Stem Cell Reports, Volume *1*

Supplemental Information

WNT3 Is a Biomarker Capable of Predicting the Definitive Endoderm Differentiation Potential of hESCs

Wei Jiang, Donghui Zhang, Nenad Bursac, and Yi Zhang

Inventory of Supplemental Information

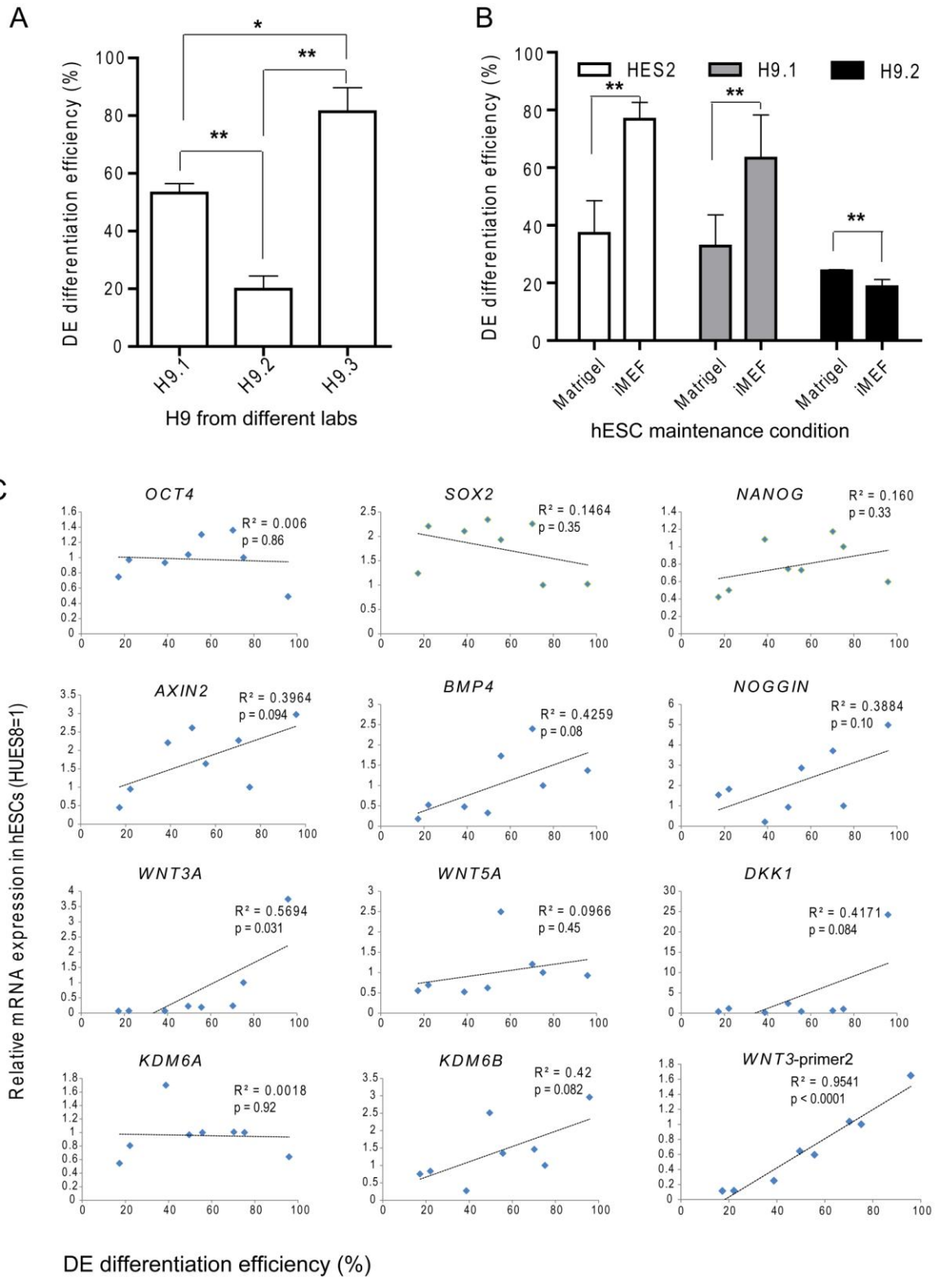
A
DE differentiation efficiency (%)
H9.1
H9.2
H9.3
H9 from different labs
B
HES2
H9.1
H9.2
DE differentiation efficiency (%)
Matrigel
iMEF
hESC maintenance condition
C
OCT4
R² = 0.006
p = 0.86
SOX2
R² = 0.1464
p = 0.35
NANOG
R² = 0.160
p = 0.33
AXIN2
R² = 0.3964
p = 0.094
BMP4
R² = 0.4259
p = 0.08
NOGGIN
R² = 0.3884
p = 0.10
WNT3A
R² = 0.5694
p = 0.031
WNT5A
R² = 0.0966
p = 0.45
DKK1
R² = 0.4171
p = 0.084
KDM6A
R² = 0.0018
p = 0.92
KDM6B
R² = 0.42
p = 0.082
WNT3-primer2
R² = 0.9541
p < 0.0001
Relative mRNA expression in hESCs (HUES8=1)
DE differentiation efficiency (%)

Figure S1 (related to Figure 1). Relationship between gene expression and efficiency of endoderm differentiation

(A) Three H9 sub-lines maintained in different laboratories showed different definitive endoderm (DE) differentiation efficiency. H9.1, H9.2, and H9.3 are respectively from the Zhang lab, Bursac Lab, and Deng lab (Peking University). All of them are cultured with mitomycin C- inactivated mouse embryonic fibroblasts (iMEF). Data was shown by value with standard deviation from three biological replicates.

(B) The same cell line maintained under different conditions showed different definitive endoderm differentiation efficiency. HES2 and H9.1, but not H9.2, showed better differentiation efficiency when cultured with iMEF. Data was shown by value with standard deviation from three biological replicates. The p value by student *t* test was shown as *: $p<0.05$; **: $p<0.01$.

(C) RT-qPCR analysis demonstrates that in contrast to *WNT3* (Figure 1B, and last panel with another primer pair), the expression levels of many genes including pluripotent transcription factors (*OCT4, SOX2, NANOG*), signaling factors (*WNT3A, WNT5A, DKK1, AXIN2, BMP4, NOGGIN*) and epigenetic factors (*KDM6A, KDM6B*), showed poor correlation with endoderm differentiation potential.

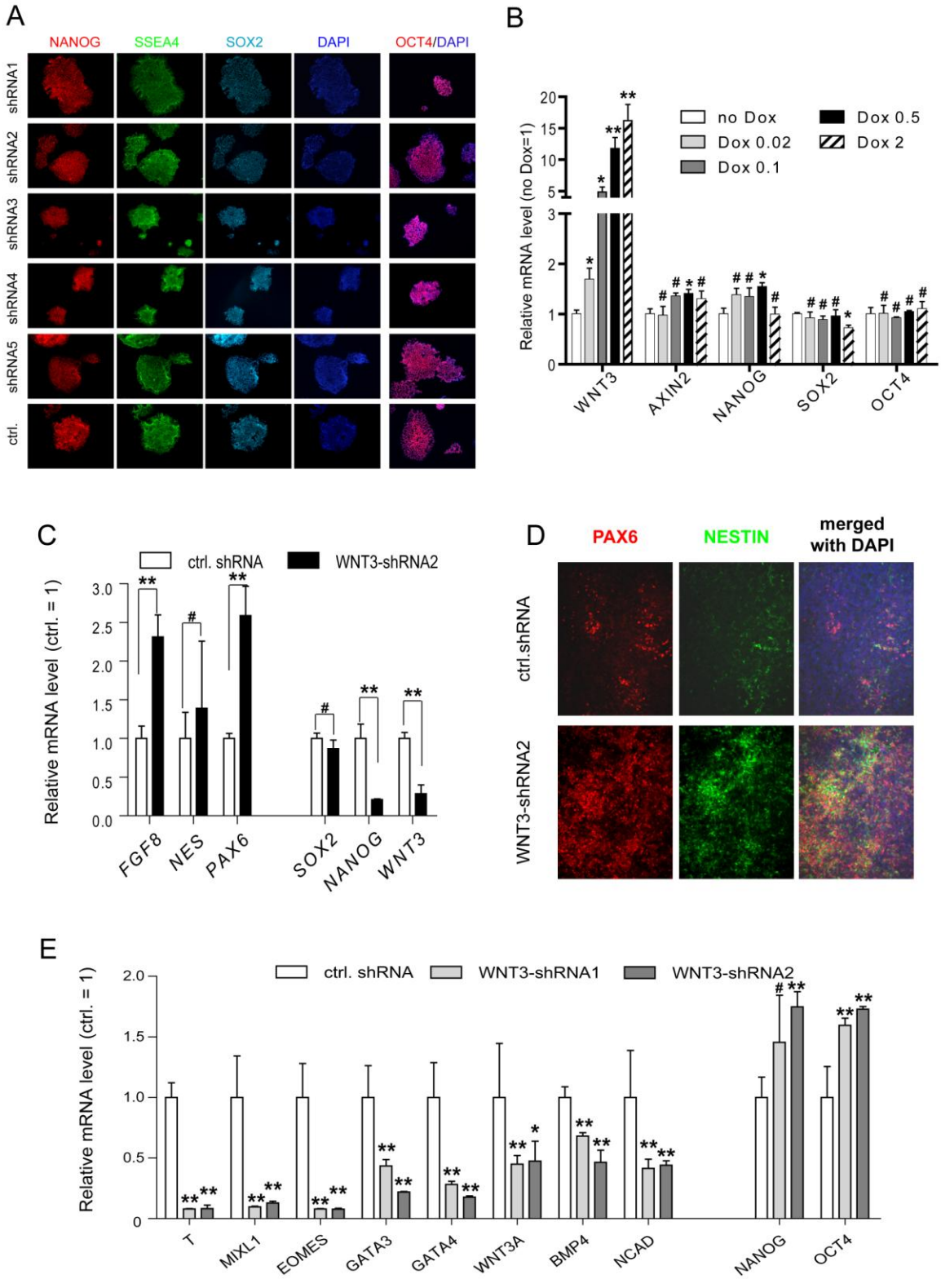
A
NANOG
SSEA4
SOX2
DAPI
OCT4/DAPI
shRNA1
shRNA2
shRNA3
shRNA4
shRNA5
ctrl.
B
Relative mRNA level (no Dox=1)
no Dox
Dox 0.02
Dox 0.1
Dox 0.5
Dox 2
WNT3
AXIN2
NANOG
SOX2
OCT4
C
ctrl. shRNA
WNT3-shRNA2
Relative mRNA level (ctrl. = 1)
FGF8
NES
PAX6
SOX2
NANOG
WNT3
D
PAX6
NESTIN
merged with DAPI
ctrl.shRNA
WNT3-shRNA2
E
ctrl. shRNA
WNT3-shRNA1
WNT3-shRNA2
Relative mRNA level (ctrl. = 1)
T
MIXL1
EOMES
GATA3
GATA4
WNT3A
BMP4
NCAD
NANOG
OCT4

Figure S2 (related to Figure 2). Change in WNT3 levels does not affect hESC maintenance, but affects neuroectoderm and mesendoderm differentiation

(A) WNT3 knockdown in HUES8 does not affect hESC pluripotent gene protein expression (NANOG, SOX2, SSEA4 and OCT4) and colony morphology.

(B) Dox-induced WNT3 expression in H9.2 does not affect expression of pluripotent factors or the canonical WNT signal marker, AXIN2. RT-qPCR analysis of *WNT3*, pluripotent factors (*NANOG, SOX2* and *OCT4*) and a canonical WNT signal marker *AXIN2* under different Dox concentration. The p value by student t test compared to no dox was shown as: #: $p>0.05$; *: $p<0.05$; **: $p<0.01$.

(C) After 5 days' neuroectoderm differentiation, WNT3-knockdown hESC HUES8 exhibited higher levels of neural and ectodermal marker gene expression (*PAX6, NESTIN, FGF8*) but with lower *NANOG* expression compared to control. The p value by student *t* test was shown as: #: $p>0.05$; *: $p<0.05$; **: $p<0.01$.

(D) After 11 days' neural differentiation, WNT3-knockdown HUES8 hESCs exhibited higher percentage of PAX6 and NESTIN positive cells compared to control.

(E) After 1.5 days' mesendoderm differentiation, WNT3-knockdown HUES8 hESCs exhibited impaired activation of mesendoderm marker genes and maintained slightly higher expression of pluripotent markers when compared with the control hESCs. The p value by student *t* test compared to control shRNA was shown as: #: $p>0.05$; *: $p<0.05$; **: $p<0.01$.

Data was shown by value with standard deviation from three biological replicates.

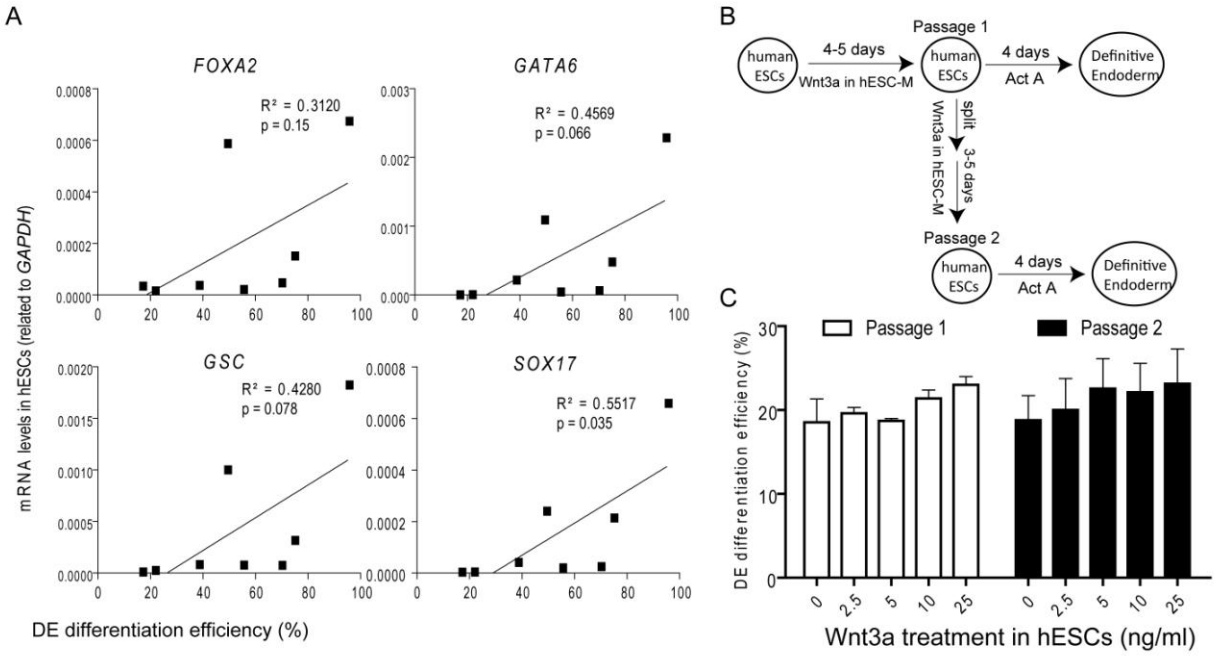

Figure S3 (related to Figure 3).

(A) Correlation between the gene expression levels of endoderm makers in hESCs and their definitive endoderm differentiation efficiency. The coefficient of determination R^2 and the *t*-statistic values are shown.

(B) Diagrammatic explanation of the experimental procedure. After culturing in regular hESC medium (hESC-M) supplemented with recombinant protein Wnt3a for either one or two passages, hESC line H9.2 was used to evaluate the definitive endoderm differentiation efficiency.

(C) Treatment with various concentrations of exogenous Wnt3a protein failed to significantly alter the definitive endoderm differentiation capacity of H9.2 hESCs. Two passages of Wnt3a treatment were analyzed. Data was shown by value with standard deviation from two biological replicates.

Table S1: Primers for quantitative expression analysis of RT-PCR.

Genes	Forward	Reverse	Product/bp
GAPDH	AATGAAGGGGTCATTGATGG	AAGGTGAAGGTCGGAGTCAA	108
WNT3	AGTTGCTTGGGGACCAGG	CTCGCTGGCTACCCAATTT	110
WNT3	GAGCCCAGAGATGTGTACTGC	CTTCTAATGGAGCCCCACCT	125
OCT4	CAAAGCAGAAACCCTCGTGC	TCTCACTCGGTTCTCGATACTG	64
SOX2	GTCATTTGCTGTGGGTGATG	AGAAAAACGAGGGAAATGGG	120
NANOG	CCCCAGCCTTTACTCTTCCTA	CCAGGTTGAATTGTTCCAGGTC	97
NODAL	ATGCCAGATCCTCTTGTTGG	AGACATCATCCGCAGCCTAC	104
WNT3A	CTTCTGCACATGAGCGTGTC	AACTGCACCACCGTCCAC	133
WNT5A	GCATCCCAGCTCTGCCCCAAC	GCGCAGTGAACCGGAGCTGAA	74
BMP4	GCATTCGGTTACCAGGAATC	TGAGCCTTTCCAGCAAGTTT	106
KDM6A	TACAGGCTCAGTTGTGTAACCT	CTGCGGGAATTGGTAGGCTC	99
KDM6B	GGAGGCCACACGCTGCTAC	GCCAGTATGAAAGTTCCAGAGCTG	112
AXIN2	CTGGTGCAAAGACATAGCCA	AGTGTGAGGTCCACGGAAAC	103
DKK1	TTCCATTTTTGCAGTAATTCCC	AGTACTGCGCTAGTCCCACC	126
NOGGIN	CATGAAGCCTGGGTCGTAGT	TCGAACACCCAGACCCTATC	98
SOX17	GCATGACTCCGGTGTGAATCT	TCACACGTCAGGATAGTTGCAGT	103
GATA6	ACTTGAGCTCGCTGTTCTCG	CAGCAAAAATACTTCCCCCA	107
FOXA2	GGAGCAGCTACTATGCAGAGC	CGTGTTCATGCCGTTCATCC	83
GSC	AGGAGAAAGTGGAGGTCTGGTTTA	GACGTCTTGTTCCACTTCTCCG	106
EOMES	CACATTGTAGTGGGCAGTGG	CGCCACCAAACTGAGATGAT	102
CXCR4	TACACCGAGGAAATGGGCTCA	AGATGATGGAGTAGATGGTGGG	112
MIXL1	GAGACTTGGCACGCCTGT	GGTACCCCGACATCCACTT	99
PAX6	TCCGTTGGAACTGATGGAGT	GTTGGTATCCGGGGACTTC	101
NES	GAGGGAAGTCTTGGAGCCAC	AAGATGTCCCTCAGCCTGG	99
FGF8	CTCTGCTTCCAAAGGTGTCC	CAGGTCCTGGCCAACAAG	94
GATA3	TTCCTCCTCCAGAGTGTGGT	AAAATGAACGGACAGAACCG	119
GATA4	CAGGCGTTGCACAGATAGTG	CCCGACACCCCAATCTC	124
NCAD	GAGGAGTCAGTGAAGGAGTCA	GGCAAGTTGATTGGAGGGATG	122

Supplemental Experimental Procedures

Generation of WNT3 knockdown and overexpression cell lines

The lentiviral vector system is obtained from the NIH AIDS Research and Reference Reagent Program. Five *WNT3* knockdown vectors were purchased from Open Biosystems (clone ID: TRCN0000033379 (C12), TRCN0000033380 (D1), TRCN0000033381 (D2), TRCN0000033382 (D3), and TRCN0000033383 (D4)). For knockdown, HUES8 was used as host. After lentiviral transfection, stable knock-down cells were selected by using 2 μg/ml puromycin based on the construct containing puromycin-resistant cassette. D2 and D3 show the best knockdown efficiency (more than 75%) and D4 show moderate knockdown efficiency (around 50%). For inducible overexpression, human WNT3 was amplified from complementary DNA from human ESCs and cloned into a doxycyclin-inducible lentiviral vector pTYF-TRE followed by sequencing to confirm, as described previously (Liang et al., 2012). For overexpression assay, H9.2 was used based on the relatively low endogenous *WNT3* expression level.

Flow cytometry analysis

Cells were dissociated to a single-cell suspension using 0.05% trypsin-EDTA or Accutase (StemCell Technologies). For labeling of cell surface antigens, cells were incubated with primary antibody (APC-conjugated mouse anti-human CD184 IgG, BD; PE-conjugated mouse anti-human CD117 IgG, BD) or control isotype in ice-cold FACS buffer (2% (vol/vol) FBS in PBS) for 30-45 minutes and washed twice. Then cell labeling was analyzed using the flow cytometer FACSAria II with FACSDiva software version 6.0 (BD Biosciences). All data presented in the manuscript was analyzed by FlowJo 7.6.1.

Neuroectoderm differentiation and mesendoderm differentiation

Neuroectoderm differentiation was performed according to the dual inhibition protocol (Chambers et al., 2009) with minor modifications. Briefly, cultured HUES8 cells with 80% confluence were treated with 2uM SB431542 and 250ng/ml Noggin in KSR-Medium (human ES medium without bFGF) for five days and then RNA was extracted to examine neuroectoderm gene expression by quantitative RT-PCR. For PAX6 and NESTIN

immunostaining, another six days' treatment with 250ng/ml Noggin only in KSR-N2 medium (gradually increasing the ratio of N2 medium from 25% to 75%) was performed.

For mesendoderm differentiation, cultured HUES8 cells with 80% confluence were washed twice by DMEM/F12 followed by 1.5 days' treatment of 100 ng/ml activin A in DMEM/F12-N2B27 medium as the same as that used for definitive endoderm differentiation.

RNA Extraction and quantitative RT-PCR

Total RNA was extracted from cell cultures using Qiashredder and RNeasy mini kits (Qiagen) and treated with RNase-free DNase I set according to the manufacturer's instructions. Total RNA (about 1-2 μg per 20μl reaction) was reverse transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen) primed with random hexamer oligonucleotides (Promega). Quantitative PCR was carried out on CFX384 Real-Time PCR system (Bio-Rad) using Sso-Fast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. Three technical replicates were performed for each sample/primer. Relative expression levels were normalized to *GAPDH* and calculated using the $2^{-\Delta Ct}$ method. To confirm the correlation between *WNT3* expression level and endoderm differentiation efficiency, two pairs of *WNT3* primers were used. All primers were purchased from Invitrogen and listed in Table S1.

Immunofluorescence staining

Culturing cells in chambered slides (Millipore) were fixed by 4% PFA for 15 minutes and blocked with 10% (vol/vol) donkey serum plus 0.2% Triton X-100 in PBS for 2 hours. Then samples were incubated at 4 °C for overnight with primary antibody or irrelevant IgG and then incubated at room temperature for 1 hour with appropriate secondary antibody (donkey anti-mouse IgG Alexa-488 or donkey anti-goat IgG Tritc or donkey anti-rabbit IgG Cy5) in blocking buffer. Cells were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) for 5 min before mounting with Vectorshield (Vector Laboratories). The used primary antibodies and dilutions were listed as follows: Goat anti-human NANOG (R&D, 1:400), Rabbit anti-SOX2 (Millipore, 1:400), Mouse anti-human SSEA4 (Millipore, 1:200), Goat anti-human OCT4 (Santa Cruz, 1:400), Goat anti-human FOXA2 (R&D, 1:500), Goat anti-human SOX17 (R&D, 1:500), Rabbit anti-human PAX6 (Covance, 1:500), Mouse anti-NESTIN (R&D, 1:500).

Supplementary references

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature biotechnology *27*, 275-280.
Liang, G., He, J., and Zhang, Y. (2012). Kdm2b promotes induced pluripotent stem cell generation by facilitating gene activation early in reprogramming. Nature cell biology *14*, 457-466.