

Supporting Information

Davidson et al. 10.1073/pnas.1118777109

SI Materials and Methods

hESC Culture. For experiments where treatments were continued over several weeks, an identical sister plate was harvested as single cells via TrypLE Express (Invitrogen) to determine an accurate cell count for seeding. The experimental plate was harvested with dispase and small cell clusters were seeded at a density of 35,000 cells per centimeter squared. For multiweek experiments, triplicate sister wells were set up for each condition in 12-well plates: 1 well was harvested with TrypLE Express to determine the cell count for each treatment and used for flow cytometric analysis; a second well was passaged with dispase and seeded at 35,000 cells per centimeter squared on the basis of the single cell count; the third well was harvested with TRIZOL for RNA isolation.

To obtain total cell number of GCTM2⁺/CD9⁺ stem cells per well, the percentage of GCTM2⁺/CD9⁺ cells (determined by flow cytometric analysis) was multiplied by the number of cells counted per well of a 12-well plate each week.

Flow Cytometry. hESCs were harvested as single cells via TrypLE Express and counted using a Nucleocounter (New Brunswick Scientific). For live flow cytometric analysis, 500,000 cells were immunolabeled with 100 μ L primary antibodies, GCTM2 (hybridoma supernatant 1:2, a kind gift from Martin Pera, University of Southern California, Los Angeles, CA) and CD9 (TG30 clone, 1:100; Millipore), followed by isotype-specific secondary antibodies: goat antimouse IgM-Alexa 647 (1:1,000) and goat antimouse IgG2a-biotin (1:5,000), followed by Alexa 750-R-PE-streptavidin or PE-Cy7-streptavidin (both 1:250) (all secondary antibodies and streptavidin conjugates from Invitrogen). hESCs were resuspended in 140 ng/mL DAPI in culture medium and transferred through a cell strainer before analysis on a BD FACSCanto II. Results were analyzed using FlowJo software.

For H1-BAR-VENUS hESCs, the percentage of VENUS-positive cells was determined from the DAPI-negative/DsRED-positive gated population relative to H1-FUBARV-negative control line. Average mean fluorescent intensity (MFI) was measured from the VENUS-positive subpopulation.

For intracellular flow analysis, 1×10^6 hESCs were fixed in 4% PFA for 20 min at room temperature, then permeabilized with 0.1% Triton for 2 min on ice. Cells were immunolabeled with 100 μ L of Oct4 antibody (C-10 clone, 1:30; Santa Cruz) for 45–60 min on ice, followed by goat antimouse IgG2b-Alexa 647 (1:1,000; Invitrogen).

Lentiviral Transduction and Selection of hESCs. H1 hESC colonies were isolated as small clusters using dispase in the same manner as for weekly passage, then exposed to ultraconcentrated lentivirus before seeding onto Matrigel in mouse embryonic fibroblast (MEF) conditional medium (CM). Transduced hESCs were cultured for a week on Matrigel, then expanded onto several 3.5-cm plates with MEFs. Each lentiviral construct contained DsRED driven by the Ubiquitin promoter as a selectable marker. After a week of expansion on MEFs, DsRED-positive areas of individual colonies were mechanically isolated and transferred onto fresh MEFs. This positive selection method was repeated for another week or two as needed until entirely DsRED-positive lines were isolated, after which time the stable lines were passaged with dispase as per usual. In some cases, a final negative selection step was performed where DsRED-negative areas of colonies were mechanically cut and discarded, then the remainder of the colonies in the plate were passaged with dispase (Fig. S7).

Fluorescent-Activated Cell Sorting. H1-BAR-VENUS hESCs were harvested as single cells with TrypLE Express. The DAPI-negative/DsRED-positive gated population was sorted on the basis of VENUS expression. A minimum of 1×10^6 sorted cells was collected for downstream RNA isolation. For each experiment, samples were normalized to the same cell number on the basis of postsort cell counts before freezing in TRIZOL.

siRNA Reverse Transfection of hESCs. hESCs were harvested into a single cell suspension with TrypLE Express, then counted on a NucleoCounter. Reverse transfection was carried out with 0.125 μ L RNAiMAX (Invitrogen) per centimeter squared plus 20 nM siRNA according to the manufacturer's instructions (this is equivalent to 0.5 μ L RNAiMAX per well of a 12-well plate, or 1.25 μ L per well of a 6-well plate). hESCs were seeded on Matrigel-coated plates at a density of 50,000 cells/cm² in MEF CM supplemented with 8 ng/mL bFGF, 5 μ M Y-27632 and 0.5 μ M thiazovivin (both ROCK inhibitors from Tocris), and then allowed to attach overnight. The following day, cells were gently washed with knockout serum replacement (KSR) medium to remove dead cells and debris, and the culture medium was replaced with fresh MEF CM + bFGF. Cells received daily media changes thereafter for the duration of the experiment. If L or Wnt3A CM was added, cells were stimulated 48 h post-siRNA transfection. Cells were harvested for analysis at 72 h post-siRNA transfection.

siRNA sequence information (from Invitrogen):

Silencer Select Negative Control 1
AXIN1, Silencer Select ID s15814
AXIN2, Silencer Select ID s15818
CTNNB1, Silencer Select ID s437
POU5F1-A, Silencer Select ID s10873
POU5F1-B, (sense) 5'-AGCAGCUUGGGCUCGAGAA-3'*
POU5F1-C, (sense) 5'-CAUGUGAAGCUGCGGCC-3'*
NANOG-A, (sense) 5'-AACCAGACCUGGAACAAUUCA-3'[†]
Scrambled-*NANOG*, (sense) 5'-AACGAGACCAUGAACGAUUCA-3'[†]
NANOG-B, (sense) 5'-AAGGGUUAAGCUGUAACAUAC-3'[‡]

L and Wnt3A Conditioned Medium. L and L-Wnt3A cells (ATCC) were cultured in 10% FBS/DMEM until ~90% confluent, then given a minimal volume of fresh medium (18 mL medium per 15-cm plate). This medium was incubated on cells for 48 h, then collected. A second batch of medium was incubated on the same cells for 48 h, then collected. This process was repeated a third time to yield three batches of conditioned medium (CM). Biological activity of secreted Wnt3A in individual batches of CM was confirmed in 293T-BAR or A375-BAR reporter cells, then batches were pooled and filtered. L and Wnt3A CM were stored at 4 °C for up to 3 mo.

Recombinant Wnt3a and Inhibitors. Recombinant Wnt3a (Millipore or Peprotech) was reconstituted at 100 μ g/mL in a final concentration of 1% Chaps in PBS and bioactivity was confirmed for each lot number in 293T-BAR or A375-BAR cells. The follow-

**POU5F1-B* and *POU5F1-C* were previously published by Matin et al. (1).

[†]*NANOG-A* and Scrambled-*NANOG* were previously published by Hyslop et al. (2).

[‡]*NANOG-B* was previously published by Wong et al. (3).

ing compounds were reconstituted in DMSO: XAV939 (Tocris), CHIR99021 (AxonMedChem), and BIO (Calbiochem).

Luciferase Reporter Assays. The lentiviral constructs used to generate reporter 293T-BAR or A375-BAR are described elsewhere (4). Nonpluripotent cells were seeded in triplicate for each condition in 48-well plates and stimulated with Wnt3A or other treatments for 16–18 h. Each well was then lysed in 50 μ L 1 \times passive lysis buffer (Promega). Ten microliters per well of lysate was assayed using the Dual Luciferase Assay kit (Promega) and normalized to Renilla counts (Renilla is driven constitutively by EF1- α promoter in these cells). For H1-BAR-Luciferase hESCs, cells were seeded in sextuplicate for each condition and stimulated for 72 h before being assayed for Firefly luciferase. Attempts to integrate EF1- α driven Renilla into H1-BAR-Luciferase cells resulted in rapid silencing of the EF1- α promoter within 3 wk postlentiviral transduction; thus Renilla normalization was not performed for hESC luciferase reporter lines.

RNA Isolation, cDNA Synthesis, and Quantitative PCR. Total RNA was extracted via TRIZOL according to the manufacturer's protocol. A total of 2.5 μ g RNA was used to generate cDNA using RevertAid First Strand cDNA Synthesis kit (Fermentas). cDNA was diluted to 25 ng/ μ L, and quantitative PCR (qPCR) was carried out using 2 μ L of cDNA per 10 μ L reaction (in duplicates for each run). qPCR was performed using Applied Biosystems SYBR Green-based detection (Applied Biosystems) according to the manufacturer's protocol on a Roche Lightcycler 480 instrument. All primer pairs were validated for specificity to human and confirmed to not yield a signal with MEF cDNA. Transcript copy numbers were normalized to GAPDH for each sample, then fold expression over control was calculated for each gene of interest. Primer sequences are listed in Table S1.

Western Blots. Cells were lysed on ice in 1 \times RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 0.2% deoxycholate) freshly supplemented with protease and phosphatase inhibitor mixtures (Roche). Cleared lysates were normalized by protein concentration as determined by BCA assay (Pierce) before diluting with sample buffer + 20 mM DTT. To remove

junctional (cadherin-bound) β -catenin, some lysates were also ConA stripped. Cleared lysates containing 750 μ g protein in 200 μ L were incubated with 50 μ L (packed volume) of ConA-sepharose beads (Amersham) overnight at 4 $^{\circ}$ C with gentle end-over-end agitation. Lysates were normalized again by protein concentration using a BCA assay before adding sample buffer. A total of 10 μ g protein was loaded per lane for Westerns. Blots were blocked in 4% (wt/vol) skim milk powder, then incubated with the following primary antibodies: goat antihuman Axin1 (1:1,000; R&D Systems), rabbit antihuman Axin1 (1:1,000; Cell Signaling; no. 2087), rabbit antihuman β -catenin (1:1,000; Cell Signaling), mouse antihuman β -catenin (1:1,000; BD Transduction Laboratories), rabbit anti-GFP (1:50,000; Abcam), rat antihuman HSP90 (1:20,000; Abcam), rabbit antihuman Nanog (1:1,000; Cell Signaling), goat antihuman Oct4 (1:500; R&D Systems), mouse antihuman Oct4 (C-10 clone, 1:1,000; Santa Cruz), and mouse antihuman β -tubulin I (1:20,000; Sigma). Primary antibodies were diluted in 1% (wt/vol) BSA, 0.02% (wt/vol) sodium azide in TBS-T. Species-specific HRP-conjugated secondary antibodies were used, followed by ECL-based detection (Pierce).

Plasmid DNA Transient Transfections. HEK293T cells were transfected in a 6-well plate format with 10 μ L per well lipofectamine 2000 according to the manufacturer's protocol (Invitrogen) using the following combination of plasmids per well:

240 ng pGL3-BARL,
240 ng pRL-TK-Renilla,
0.8–1,000 ng pLM-vexGFP-Oct4, pLenti-PGK-GFP-Puro, and/
or pMXs-hNANOG, and
pCS2+ up to 2.4 μ g total DNA.

Twenty-four hours posttransfection, cells from each well were split into duplicate wells of a 12-well plate and sextuplicate wells of a 96-well plate. Three to four hours later, cells were stimulated with 50% L or Wnt3A CM for an additional 15–18 h. The 96-well plate was then lysed in 25 μ L per well of 1 \times passive lysis buffer for the dual luciferase assay. The 12-well plate was lysed in 80 μ L per well of 1 \times RIPA buffer for Western blots.

1. Matin MM, et al. (2004) Specific knockdown of Oct4 and beta2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. *Stem Cells* 22:659–668.
2. Hyslop L, et al. (2005) Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. *Stem Cells* 23:1035–1043.

3. Wong RC, et al. (2011) L1TD1 is a marker for undifferentiated human embryonic stem cells. *PLoS ONE* 6:e19355.
4. Biechele TL, Moon RT (2008) Assaying beta-catenin/TCF transcription with beta-catenin/TCF transcription-based reporter constructs. *Methods Mol Biol* 468: 99–110.

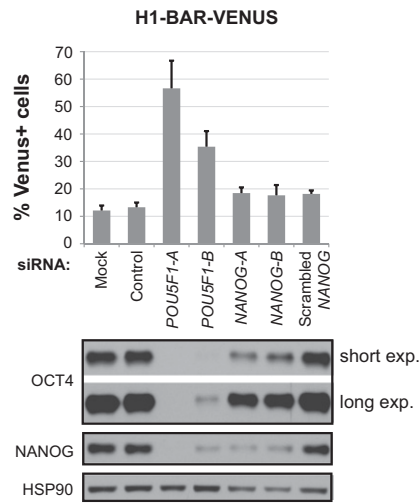


Fig. S6. NANOG does not contribute to repression of β -catenin reporter activity in hESCs. H1-BAR-VENUS cells were transfected with siRNAs, then analyzed for VENUS expression by flow cytometry at 72 h posttransfection. Graph of mean + SEM from three independent experiments. Western blots below show OCT4 and NANOG protein reduction resulting from RNAi-mediated knockdown for each siRNA from a representative experiment.

