.94	0.96	0.96	0.97	0.97	0.99	0.99	0.95	0.95	0.98	0.97	0.95	0.95	0.96	0.96	0.98	0.98	1.00	1.00
B/SB 5D_2	0.58	0.58	0.95	0.95	0.95	0.95	0.95	0.96	0.97	0.97	0.96	0.96	0.94	0.94	0.96	0.96	0.97	0.97	0.99	0.99	0.95	0.95	0.98	0.97	0.96	0.95	0.96	0.96	0.98	0.98	1.00	1.00

Supplementary Table S4. Overlap with Placental-Regulated Genes

	Numbers		
	Upregulated	*Downregulated*	*Total*
Placenta	2782	3537	6319
B	415	165	580
B/SB/F	895	865	1760
B/SB/SU	1541	1728	3269
B/SB	1269	1020	2289

Total number of significantly regulated genes (minimum 2-fold up or down; P value ≤ 0.05).

	Numbers			*Percentages*		
	Upregulated	*Downregulated*	*Total*	*Total*	*Upregulated*	*Downregulated*
B	239	116	355	7,23	10,83	4,29
B/SB/F	441	597	1038	21,14	19,98	22,08
B/SB/SU	910	1263	2173	44,25	41,23	46,71
B/SB	617	728	1345	27,39	27,96	26,92
	2207	2704	4911			

Overlap of regulated genes with the same trend as that in placenta.

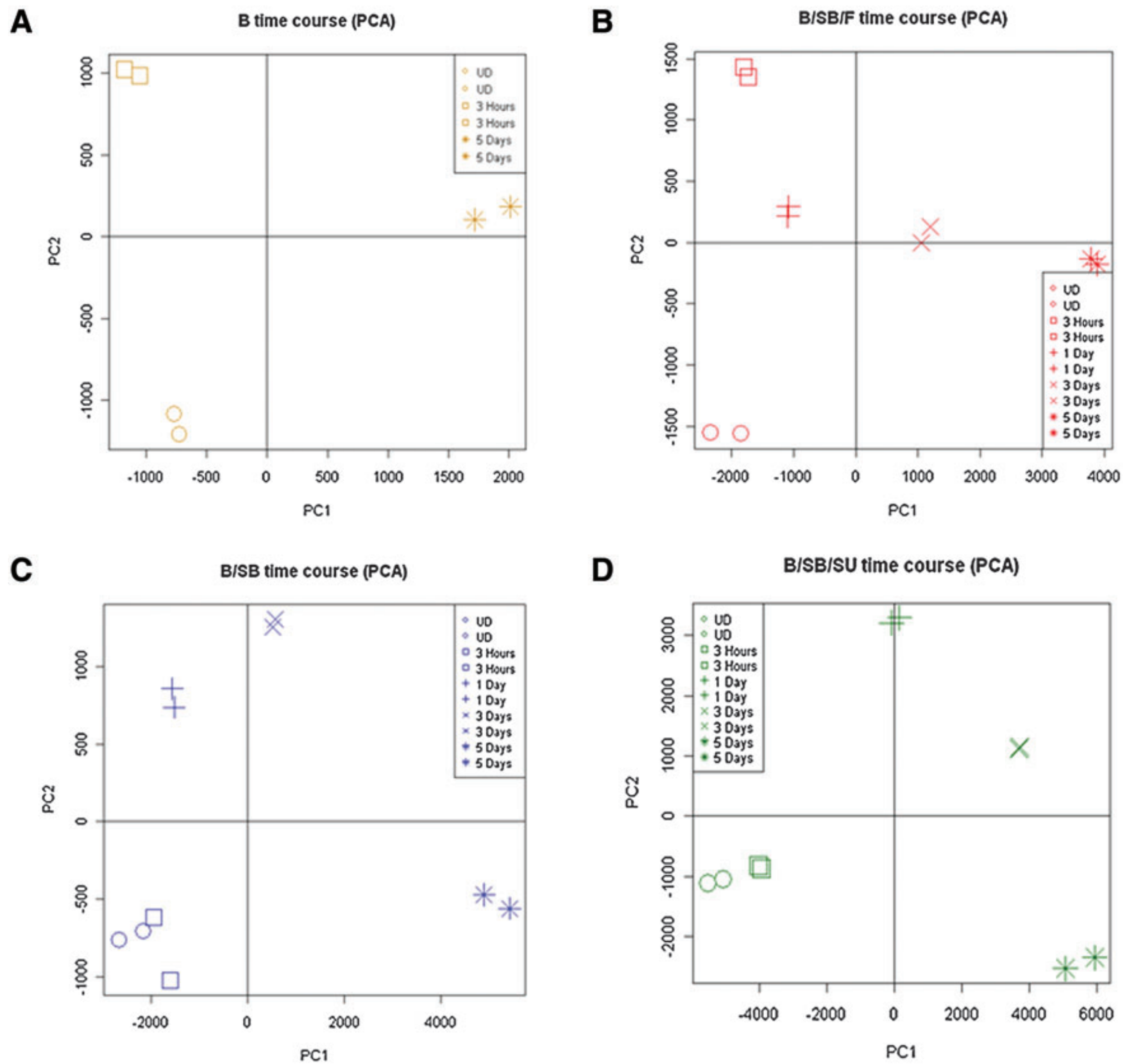
Supplementary Table S5. Lineage Tracing: Expression of Lineage-Specific Markers in Comparison to That in Control (Undifferentiated hESCs): Log2 Ratio (Treated vs. hESCs) (Microarray Data)

Symbol	*Placenta*	*SU 5 D*	*B/SB/SU 5 D*	*B/SB 5 D*	*B/SB/F 5 D*	*B 5 D*	P *value*
FGF2	−4,68342	−1,80741	−1,76365	−1,46699	−1,5247	−0,84628	4,73E-10
POU5F1	−10,2642	−3,26278	−6,57956	−3,73773	−3,35624	−0,49278	1,01E-12
SOX2	−9,64657	−3,33198	−5,89149	−2,01419	−2,02388	−0,78037	1,01E-13
NANOG	−6,13888	−3,10917	−3,77439	−4,59148	−5,01166	−0,30038	2,82E-11
MYC	−0,77646	−0,49965	−1,25271	−1,63256	−1,51055	−0,90093	6,08E-05
TERF1	−5,64624	−2,18436	−3,10762	−2,391	−2,40945	−0,17079	7,57E-11
LEFTY1	−3,88114	−2,87325	−1,86256	−2,63437	−7,11147	−0,62879	1,60E-13
LEFTY2	1,759726	0,822152	−1,3185	−2,17293	−2,93758	0,989163	7,48E-10
DPPA4	−10,477	−2,76053	−4,00661	−2,8904	−2,62282	−0,55373	8,43E-14
ZNF206	−10,4343	−3,53798	−7,5553	−2,95936	−2,48667	−0,76211	7,61E-13
GATA2	4,946364	2,937983	5,053814	3,547769	3,250321	2,281567	0
GATA3	4,024619	3,468135	4,425259	3,722087	3,563834	2,497059	0
KRT7	4.46134	4.657160	4.59051	2.130890	1.317782	1.510547	0
HAND1	−2,45702	3,712959	4,262864	4,799032	4,450249	4,268164	0
ID2	−0,97725	1,192766	2,016213	2,663504	2,840221	1,882119	8,08E-12
CDX2	−3,48776	0,309484	0,795828	1,145822	2,470481	0,776376	5,66E-10
PHLDA2	4,352175	2,261826	4,738734	0,33307	0,466393	1,185742	0
CGA	8,522905	5,128186	8,491924	2,101571	0,96583	2,31131	0
CGB	5,006099	1,237962	2,645981	0,337308	−7,45E-02	−0,35792	0
CGB1	6,852427	4,276995	5,132714	1,213658	0,45931	−0,18327	0
CGB5	7,961934	3,777384	5,380853	0,779345	0,180226	−0,23286	0
CRH	8,101506	3,415849	4,447988	4,665436	2,514518	1,926187	0
GCM1	6,697265	3,924444	4,416164	5,18E-02	0,359705	−0,29883	0
ERVWE1	5,849227	3,489618	4,776334	−2,79406	−1,9857	−1,05964	0
HERV-FRD	3,339363	3,812814	4,5532	0,492018	−0,11147	−0,86552	0
HOP	7,500087	6,716345	6,932379	1,400718	1,655771	3,409946	0
CYP1B1	−0,67822	2,923567	3,714562	0,464956	0,204753	−1,84E-02	2,16E-10
CYP19A1	8,048867	2,695707	5,142028	0,47148	−2,39947	−0,45118	0
CYP1A1	−2,19317	0,630285	5,067099	−1,91E-02	0,290314	5,14E-02	0
CYP11A1	6,296717	3,730653	3,963662	0,355185	0,116272	0,290553	0
HSPB1	1,290682	0,980136	−0,3149	−1,58403	−1,43169	−1,30E-02	0
HSPB2	1,493293	−0,13832	6,55E-02	−0,92315	−0,64224	0,300611	2,11E-06
PSG4	9,643541	2,093441	2,723941	−0,23732	−4,06129	−1,85644	0
PLAC2	3,191014	1,931765	2,088245	0,801386	0,588876	−1,37E-02	0
CDH1	−0,79837	0,647736	1,344357	1,371662	0,938529	0,500272	6,55E-15
VIM	1,065665	0,950372	−0,46583	1,501622	1,187414	0,754862	1,27E-10
SNAI2	1,753054	3,502417	1,012534	2,906466	2,234387	3,190419	4,83E-13
SOX7	1,117437	0,925025	0,455654	1,150606	1,012	0,639329	3,15E-07

Supplementary Table S6. Expression of Cyclins and CDKs in Comparison to That in Control (Undifferentiated hESCs): Log2 Ratio (Treated vs. hESCs)

Symbol	*SU 5 D*	*B/SB/SU 5 D*	*B/SB 5 D*	*B/S/F 5 D*	*B 5 D*	P *value*
CCNB1	1,359348	−1,11468	−1,19972	−0,19141	−0,74416	9,03E-05
CCNA1	−2,36207	−1,76368	−1,78762	−1,45728	−0,60153	2,66E-12
BAD	1,096672	1,829091	0,482073	0,401093	0,298574	9,35E-11
BCL2	−0,1897	1,602034	1,614585	−8,1202	−1,32322	0
BCL2L1	0,341411	1,038772	1,42E-03	−0,45084	−0,20707	2,10E-09
MDM2	0,487885	1,472758	0,33975	−0,1511	−0,30513	1,06E-05
GMNN	0,254093	−1,00837	−0,80304	−0,76855	−0,49418	4,57E-09
TP53	−1,17638	−0,91928	−0,26412	−0,46814	0,475922	0,000133
GADD45G	2,29848	2,460617	0,726594	0,156654	0,02172	4,39E-12
CHEK1	−0,70849	−1,8281	−0,75326	−0,41782	−0,38554	4,02E-14
CHEK2	−1,51372	−2,14292	−1,78922	−1,5431	−0,40427	1,33E-10
CDKN1A (p21)	1,672959	0,932918	−0,84086	−5,50E-02	−1,07027	1,41E-12
CDKN1C (p57)	3,684214	3,774775	1,64516	0,713853	0,889779	7,22E-15
CDKN2A (p16)	−0,11742	0,171339	0,863412	−0,37113	−8,66E-02	0,018389
CDK1 (CDC2)	0,723968	−1,26318	−0,64477	−0,48479	−0,28541	1,38E-09
CCNA1	−2,36207	−1,76368	−1,78762	−1,45728	−0,60153	2,66E-12
CCNA2	−0,22874	−1,13381	−0,54501	−0,29097	−0,12975	4,66E-12
CDK2	1,298116	1,240756	0,506014	0,320186	9,66E-02	0
CCNB1	1,359348	−1,11468	−1,19972	−0,19141	−0,74416	9,03E-05
CCNB2	0,149391	−1,27985	−0,73502	−0,50096	−0,40716	2,74E-10
CCND3	1,940099	1,728964	−0,02539	−0,1144	−7,40E-02	8,00E-13
CDK4	0,23922	−0,54982	−0,29582	−1,48E-02	−0,16833	0,000208
CDK6	1,170303	1,989741	1,614451	0,829038	0,883646	3,21E-11
CCNE1	0,80397	1,092275	−0,28459	−0,33633	5,31E-02	1,33E-13

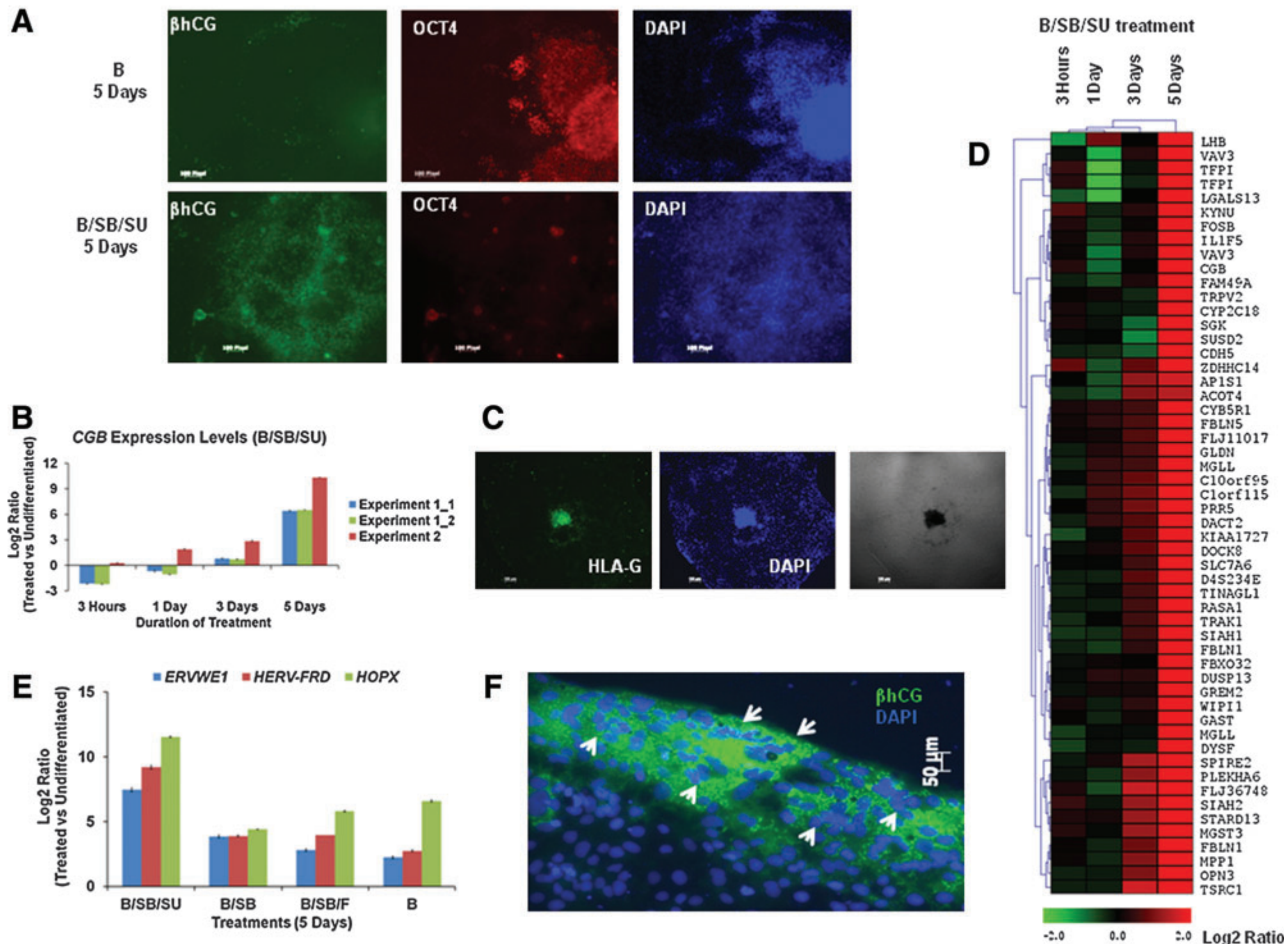
SUPPLEMENTARY FIG. S1. Experimental reproducibility and inhibition of ACTIVIN/NODAL and FGF pathways, coupled with BMP activation prevent differentiation into embryonic lineages, but support trophoblast differentiation. **(A)** Dendrogram representing the Pearson's correlation coefficient between the replicates of the indicated samples (_1 and _2 represent replicates for the respective samples). **(B)** Analysis of the indicated genes in B/SB/SU-treated samples (5 days) (H9). **(C)** Western blot analysis of NANOG expression in the indicated samples (5 days).



SUPPLEMENTARY FIG. S2. BMP-induced differentiation is accelerated and accentuated with additional inhibition of ACTIVIN/NODAL and FGF signaling. **(A–D)** PCA on the regulated genes (2-fold) for the mentioned treatments, with respect to their expression in hESCs. Genes that were significantly regulated in at least one of the time points of the indicated samples were included in this analysis. hESCs, human embryonic stem cells; PCA, Principal Component Analysis.

for western blot analysis. Protein concentrations were measured by employing the Bradford assay, using standard protein solutions (Bovine gamma globulin) and Bradford reagent (Bio-Rad). Protein samples (20 μg) were run on 10% acrylamide gels (10%—resolving; 5%—stacking) and transferred to a nitrocellulose membrane (GE Helthcare) blocked with 3% milk [low-fat milk powder in TBST (8 g NaCl, 0.02 M Tris–HCl, pH7.6, in double-distilled water)]. Further, primary antibodies were diluted in 1% bovine serum albumin (BSA)-1× TBST or milk-TBST, and the blots were incubated in them overnight at 4°C or for 1 h at room temperature, according to the manufacturer's instructions, for each antibody: CDX2 (Chemicon International AB4123), HLA-G (Santa Cruz Biotechnology, Inc., sc-17958), HistoneH3 pS10 (Novus Biologicals 06570), pSMAD2 (Cell Signalling Technology 3108; 1/1000), SMAD2/3 (Cell Signalling Technology 3102; 1/1000), pSMAD1 (Cell Signalling Technology 9511; 1/1000), SMAD1 (Cell Signaling Technology 9512; 1/1000), pGSK3β (Cell Signalling Technology 9336; 1/1000), GSK3β (Cell Signalling Technology 9315; 1/1000), NANOG (Abcam, ab62734; 1/1000), GAPDH (Ambion 4300; 1:5000). Further, the membranes were rinsed thrice in 1× TBST with gentle shaking and incubated in the respective secondary antibodies for 45 min. HRP-linked secondary antibodies (Molecular Probes, Invitrogen), diluted in milk/TBST were used. Luminescence detection was done with ECL reagents (GE Healthcare) on Hyperfilm ECL (Amersham) using the Curix 60 film-developing machine (Agfa).

Monolayer culture of cells were washed with PBST (PBS with 0.05% Tween 20) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Then, the cells were washed twice for 5 min with PBST with gentle shaking. After washing, cells were permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. Afterward, the cells were blocked in PBST containing 1% BSA (Fraction V, 99% purity; Sigma) and 5% normal chicken serum (Vector Laboratories) for 45 min at room temperature with gentle shaking. Thereafter, cells were incubated with primary antibodies for 1 h (diluted to working concentration in PBST containing 1% BSA and 1% normal chicken serum): β hCG



SUPPLEMENTARY FIG. S3. B/SB/SU treatment induces differentiation of hESCs to βhCG-secreting trophoblast cells, preventing extravillous trophoblast differentiation. **(A)** Representative immunostains for βhCG and OCT4 expression in the indicated samples. **(B)** Analysis of *CGB* expression in B/SB/SU-treated samples of 2 different experiments (5 days) (_1 and _2 represent biological replicates). **(C)** Representative immunostains for HLA-G in the B/SB/SU-treated samples. **(D)** Heat map depicting the regulation of genes with respect to hESCs in the indicated samples. **(E)** Expression analysis of indicated genes and samples. **(F)** Representative immunostains for βhCG in B/SB/SU-treated cells, exhibiting multinucleated βhCG-positive regions (marked with *white arrows*).

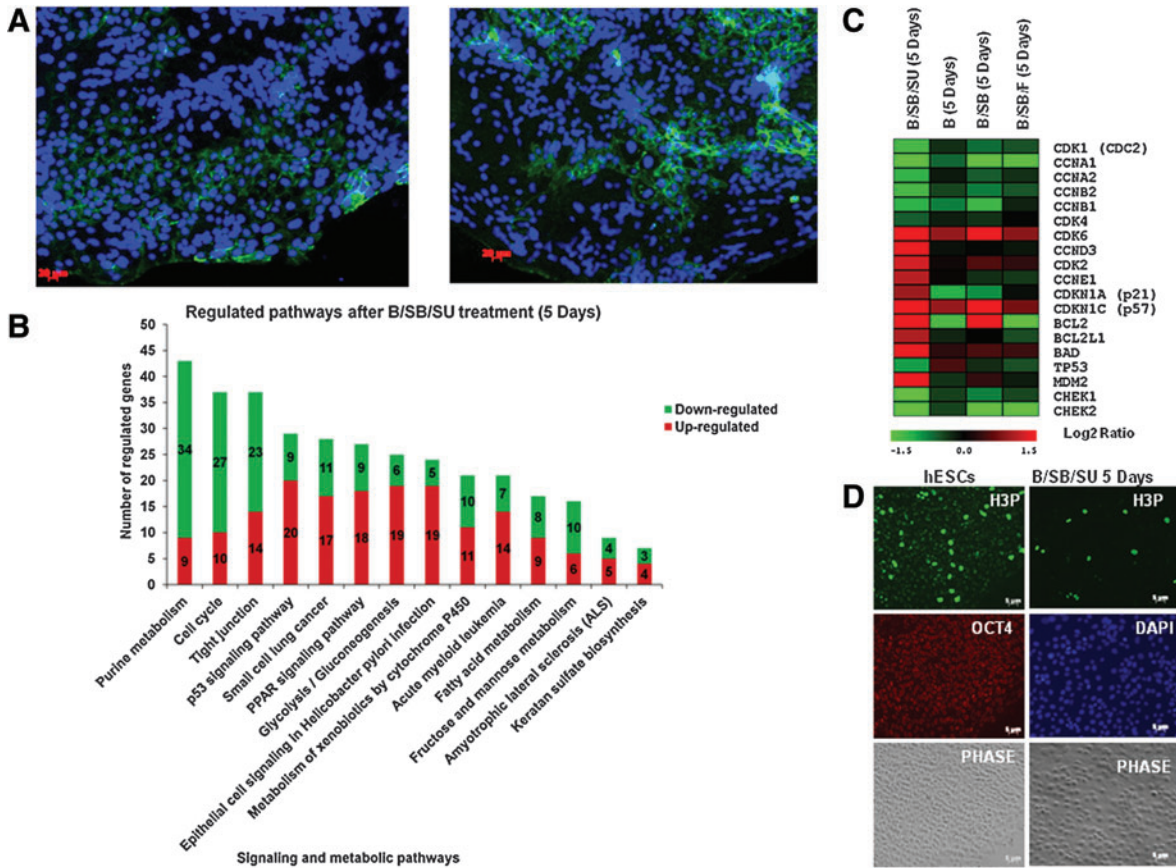
(Abcam, ab763; 1/100), OCT4 (Santa Cruz Biotechnology, sc-8629, 1/100), HLA-G (Santa Cruz Biotechnology, Inc., sc-17958; 1/100), KRT7 (DAKO M701829, 1/100), CDH1 (BD Biosciences 610182; 1/100), Syncytin1 (Santa Cruz, sc-50369), VIM (Sigma-Aldrich, V6630), ACTB (Sigma A5316), Histone H3 pS10 (Novus Biologicals 06570; 1/100). Then, cells were washed 3 times for 5 min each in PBST with 0.1% BSA and incubated with secondary antibodies (diluted in PBST with 1% BSA) for 1 h in dark. Afterward, 3 washes (PBST with 0.1% BSA) were carried out, with the third wash containing the DAPI solution (Molecular Probes, Invitrogen). Fluorescence was examined, and images were captured using a confocal microscope (LSM510 Meta; Zeiss).

Enzyme-linked immunosorbent assay

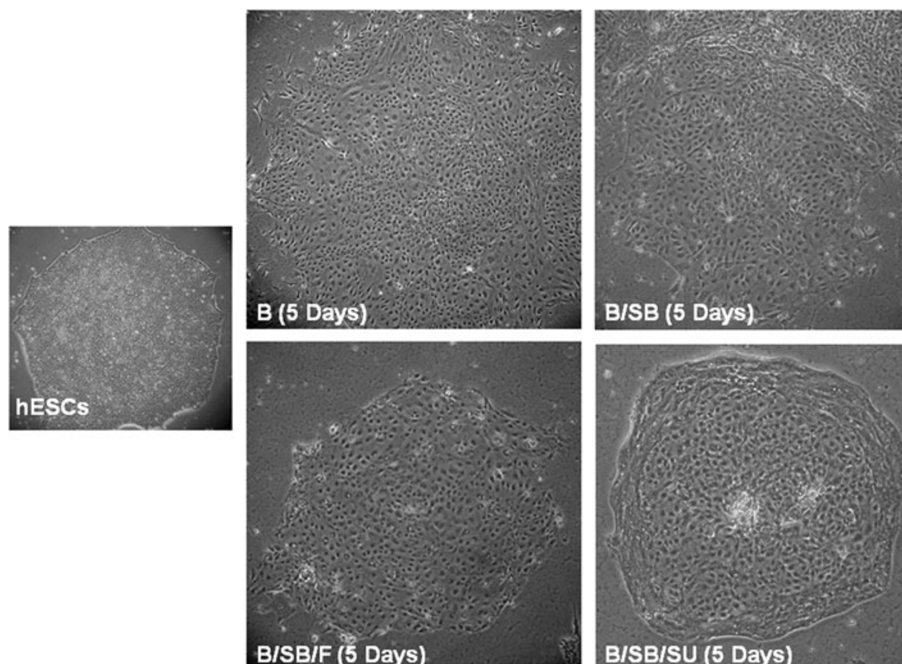
The cells were treated for 5 days, and the medium was changed every day, supplemented with the respective treatments (fresh recombinant proteins or small molecules). After 5 days, the cell culture medium was collected and stored at −80°C. Before performing enzyme-linked immunosorbent assay (ELISA), the samples were thawed and brought to room temperature. ELISA was carried out using the Human Chorionic Gonadotropin (HCG) ELISA Kit (Bio-Quant, BQ 047F), according to the manufacturer's instructions.

Transmission electron microscopy

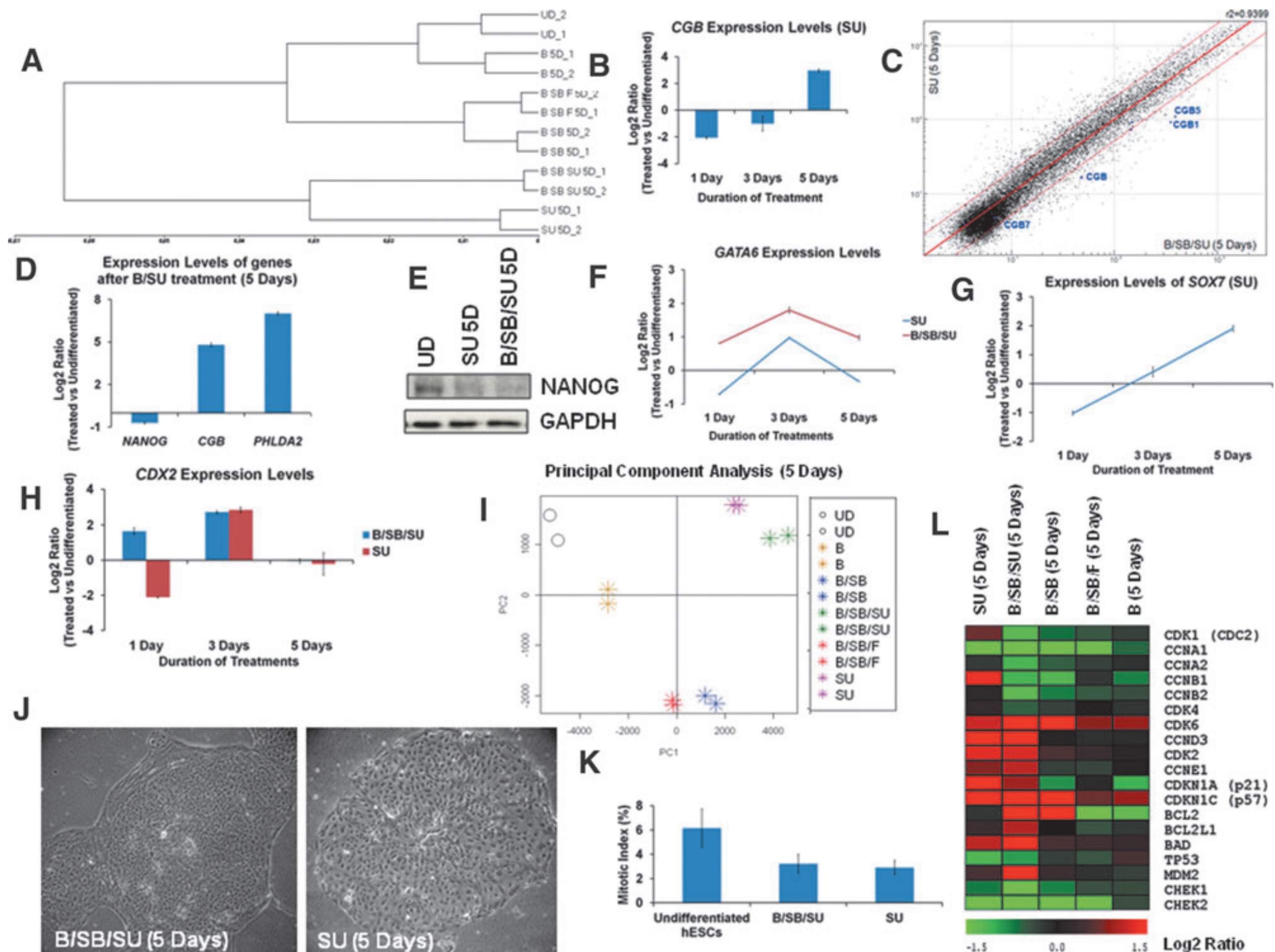
Undifferentiated hESCs were grown on Matrigel-coated Thermanox plastic coverslips (Nunc; www.nuncbrand.com) till they reached ∼50% confluency, and B/SB/SU treatment was carried out for 5 days. Cells were then rinsed with pre-warmed PBS and fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4), supplemented with 50 mM sodium chloride for at least 30 min at room temperature. Specimens were washed in the same buffer and postfixed for 1.5 h in 0.5% osmium tetroxide at room temperature, followed by 0.1% tannic acid for 30 min and 2% uranyl acetate for 1.5 h. Samples were dehydrated in a graded series of ethanol, embedded in Spurr's resin (Low Viscosity Spurr Kit; Ted Pella, www.tedpella.com), and polymerized at 60°C. Ultrathin sections (70 nm) were prepared with an ultramicrotome (Reichert



SUPPLEMENTARY FIG. S4. B/SB/SU treatment induces differentiation of hESCs to multinucleated syncytiotrophoblast. **(A)** Representative immunostain for E-cadherin expression in the B/SB/SU-treated samples, exhibiting multinucleated regions in the colony edges. **(B)** The signaling and metabolic pathways that are regulated in B/SB/SU-treated cells (5 days). The regulated (2-fold) genes were subjected to the DAVID pathway analysis, and the genes included in each pathway were further divided into up- or downregulated genes, based on the microarray data and plotted. **(C)** Heat map depicting the expression of cell cycle-related genes in the indicated samples. **(D)** Representative immunostains for H3P expression in the indicated samples.



SUPPLEMENTARY FIG. S5. B/SB/SU-treated colonies looked prominently different from those of the other treatments after 5 days. Representative phenotypes of undifferentiated hESCs and those treated with B, B/SB, B/SB/F, and B/SB/SU for 5 days.



SUPPLEMENTARY FIG. S6. FGF inhibition in hESC supports differentiation to hCG-secreting trophoblast cells. **(A)** Dendrogram based on Pearson's correlation coefficient between samples (5D: 5 days). **(B)** Real-time PCR-based analysis of *CGB* expression during the course of SU treatment. **(C)** Scatter plot, depicting the comparative gene expression levels of genes between B/SB/SU- and SU-treated samples (5 days) (microarray data). The βhCG-encoding genes (*CGB*, *CGB1*, *CGB5*, and *CGB7*) have been marked. **(D)** Analysis of the indicated genes in B/SU-treated samples (5 Days). **(E)** Western blot analysis of NANOG expression in hESCs and the indicated samples (5 days). **(F)** Expression analysis for *GATA6* during B/SB/SU and SU treatments. **(G)** Analysis of *SOX7* expression during the course of SU treatment. **(H)** Analysis of *CDX2* expression during B/SB/SU and SU treatments. **(I)** PCA on the regulated genes. Those genes were included that were significantly regulated (2-fold) in at least one of the indicated treatments (5 days) in comparison to their expression in hESCs. **(J)** Representative phenotypes of hESCs treated with B/SB/SU or SU for 5 days. **(K)** Mitotic index for the indicated samples. **(L)** Heat map depicting the expression of cell cycle-related genes in the indicated samples.

Ultracut E; Leica, www.leica-microsystem.com) and mounted on electron microscopy copper grids, 300 mesh. Sections were counterstained with uranyl acetate and lead citrate for 20 s. Micrographs were made with a Philips CM100 using a 1K CCD camera (Tietz Video and Image Processing Systems, www.tvips.com).

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