Supporting Information

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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 VENUSpositive cells was determined from the DAPI-negative/DsREDpositive 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 µL 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 3.5cm 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 µL RNAiMAX (Invitrogen) per centimeter squared plus 20 nM siRNA according to the manufacturer's instructions (this is equivalent to 0.5 µL RNAiMAX per well of a 12-well plate, or 1.25 µL per well of a 6-well plate). hESCs were seeded on Matrigelcoated plates at a density of 50,000 cells/cm² in MEF CM supplemented with 8 ng/mL bFGF, 5 µM Y-27632 and 0.5 µM 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'-CAUGUGUAAGCUGCGGCCC-3'* NANOG-A, (sense) 5'-AACCAGACCUGGAACAAUUCA-3'† Scrambled-NANOG, (sense) 5'-AACGAGACCAUGAAC-GAUUCA-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× 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

- 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.
- Hyslop L, et al. (2005) Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. *Stem Cells* 23:1035–1043.

junctional (cadherin-bound) β-catenin, some lysates were also ConA stripped. Cleared lysates containing 750 µg protein in 200 µL were incubated with 50 µL (packed volume) of ConA-sepharose beads (Amersham) overnight at 4 °C with gentle endover-end agitation. Lysates were normalized again by protein concentration using a BCA assay before adding sample buffer. A total of 10 µg 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 µg 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× passive lysis buffer for the dual luciferase assay. The 12-well plate was lysed in 80 μ L per well of 1× RIPA buffer for Western blots.

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

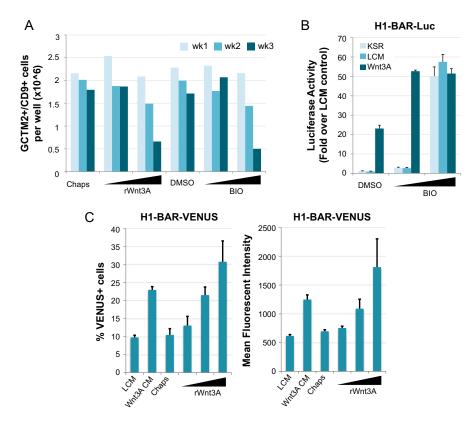


Fig. S1. Recombinant Wnt3A and GSK3- β inhibitor, BIO, both fail to maintain undifferentiated hESCs for 3 wk. (A) The number of GCTM2⁺/CD9⁺ cells per well was calculated by multiplying the percent of GCTM2⁺/CD9⁺ cells by the number of cells counted each week from 1 well of a 12-well plate. For recombinant Wnt3A (rWnt3A) treated cells: concentrations tested were 100 ng/mL and 400 ng/mL; vehicle control was 1% (wt/vol) Chaps. For BIO-treated cells: concentrations tested were 2 μ M and 4 μ M; vehicle control was DMSO. (*B*) H1-BAR-Luciferase reporter hESCs treated with BIO (2 μ M and 4 μ M) with or without 50% CM. Graph is mean + SEM of experimental replicates from a representative experiment. (*C*) Flow cytometric quantification of VENUS expression and mean fluorescent intensity for H1-BARV cells after 72-h stimulation with CM or with recombinant Wnt3A. CM yields a similar reporter response as 400 ng/mL rWnt3A.

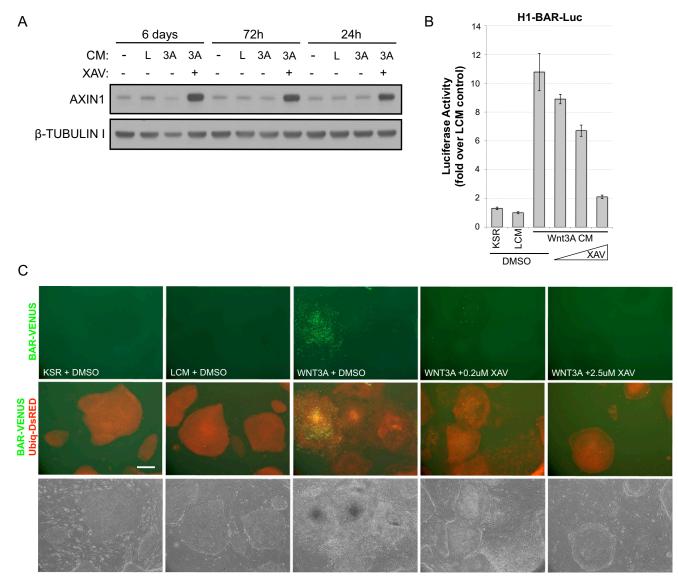


Fig. S2. XAV939 stabilizes AXIN and blocks Wnt3A-induced activation of β -catenin reporter in hESCs. (*A*) Western blot showing AXIN1 expression in hESCs cultured with KSR medium, 50% LCM, 50% Wnt3A CM, or 50% Wnt3A CM + 2.5 μ M XAV for the period noted. All conditions without XAV received DMSO vehicle control. (*B*) XAV inhibits Luciferase reporter activation induced by Wnt3A in H1-BAR-Luc hESCs in a dose-dependent manner. Wnt3A CM, 50%; XAV, 100 nM, 250 nM, and 2.5 μ M). (*C*) Representative images of H1-BAR-Venus hESCs cultured in the conditions listed for 6 d. XAV inhibits VENUS reporter expression (green) and morphological differentiation (brightfield) in a dose-dependent manner. L and Wnt3A CM both are 50% in KSR medium. (Scale bar, 400 μ m.)

DNA C

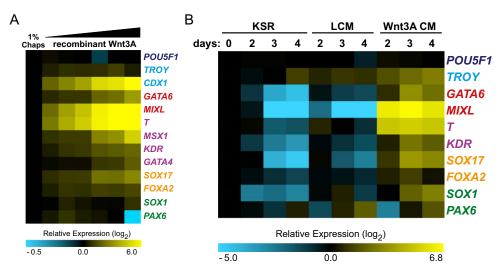


Fig. S3. Similar gene expression profiles obtained for hESCs cultured with recombinant Wnt3A versus Wnt3A CM. (*A*) qRT-PCR results for H1 cells cultured for 72 h with increasing concentrations of recombinant Wnt3A protein (50, 100, 200, 400, 800, and 1200 ng/mL). Heatmaps represent fold expression over vehicle control. (*B*) Transcriptional effects of Wnt3A CM on hESCs cultured for 2, 3, or 4 d.

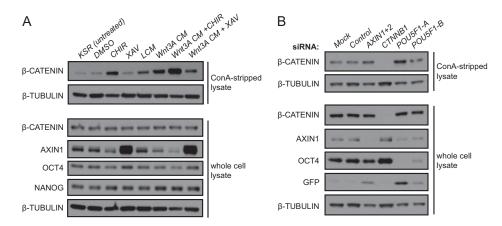


Fig. 54. Western blots showing protein expression in hESC following activation or inhibition of Wnt/ β -catenin pathway. (A) Western blots from H1 cells cultured for on MEFs in KSR media for 72 h with the following treatments: untreated (control), DMSO (vehicle control), 5 μ M CHIR99021 (CHIR), 2.5 μ M XAV939 (XAV), 50% LCM, 50% Wnt3A CM, Wnt3A CM + 5 μ M CHIR, and Wnt3A CM +2.5 μ M XAV. Results show increased levels of nonmembrane-bound β -catenin (ConA-stripped lysates) under conditions that activate the Wnt pathway compared with the respective controls. Conversely, Axin levels are reduced under conditions that activate Wnt/ β -catenin gand increased by the inhibitor, XAV939. (B) Western blots from H1-BAR-VENUS cells at 72 h post-siRNA transfection on Matrigel in MEF CM. *POUSF1-A* siRNA results in a greater level of stabilized β -catenin protein in the nonmembrane-bound fraction (ConA-stripped) compared with control siRNA. The GFP antibody detects VENUS protein levels, indicative of BAR reporter activation.

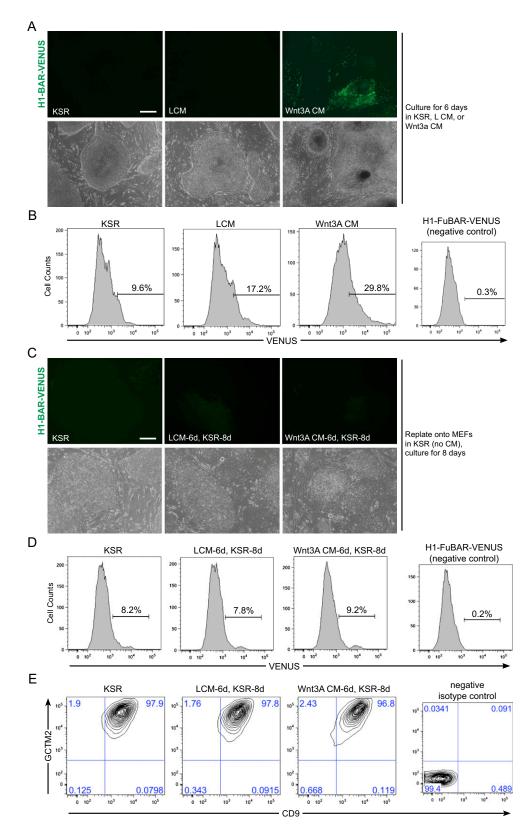


Fig. S5. Wnt-induced BAR-VENUS expression is reversible in hESCs. (*A*) Images of H1-BAR-VENUS cells cultured on MEFs for 6 d in the following conditions: KSR media (untreated), 50% LCM in KSR (control), or 50% Wnt3A CM in KSR. (*Upper*) VENUS fluorescence. (*Lower*) Brightfield images. (Scale bar, 400 µm.) (*B*) Flow cytometric quantification of percent VENUS expression in the samples pictured in *A*, with H1-FuBAR-VENUS as gating reference. (*C*) Following 6 d of culture with KSR, LCM, or Wnt3a CM, the H1-BAR-VENUS cells were replated onto MEFs in KSR media (no CM) and subsequently cultured for 8 d. Images show that hESCs previously cultured with Wnt3a CM revert back to VENUS-negative colonies. (*D*) Flow cytometric quantification of percent VENUS expression in the samples pictured in *C*, with H1-FUBAR-VENUS as a gating reference. (*E*) Flow cytometric plots showing GCTM2/CD9 coexpression of hESCs from *C* and *D*.

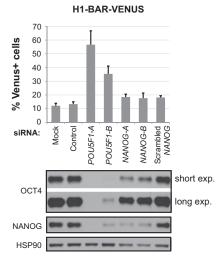


Fig. S6. NANOG does not contribute to repression of β -catenin reporter activity in hESCs. H1-BAR-VENUS cells were transfected with siRNAs, then analyzed for VENUS expression by flow cytometry at 72 h posttransfection. Graph of mean + SEM from three independent experiments. Western blots below show OCT4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

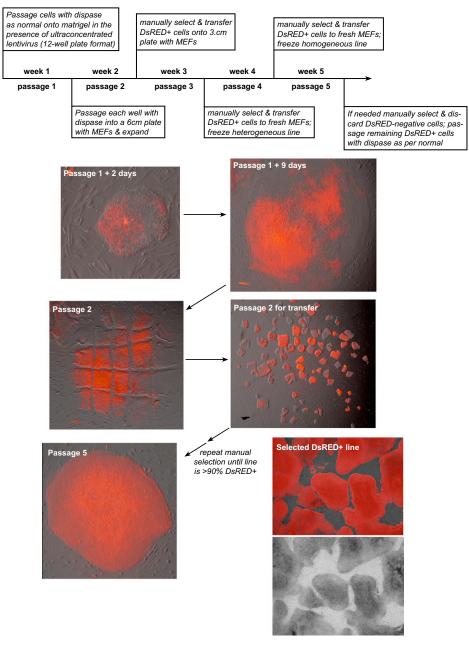


Fig. S7. Method of generating and mechanically selecting stable hESC lines using fluorescent protein expression.

DNAS

Table S1. Primer sequences used for real-time quantitative PCR

PNAS PNAS

Gene	Primer –forward	Primer -reverse	Reference
AFP	GAAGCAAGAGTTTCTCATTAACC	AATTTAAACTCCCAAAGCAGCA	
AXIN2	GCGATCCTGTTAATCCTTATCAC	AATTCCATCTACACTGCTGTC	
CD34	GCGCTTTGCTTGCTGAGTTT	GCCATGTTGAGACACAGGGT	
CDX1	GGTAAGACTCGGACCAAGGA	GATCTTCACCTGCCGTTCAG	
FOXA2	GACAAGTGAGAGAGCAAGTG	ACAGTAGTGGAAACCGGAG	(1)
FST	AGACCAATAATGCCTACTGTG	ACTTTGCTTTGATACACTTTCC	
GAPDH	TGAAGGTCGGAGTCAACGGA	CCATTGATGACAAGCTTCCCG	
GATA4	CCAATCTCGATATGTTTGACGA	TTGATGCCGTTCATCTTGTG	
GATA6	AACTTCCACCTCTTCTAACTCAG	CATCTTGACCCGAATACTTGAG	
KDR	CAAAGACTTGCTCTGGTAATAGC	GAGACTGACGCCTATGTAGAG	
MIXL1	GGTACCCCGACATCCACTT	GCCTGTTCTGGAACCATACCT	(2)
MSX1	TTAACCCTCACACTGCTCC	CTCTAGCTCTGTTCAACTGTC	
NANOG	AGAAGGCCTCAGCACCTAC	GGCCTGATTGTTCCAGGATT	(3)
PAX6	CTTCACCATGGCAAATAACC	GAAATGAGTCCTGTTGAAGTG	
POU5F1	GGGAAGGTATTCAGCCAAACG	GGTTCGCTTTCTCTTTCGGG	
SOX1	GAAACACAATCGCTGAACCA	TCGTCAGGAATAATGAACAAGG	
SOX17	CCTGGGTTTTTGTTGTTGCT	TCGTCAGGAATAATGAACAAGG	
Т	CTCCTTCAGCAAAGTCAAGC	TTAAGAGCTGTGATCTCCTCG	
TROY	GGAGTTGTCTAAGGAATGTGG	GCTGAACAATTTGCCTTCTG	
TUBB3	CGGTGGTGGAGCCCTACAAC	AGGTGGTGACTCCGCTCAT	

Cai J, et al. (2006) Assessing self-renewal and differentiation in human embryonic stem cell lines. Stem Cells 24:516–530.
Rosa A, Spagnoli FM, Brivanlou AH (2009) The miR-430/427/302 family controls mesendodermal fate specification via species-specific target selection. Dev Cell 16:517–527.
Hyslop L, et al. (2005) Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells 23:1035–1043.