

Defining the Role of Wnt/ β -Catenin Signaling in the Survival, Proliferation, and Self-Renewal of Human Embryonic Stem Cells

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ABSTRACT

We used a panel of human and mouse fibroblasts with various abilities for supporting the prolonged growth of human embryonic stem cells (hESCs) to elucidate growth factors required for hESC survival, proliferation, and maintenance of the undifferentiated and pluripotent state (self-renewal). We found that supportive feeder cells secrete growth factors required for both hESC survival/proliferation and blocking hESC spontaneous differentiation to achieve self-renewal. The antidifferentiation soluble factor is neither leukemia inhibitory factor nor Wnt, based on blocking experiments using their antagonists. Because Wnt/ β -catenin signaling has been implicated in cell-fate determination and stem cell expansion, we further examined the effects of blocking or adding recombinant Wnt proteins on undifferentiated hESCs. In the absence of feeder cell-derived factors, hESCs

cultured under a feeder-free condition survived/proliferated poorly and gradually differentiated. Adding recombinant Wnt3a stimulated hESC proliferation but also differentiation. After 4–5 days of Wnt3a treatment, hESCs that survived maintained the undifferentiated phenotype but few could form undifferentiated hESC colonies subsequently. Using a functional reporter assay, we found that the β -catenin-mediated transcriptional activation in the canonical Wnt pathway was minimal in undifferentiated hESCs, but greatly upregulated during differentiation induced by the Wnt treatment and several other methods. Thus, Wnt/ β -catenin activation does not suffice to maintain the undifferentiated and pluripotent state of hESCs. We propose a new model for the role of Wnt/ β -catenin signaling in undifferentiated hESCs. *STEM CELLS* 2005;23:1489–1501

INTRODUCTION

Pluripotent human embryonic stem cell (hESC) lines offer unprecedented opportunities for investigating human cell biology and for developing novel cell-based therapies [1, 2]. All the currently available hESC lines were derived and propagated by coculture with primary mouse embryonic fibroblasts (pMEFs). It was recently found that direct cell-cell contact with pMEFs is not necessary [3]. If an extracellular matrix mixture such as Matrigel is provided, undifferentiated hESCs can be propagated using

the conditioned medium (CM) from pMEFs in either serum-containing or serum-free media. Under the latter condition, adding basic fibroblast growth factor (bFGF) and insulin is necessary [4]. However, bFGF is insufficient to prevent hESC differentiation, and the presence of soluble factors made by pMEFs is also required. In contrast to many mouse embryonic stem cell (mESC) lines, adding leukemia inhibitory factor (LIF) and the activation of the gp130/JAK/STAT3 signaling pathway fail to block spontaneous differentiation of hESCs [1, 2, 5]. The nature of soluble

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factors within pMEF-CM remains undefined, which presents a challenge for improving culture conditions for the propagation (self-renewing proliferation) of hESCs.

Recently, we and others have found that selected human cells, including adult human marrow stromal cells (hMSCs), can substitute pMEFs in supporting the growth of undifferentiated hESCs [6–10]. After many passages, the expanded hESCs on these supportive human feeder cells continue to form compact colonies of cells expressing markers such as alkaline phosphatase (AP), which is associated with undifferentiated hESCs and mESCs. These studies also found, however, that many human cell types were not supportive: AP-positive (AP⁺) compact colonies of undifferentiated hESCs disappeared after two passages. The pace of downregulation in expression of AP and other undifferentiated markers in differentiating hESCs is much slower than mESCs [11], probably due to the fact that hESCs proliferate and differentiate much slower [4]. Therefore, a longer timeframe is required in assays to distinguish undifferentiated or differentiating hESCs. For example, we analyzed hESCs cultured on hMSCs after each passage, which amplified more than 100-fold during the 30-day continuous cultures in five passages [7]. During 2 months in continuous culture, the expanded hESCs expressed a high level of AP and stage-specific embryonic antigen (SSEA)-4, and maintained developmental pluripotency [7]. In the current study, we compared supportive with nonsupportive feeder cells to delineate molecular differences between the two types of feeder cells. We found that all feeder cells, supportive or nonsupportive, produce soluble factors stimulating hESC survival/proliferation. However, cytokines (approximately ≥ 30 kDa) secreted from the supportive feeder cells are necessary and sufficient to block differentiation of hESCs when cultured on nonsupportive feeders. Thus, our comparative studies using the two types of feeder cells revealed that an antidifferentiation soluble factor preferentially made by supportive feeder cells is essential to maintain and expand undifferentiated hESCs. The paradigm that an antidifferentiation factor is required to block hESC spontaneous differentiation and to achieve self-renewing proliferation appears similar to that in mESCs, but the antidifferentiation soluble factor for hESCs is not LIF [1, 2, 5]. Because members of the pleiotropic Wnt cytokine family (~ 40 kDa) play important roles in many developmental events and affect stem cell fates in several systems [12–14], we examined directly whether Wnt is an antidifferentiation factor for keeping hESCs undifferentiated and pluripotent. The results of our extended cell culture (lasting up to 26 days) and functional analyses, however, are contradictory to a recent report concluding that the extracellular Wnt3a signal is sufficient to maintain undifferentiated and pluripotent hESCs, based on the 5-day cell-culture study [15]. To investigate and understand this discrepancy, we decided to further examine the effects of adding Wnt antagonists as well as the same Wnt3a recombinant protein on H1 hESC line as previously described [15]. In addition, we analyzed

the Wnt intracellular signaling in hESCs, including the activation of the canonical Wnt signaling pathway mediated by β -catenin. Based on cell culture and molecular analyses, we propose a new model for the role of Wnt/ β -catenin signaling in hESCs.

MATERIALS AND METHODS

hESC Cultures

The H1 and H9 hESC lines were obtained from WiCell Research Institute, Inc. (Madison, WI, <http://www.wicell.org>) at passage (p) 22 or p23 [1]. We propagated hESCs on pMEFs and hMSCs as previously described [7, 16]. Undifferentiated H1 hESCs from p35–80, and H9 cells of p29–34 with a normal karyotype were used in this study. Approximately once every 3 months, hESCs were tested for karyotyping and the ability to form teratomas in severe combined immunodeficiency (SCID)–beige mice [7, 16]. hESCs transduced with a lentiviral vector expressing green fluorescent protein (GFP) [17] were used for easy determination of cell fate in cocultures in a few experiments.

Human Feeder Cells and Coculture with hESCs

hMSCs were cultured as previously described [7]. Several other types of human fibroblasts were also used. These included ccd-1087sk cells (normal breast skin, CRL-2104), Hs27 (newborn foreskin, CRL 1634), BJ (newborn foreskin, CRL-2522), and WI-38 (fetal lung, CCL-75), all purchased from the American Type Culture Collection (Manassas, VA, <http://www.atcc.org>). These human fibroblast cells were cultured as instructed. The immortalized 1087sk cells, designated as Human Adult Fibroblast, immortalized (HAFi), were also used [16]. To use as feeders, human fibroblast cells were irradiated (50 Gy) and plated at a density of approximately 2×10^4 cells per cm^2 . Stock ESCs ($\sim 10^4$ cells/ cm^2) from cocultures on pMEFs were seeded on irradiated human feeder cells at low density (to reduce the carryover of pMEFs from the previous coculture). The culture medium was changed daily. After 3–7 days, the cocultures were either passaged along after a 1:3 split or stained for AP activity. All the feeder cells were negative for AP, whereas undifferentiated hESC colonies stains densely for AP.

Testing Soluble Factors in CM and Its Fractionation

CM was collected from both pMEFs and human feeder cells [16]. The CM from supportive feeder cells such as hMSCs, 1087sk, HAFi, and pMEFs was used to replace the basal hESC medium for hESCs cultured on nonsupportive Hs27 feeder cells. The culture medium was changed daily. For CM fractionation experiments, CM was passed through the filtration unit containing the cut-off membrane of approximately 30-kDa molecule weight (Centricon or Amicon Ultra; Millipore, Bedford, MA, <http://www.millipore.com>). hESCs were cultured on Hs27 cells with either <30 -kDa fraction in the flow through, or the ≥ 30 -kDa concentrated frac-

tion after dilution or the two fractions were mixed back (reconstituted), along with the basal ESC medium and unfractionated CM. The numbers of AP⁺ compact colonies were counted to quantify the biological activity of CM and its fractions.

Using Soluble Wnt3a and Wnt Antagonists

Recombinant Wnt3a and two types of soluble Wnt antagonist proteins were purchased from R&D Systems (Minneapolis, <http://www.rndsystems.com>). The sFRP2 blocks the binding of Wnt to its cell-surface receptor Frizzled. Dickkopf-1 (Dkk-1) binds the LRP-5 or LRP-6 that is a coreceptor crucial for Wnt signaling and thus blocks the Wnt signaling in target cells. In the feeder-cell coculture system, hESCs were first incubated with 250 ng/ml of sFRP2 or Dkk-1 for 1 hour and then seeded on the HAFi feeder cells. The sFRP2 or Dkk-1 was continuously present after each medium change. Cells were passed onto fresh feeders every 3–5 days (up to a total of 26 days of culture) to determine the effect of blocking Wnt signaling in terms of the ability for hESCs to form AP⁺ compact colonies. The resulting hESCs were further analyzed by the expression of the SSEA-4 marker by flow cytometry [7]. The two types of Wnt antagonists were selected based on their best potency assayed by R&D Systems and previous studies [18–20]. We further confirmed the biological activities of the Wnt3a and Dkk-1 proteins using human 293T cells as well as hESCs.

The Feeder-Free Culture of hESCs Using Matrigel and pMEF-CM

We often used the feeder-free culture system described previously with modifications [3], to reduce the numbers of carryover pMEFs from the previous coculture or to assess the biological activity in CM. Matrigel (BD Biosciences, Bedford, MA, <http://www.bdbiosciences.com>) was diluted at 1:30 (instead of 1:20) with the knockout Dulbecco's modified Eagle's medium. After coating for ≥ 45 minutes, the coated plates were washed twice before use. hESCs were cultured on Matrigel with pMEF-CM, which provides soluble factors required for cell survival/proliferation and maintenance of undifferentiated hESCs.

For cell-culture assays of growth factor-dependence, we harvested hESCs (with minimal numbers of pMEFs) after one passage on Matrigel with pMEF-CM. After harvesting by 0.05% trypsin/0.5 mM EDTA, hESCs in small clumps (~10–20 cells) were seeded on Matrigel-coated 24-well plates (15 \times 10³ cells per well) with pMEF-CM or the plain hESC medium, in the presence or absence of Wnt3a (100 ng/ml unless otherwise indicated) or sFRP2 or Dkk-1 (250 ng/ml). The culture medium was changed daily. At day 4–5, when the largest colonies in the positive control reached confluency, the cells in each group were harvested, counted on a hemocytometer using 0.4% trypan blue, and passed as equal fractions (1:3 split) or fixed numbers onto the Matrigel-based culture again. Once confluent, the same process was repeated at day 8–9. A fraction of harvested cells were also

seeded on pMEF feeder cells to assess numbers of hESCs capable of subsequently forming colonies. The undifferentiated hESC colonies formed on pMEFs were counted after AP staining. The AP⁺ colony-forming units (AP⁺CFUs) are used to quantify numbers of undifferentiated hESCs in the test population. Therefore, the numbers of cells recovered and underlying AP⁺CFUs are two measurable parameters. The relative frequency (RF) of AP⁺CFUs, calculated after dividing the number of AP⁺CFUs by the number of cells used for the AP⁺CFU assay, is used to estimate the richness of undifferentiated hESCs in the population. The RF in the pMEM-CM control group (optimal growth) is defined as 1.00 and used to normalize the RF of other samples in the same experiment. Similarly, the effects of the addition of bone-morphogenetic protein 4 (BMP4, 10 ng/ml; R&D Systems) or retinoic acid (RA, 10⁻⁶ M; Sigma, St. Louis, <http://www.sigmaaldrich.com>) to pMEF-CM were measured.

Quantitative Reverse Transcription–Coupled Polymerase Chain Reaction

Real-time quantitative reverse transcription–coupled polymerase chain reaction (qRT-PCR) was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, <http://www.appliedbiosystems.com>). Total RNA (0.5 μ g) from each sample was used for first-strand cDNA synthesis by SuperscriptTM First Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad, CA, <http://www.invitrogen.com>). The cDNA in each sample was used as PCR templates to detect the expression of *Oct3/4*, Brachyury, and β -actin genes. At least four duplicates were included in each PCR reaction lasting for 45 cycles. The primers and probe sequences for the human *Oct 3/4* (POU5F1) are: 5'-ACTGCAGCAGATCAGCCACA-3' (hOct4 For), 5'-TGCGCCGGTTACAGAAC-3' (hOct4 Rev), and 5'-CTTGGGCTCGAGAAGGATGTGGTCC-3' (hOct4 probe). Primers and probes for Brachyury (Hs00610080_m1) and β -actin (Hs99999903_m1) were purchased from Applied Biosystems, Inc. PCR data were analyzed using the $\Delta\Delta C_T$ method to obtain the relative expression levels [21], using that of β -actin as an endogenous control in each sample.

A Luciferase Reporter Assay to Measure Wnt/ β -Catenin–Mediated Transcriptional Activation

The improved superTOPflash (TOP) luciferase reporter plasmid and its negative control, superFOPflash (FOP) plasmid, were provided by Dr. Randall Moon [22]. The improved reporter system is based on the TA-luc plasmid (BD Biosciences Clontech, Mountain View, CA, <http://www.clontech.com/clontech>) with reduced basal transcription activity. Eight copies of the cognate (in TOP) of defective (in FOP) TCF transcriptional factor binding site were inserted upstream the TATA transcription initiation box. To normalize variations in the assay process, an additional plasmid EF.lacZ was included as an internal control. Undifferentiated hESCs were first

cultured on Matrigel with pMEF-CM for one passage to reduce the carryover pMEFs. For transfection, hESCs were plated on Matrigel-coated 24-well plates 1–2 days before transfection. Six-tenths μg TOP (or FOP) and 0.2 μg EF.lacZ plasmids were mixed with lipofectamine 2000 (Invitrogen) before adding to hESCs cultured on Matrigel. The transfection mixture (0.1 ml) was added to 0.5 ml culture medium (pMEF-CM or otherwise indicated). After overnight transfection, the cells were replenished with fresh medium. After an additional 24 hours, transfected cells were lysed with Glo lysis buffer (Promega, Madison, WI, <http://www.promega.com>). The luciferase activity was measured in terms of relative light units on a TopCount NXT™ (Microplate Scintillation and Luminescence counter; Packard/PerkinElmer, Wellesley, MA, <http://www.perkinelmer.com>). The β -galactosidase activity (from EF.LacZ) was estimated with the β -galactosidase enzyme assay system (Promega). The assay was done in duplicates, and the luminescence values were normalized with the β -galactosidase readout. After normalization in each sample, the mean and SD ($n = 2$) of the luciferase activity from the TOP or FOP reporter was calculated.

Cell Proliferation and Apoptosis Assays

hESCs were cultured on Matrigel in six-well plates in the presence of pMEF-CM (a positive control) or the basal ESC medium \pm 100 ng/ml Wnt3a for 2 days with a media change on day 1. On day 2, one set of wells was pulsed with 10 μM BrdU using the BrdU kit (BD Biosciences) for 30 minutes. Cells were washed free of BrdU and harvested. Cells incorporating BrdU (actively cycling cells) were determined by an anti-BrdU antibody (supplied with the kit) on a FACSCalibur™ (BD Biosciences) using the manufacturer's protocol. To assess nonspecific staining by the anti-BrdU antibody, BrdU addition was omitted in a control group that was otherwise treated the same way. To determine apoptotic cells, a second set of wells was harvested at day 2 and stained with Annexin-V (fluorescein isothiocyanate [FITC]–conjugated). Cells were also stained with 7 AAD without permeabilization, to determine percentages of early apoptotic (Annexin⁺ / 7 AAD[–]) and late apoptotic (Annexin⁺ / 7 AAD⁺) cells.

Statistical Analysis

The Microsoft Excel (version X for Macintosh; Microsoft, Redmond, WA, <http://www.microsoft.com>) software is used for data management (calculating mean and SD), histogram plots, and Student's *t*-tests. If pooled samples from several similar experiments pass the normal distribution test, then the *t*-test is used for the null hypothesis between any two groups based on unpaired, unequal variance and two-sided model. When the number of replicates (n) is small (<12), the nonparametric Mann-Whitney test (also known as Wilcoxon two-sample test) is performed. The latter test is run unsupervised by the SAS 8.2 package (SAS Institute, Inc., Cary, NC, <http://www.sas.com>). It is considered to be significant if *p* value (null hypothesis) is $< .05$.

RESULTS

Establishing a Panel of Feeder Cells with a Different Ability to Support hESCs

To identify factors made by supportive feeder cells like hMSCs, we compared the ability of fibroblastic hMSCs with other postnatal human fibroblasts such as ccd-1087sk (1087sk, from breast skin) and Hs27 (newborn foreskin) fibroblasts. The latter two displayed morphology and phenotypes similar to hMSCs but lacked the hMSCs' adipogenic, osteogenic, or chondrogenic potential [23]. The H1 hESCs were seeded on various irradiated feeders as previously described for the coculture with hMSCs [7]. Because hESCs proliferate and differentiate much slower than mESCs, we examined the fate of hESCs cultured on different feeder cells for several passages by measuring their ability to generate (more) undifferentiated colonies (self-renewing proliferation) in each subsequent passage.

We observed that 1087sk cells as well as hMSCs can support the growth of undifferentiated hESCs as determined by the formation of AP⁺ compact colonies for three consecutive passages (Figs. 1A, 1B). Unlike hMSCs and 1087sk fibroblasts, Hs27 cells could not support the formation of AP⁺ compact colonies (Fig. 1C), an assay used throughout this study to measure the hESC self-renewal after each passage. With the same assay, we found that newborn foreskin BJ and fetal lung WI-38 fibroblasts failed to support hESC growth after two passages (Fig. 1D), consistent with a recent report [10].

To obtain a sustainable source of the human feeder cells, we attempted to immortalize both hMSCs and 1087sk human fibroblasts by introducing the gene encoding hTERT, the catalytic subunit of the human telomerase. The transduced MSCs acquired a much faster growth rate, became irradiation-sensitive, and were therefore discontinued. The transduced ccd-1087sk cells had the same growth rate even after 42 passages (weekly), whereas the parental cells senesced after 32 passages [16]. We designated the resulting feeder cells as HAFi. HAFi cells can also support the growth of hESCs at least as well as the parental 1087sk cells and pMEFs. The hESCs grown on the HAFi feeders had a characteristic morphology (Figs. 1A, 1B), expressed undifferentiated markers such as SSEA-4, and formed teratomas [16]. Thus, we established a panel of supportive (MSCs, 1087sk, and HAFi) and non- (or poorly) supportive (Hs27, BJ, and WI-38) feeder cells for a unique comparative study.

The Supportive Feeder Cells Preferentially Produce a Soluble Antidifferentiation Factor

To determine whether the lack of AP⁺ hESC colonies cultured on nonsupportive cells is due to cell death or differentiation (hESCs are viable but unable to form AP⁺ compact colonies), we used hESCs that were stably transduced with a lentiviral vector expressing the GFP reporter gene. GFP⁺ compact colonies of hESCs

were observed 4–7 days after plating onto supportive feeder cells (HAFi or pMEFs) and stained positive for AP (not shown). When Hs27 (nonsupportive) cells were used, GFP⁺ cells were present (Fig. 2Aa), but fewer AP⁺ compact colonies were formed and GFP⁺ cells appeared to be differentiated (Fig. 2Ab).

To determine if the antidifferentiation activity of supportive feeder cells is cell-associated or secreted, we tested the effect of CM from supportive MSCs or 1087sk feeder cells on the growth of hESCs cultured on Hs27 feeder cells unable to support hESC growth for two or more passages (Figs. 2Ab, 2B). This experiment may also help to distinguish two possibilities: nonsupportive Hs27 cells are either lacking an antidifferentiation factor (the result should be positive) or producing a dominant differentiation-inducing factor (the result should be negative). We observed that the MSC-CM or 1087sk-CM was sufficient to convert Hs27 feeder cells to support hESCs (Fig. 2B), suggesting that the soluble factor(s) responsible for maintaining undifferentiated hESCs (or antidifferentiation) was present in MSC-CM and 1087sk-CM. Similar results were observed with HAFi-CM and pMEF-CM (Figs. 2Ac–2Af, 2C). The reciprocal experiment gave similar results when the Hs27-CM was applied to supportive feeder cells

(not shown). These data suggest that supportive feeder cells produce an extra soluble factor that blocks differentiation of hESCs, in addition to survival/proliferation factors produced by both types of feeders. Initial fractionation of CM indicated that the supportive activity in both HAFi-CM and pMEF-CM resides in the ≥ 30 -kDa fraction (Fig. 2C).

Wnt is Not the Antidifferentiation Factor Secreted by the Supportive Feeder Cells

Comparative gene microarray and proteomic analyses are ongoing to systemically identify genes that are differentially expressed by supportive (vs. nonsupportive) feeder cells. Because the activity in the supportive CM was retained in the fraction of ≥ 30 kDa,

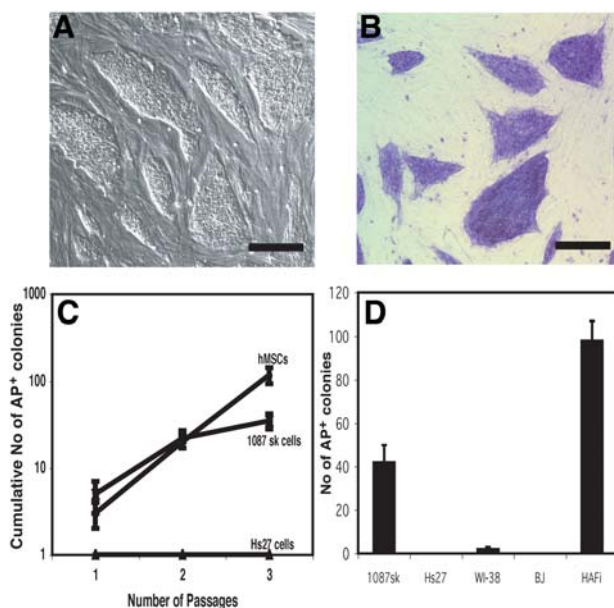


Figure 1. Identification of a panel of supportive and nonsupportive human feeder cells. **(A):** Undifferentiated H1 hESCs formed compact colonies after the third passage of culture on 1087sk breast skin fibroblasts. **(B):** The resulting H1 hESC colonies stained positive for AP⁺ as those cultured on marrow stromal cells or primary mouse embryonic fibroblasts (all feeder cells were AP⁺). Scale bars = 100 μ m. **(C):** Cumulative numbers (No) of AP⁺ compact (undifferentiated) colonies over three passages on three types of human feeder cells. The mean and SD ($n = 3$) are plotted in log scale. **(D):** The parental 1087sk and the immortalized (HAFi) human cells support growth of undifferentiated hESCs after two passages, whereas Hs27, WI-38, and BJ fibroblasts do not. Abbreviations: AP, alkaline phosphatase; HAFi, Human Adult Fibroblast, immortalized; hESC, human embryonic stem cell.

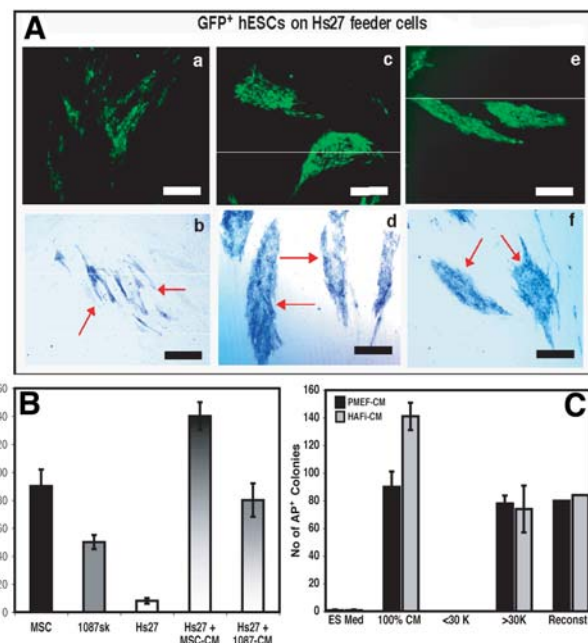


Figure 2. The antidifferentiation activity is present in the CM from supportive feeder cells. **(A):** Fate of H1 hESCs (GFP⁺) cultured on nonsupportive Hs27 feeder cells in the presence of basal ESC medium (a, b), pMEF-CM (c, d), or HAFi-CM (e, f) at day 5. GFP⁺ hESCs were traced by GFP fluorescence (upper panels) followed by AP staining (low panels). The presence of GFP⁺ hESCs and loss of AP⁺ compact colonies on nonsupportive feeder cells (a, b) indicate that hESCs survived but differentiated. pMEF-CM (c, d) and HAFi-CM (e, f) restored the AP⁺ compact colony formation (quantified in C). Scale bar = 100 μ m. **(B):** MSC-CM and 1087sk-CM can also restore the ability to support undifferentiated H1 hESCs cultured on Hs27 nonsupportive feeder cells. The numbers (No) of AP⁺ compact colonies were enumerated after staining at day 5 ($n = 3$). **(C):** Similar activities of pMEF-CM and HAFi-CM on H1 hESCs cultured on Hs27 feeder cells. Fractionation experiments show that activity is retained in the fraction of approximately ≥ 30 kDa. Abbreviations: AP, alkaline phosphatase; CM, conditioned medium; GFP, green fluorescent protein; HAFi-CM, Human Adult Fibroblast, immortalized–conditioned medium; hESC, human embryonic stem cell; MSC, marrow stromal cell; MSC-CM, marrow stromal cell–conditioned medium; pMEF-CM, primary mouse embryonic fibroblast–conditioned medium.

we assessed the possibility that the pleiotropic secreted protein Wnt (~40 kDa) is the antidifferentiation factor secreted from the supportive feeder cells. The expression of genes encoding several forms of Wnt and its soluble antagonists was detected in both human supportive and nonsupportive feeder cell types (supplemental online Table 1) and in undifferentiated and differentiated hESCs (supplemental online Table 2). To directly test whether Wnt signaling is critical to self-renewing proliferation of hESCs, we used two soluble Wnt antagonists, secreted Frizzled-Related Protein 2 (sFRP2) and Dickkopf-1 (Dkk-1), which block the binding of Wnt to its receptors, Frizzled and coreceptors Low-Density Lipoprotein Receptor Related Protein (LRP)-5 or LRP-6, respectively. Both antagonists were used previously to block diverse functions of the Wnt signaling in vivo or in vitro [18–20]. hESCs were cultured on HAFi cells in the presence of sFRP2 or Dkk-1 for up to 26 days. The presence of either Wnt antagonist slightly reduced but did not abolish the ability of hESCs to form AP⁺ compact colonies in each subsequent passage (Fig. 3A). The treated hESCs retained the expression of undifferentiated markers such as SSEA-4 after the 26-day treatment (Fig. 3B). Similar results

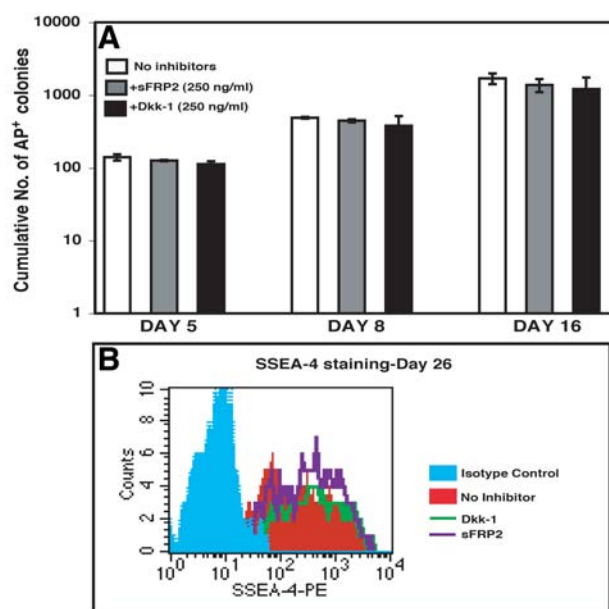


Figure 3. Blocking Wnt signaling does not abolish self-renewal of undifferentiated hESCs on supportive feeder cells. GFP⁺ H1 hESCs were cultured on HAFi cells in the presence or absence of Wnt antagonists *sFRP2* or *Dkk-1* (250 ng/ml). Continuous presence of Wnt antagonists did not abolish the AP⁺ colony-forming ability of hESCs as analyzed at days 5, 8, and 16 (the end of the third passage). (A): The cumulative numbers (No) of AP⁺ compact colonies after each passage are plotted as mean and SD ($n = 3$). (B): Flow cytometric analysis of H1 hESCs in coculture (identified as GFP⁺ cells) for the expression of SSEA-4 (a marker of undifferentiated hESCs) at day 26, after continuous treatment by the Wnt antagonists. Abbreviations: AP, alkaline phosphatase; GFP, green fluorescent protein; HAFi, Human Adult Fibroblast, immortalized; hESC, human embryonic stem cell; PE, phycoerythrin.

were observed when pMEFs were used as feeder cells (not shown). In addition, adding the purified Wnt3a protein (up to 100 ng/ml) to hESC cultures on nonsupportive Hs27 cells could not substitute the antidifferentiation activity present in the pMEF-CM or HAFi-CM in supporting the formation of AP⁺ colonies (not shown).

To block Wnt activities more effectively, we performed the blocking experiment with hESCs cultured on a feeder-free system using Matrigel supplemented with pMEF-CM [3]. Several days after seeding on Matrigel, flattened colonies were observed together with few differentiating cells outside colonies (Fig. 4Aa). The cells harvested from the Matrigel/pMEF-CM culture, however, can form compact AP⁺ colonies again after being seeded on pMEFs (Fig. 4Ab). Therefore, we decided to measure the level of undifferentiated hESCs present in the Matrigel/pMEF-CM culture based on the numbers of compact AP⁺ CFUs formed in the subsequent pMEF culture. The latter step allows us to count AP⁺ colonies more accurately and, more importantly, to measure the level of colony-forming, undifferentiated hESCs present in the feeder-free system. The feeder-free system allows us to measure precisely the total numbers of recovered hESCs and their correlation to AP⁺ CFUs. When hESCs were cultured on Matrigel (with pMEF-CM) in the presence of sFRP2 or Dkk-1 for 4 days, the total numbers of cells recovered were reduced by 86% and 80%, respectively, as compared with the control pMEF-CM alone (Fig. 4Ba). However, the numbers of AP⁺ CFUs were reduced only by 35% and 32%, respectively. Thus, the RF of AP⁺ CFUs, a quality index of undifferentiated hESCs, actually increased 4.48-fold and 3.42-fold after treatment of sFRP2 and Dkk-1, respectively.

At day 4, equal fractions of the harvested hESCs from the first passage were treated under the same condition after a 1:3 split. After an additional 4 days, sFRP2 reduced cell numbers by 58% compared with the pMEF-CM control, whereas Dkk-1 did not significantly reduce cell numbers (Fig. 4Bb). However, the RF of AP⁺ CFUs in the presence of sFRP2 or Dkk-1 was similar to the pMEF-CM control. Similar results were observed at day 13 (not shown). Representative undifferentiated hESC colonies (AP⁺ CFUs) grown with or without Wnt antagonists for 13 days are shown in Figure 4A (c, d). Thus, blocking the Wnt signaling significantly reduced the level of hESC survival/proliferation under the feeder-free condition, but the majority of AP⁺ CFUs remained. The RF of AP⁺ CFUs actually increased after sFRP2 or Dkk-1 treatment, indicating undifferentiated hESCs were likely enriched (Fig. 4B).

Purified Wnt3a Proteins Are Unable to Substitute for Soluble Factors in pMEF-CM

We next added recombinant Wnt3a proteins to the basal hESC medium to examine whether it could replace pMEF-CM in the feeder-free Matrigel culture. In the absence of pMEF-CM, only small clusters of hESCs remained after 4–5 days with the hESC medium alone (Fig. 4D). The addition of Wnt3a (up to 100 ng/ml)

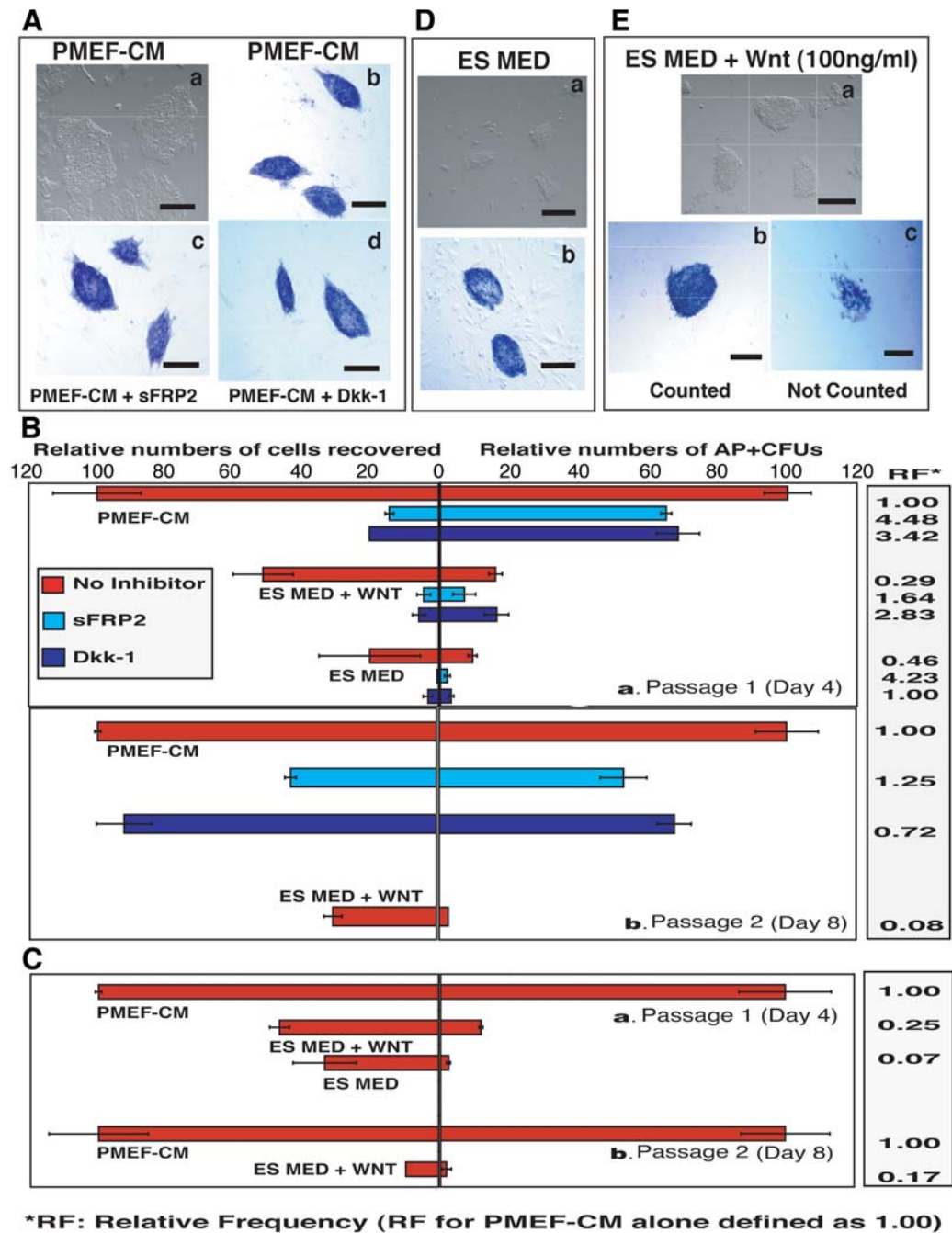


Figure 4. Effects of adding Wnt antagonists or Wnt3a on H1 and H9 hESCs cultured in the Matrigel feeder-free system. **(A):** H1 hESCs proliferated and formed flattened colonies on Matrigel at day 4 with pMEF-CM (a). The dissociated cells can form AP⁺ compact colonies again once plated on pMEFs (b). Adding *sFRP2* or *Dkk-1* to pMEF-CM did not abolish the ability to form AP⁺CFUs (c, d). **(B):** Relative numbers of harvested H1 hESCs (left) and resulting AP⁺CFUs (right) after first and second passages under various culture conditions. Four days after seeding with equal numbers of hESCs (a, passage 1), total recovered cells in each group ($n = 3$) were counted and plotted (left panels). One third of the harvested cells were seeded again under the same condition (for passage 2), and one third were seeded on pMEFs to assay for AP⁺CFUs (right panels). For a simple comparison, both parameters were normalized by the mean value of the pMEF-CM control group (defined as 100%). The RF of AP⁺CFUs of each cell population was calculated and normalized by the pMEF-CM group (defined as 1.00, far right). In the absence of pMEF-CM, Wnt3a (100 ng/ml) was added into the ES MED. After passage 2, too few cells survived at passage 2 for the ES MED or the +Wnt antagonist groups. **(C):** Similar assays with H9 hESCs. **(D, E):** Morphology of hESCs with the ESC medium alone (ES MED) or + Wnt3a at day 4 (a). Representative morphology of AP⁺CFUs (formed subsequently on pMEFs) is also shown in (b), and a colony from differentiating hESCs after Wnt3a treatment is shown in (c). Scale bar = 100 μ m. Abbreviations: AP, alkaline phosphatase; AP⁺CFU, alkaline phosphatase-positive colony-forming unit; ES MED, embryonic stem cell medium; ESC, embryonic stem cell; GFP, green fluorescent protein; HAFi-CM, Human Adult Fibroblast, immortalized-conditioned medium; hESC, human embryonic stem cell; pMEF, primary mouse embryonic fibroblast; pMEF-CM, primary mouse embryonic fibroblast-conditioned medium; RF, relative frequency.

into the basal medium (containing bFGF and insulin) provided a cell survival/proliferation advantage in a dose-dependent manner (not shown). After Wnt3a treatment of 4–5 days, surviving hESCs formed fewer, but more compact, colonies as compared with the culture with pMEF-CM (Fig. 4E), similar to the previous report [15]. We did not further examine the role of Wnt on the morphological changes, although it has been reported that Wnt also affects cell adhesion and planar polarity [13]. Instead, we harvested the surviving hESCs in the presence or absence of Wnt3a after the first passage and assayed for AP⁺CFUs. As compared with the pMEF-CM control (taken as 100%), 52% and 20% of hESCs could be recovered when Wnt3a was present or absent in the ESC medium (Fig. 4B, left panel, red bars). The Wnt3a stimulatory effect was largely blocked by sFRP2 or Dkk-1 (blue and purple bars), and the cell numbers reduced to the minimal levels both in the presence or absence of exogenous Wnt3a. Collectively, the data indicate that sFRP2 and Dkk-1 blocked the stimulatory effect of survival/proliferation by autocrine Wnt signaling in hESCs (more obvious when the basal medium alone was used) as well as paracrine Wnt signaling (when pMEF-CM or exogenous Wnt was used). Despite the significant increase in total cell numbers, adding Wnt3a neither increased AP⁺CFUs proportionally nor substituted for pMEF-CM in maintaining AP⁺CFUs. When pMEF-CM was absent, the RF of AP⁺CFUs was actually decreased to 0.29 when Wnt3a was present (Fig. 4B).

We further examined the Wnt3a effects beyond the first passage (4–5 days), as we did with pMEF-CM (Fig. 4Bb). Too few cells survived in the group without Wnt3a after an additional 4 days, preventing further analysis. In the presence of Wnt3a, the numbers of recovered cells and AP⁺CFUs were reduced further to 31% (accumulatively 16%) and 2.6% (accumulatively 0.4%), respectively, as compared with the pMEF-CM control group (100%). The RF of AP⁺CFUs in the Wnt-treated cells reduced to 0.08 at passage 2 (Fig. 4Bb), 12.5-fold lower than the pMEF-CM control.

In addition to the H1 hESCs we used extensively, we also examined the Wnt3a effects on a different hESC line, H9 (Fig. 4C). Although the growth properties of H9 are not identical to H1, we also observed that Wnt3a modestly enhanced H9 hESC survival/proliferation and colony formation at day 4. However, at the end of the second passage (day 8), $\leq 5\%$ of colonies formed if Wnt3a was used to replace pMEF-CM. Similar to H1, the RF of AP⁺CFUs in the Wnt-treated H9 cells was reduced to 0.17 at passage 2 (Fig. 4Cb), sixfold lower than the pMEF-CM control.

To examine molecular changes following the Wnt3a treatment, we assayed the expression of marker genes that are preferentially expressed in either undifferentiated or differentiated ESCs. As a control, we also used hESCs after treatment by adding two known differentiation-inducing agents, BMP4 or RA, into pMEF-CM. Consistent with a previous report [24], hESCs treated with BMP4 quickly differentiated (Fig. 5A). Cells treated with RA

also differentiated although at a much slower rate [7, 11]. By day 9, the harvested cells with BMP4 or RA treatment contained few AP⁺CFUs (Fig. 5B). Consistent with a previous report [24], the level of *Oct-4* mRNA in the BMP4-treated cells was reduced by more than fivefold (Fig. 5C). The RA-treated sample, which lost the AP⁺CFU biological activity at day 9, showed marginal reduction in *Oct-4* mRNA (Fig. 5C). The observation in the differentiated hESCs induced by RA is consistent with the notion that *Oct-4* expression is not sufficient and that other signals in the “stem cell orchestra” are also required to maintain undifferentiated ESCs [25, 26]. In Wnt3a-treated cells (when pMEF-CM was absent),

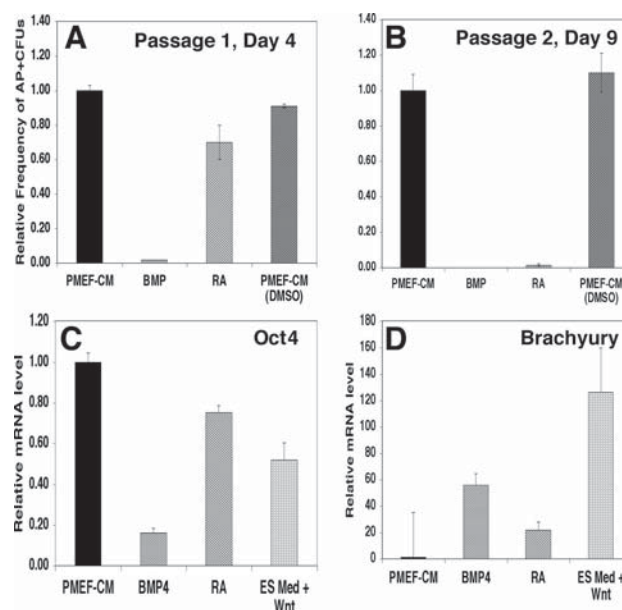


Figure 5. Analysis of differentiating hESCs induced by various culture conditions. **(A, B):** Colony-forming ability of H1 hESCs after treatment with BMP4 (10 ng/ml), RA (10^{-6} M), or DMSO (solvent control for RA) at day 4 and 9, respectively. The mean and SD ($n = 3$) of relative frequencies of AP⁺CFUs are plotted accordingly after being normalized by the mean value of the pMEF-CM group (defined as 1.00). **(C, D):** Quantitative reverse transcription–polymerase chain reaction analyses to measure mRNA levels of the *Oct-4* gene **(C)** and the *Brachyury* gene associated with mesoderm and endoderm differentiation **(D)**. In addition to the BMP4- and RA-treated samples harvested at day 9, the Wnt3a-treated sample (ES MED + Wnt) at day 8 (Fig. 4B) was also included. After reverse transcription, cDNA from each sample was amplified for 45 cycles with primers specific for *Oct-4* or *Brachyury* gene and the β -actin gene used an internal control. The relative level of *Oct-4* or *Brachyury* mRNA was first normalized by that of β -actin in each sample ($n = 4$). For a simple comparison, the mean value of relative mRNA level in the pMEF-CM control group is defined as 1.00, either for *Oct-4* **(C)** or *Brachyury* **(D)**. Note that *Oct-4* mRNA is expressed at a high level **(C)** and *Brachyury* is barely detectable **(D)** in the pMEF-CM control group. Abbreviations: AP⁺CFU, alkaline phosphatase–positive colony-forming unit; BMP-4, bone-morphogenetic protein 4; DMSO, dimethyl sulfoxide; ES MED, embryonic stem cell medium; hESC, human embryonic stem cell; pMEF-CM, primary mouse embryonic fibroblast–conditioned medium; RA, retinoic acid.

the level of *Oct-4* mRNA was reduced by 50% (Fig. 5C). We next examined the expression of genes such as Brachyury, the expression of which is associated with lineage commitment/differentiation (Fig. 5D). The expression of the Brachyury gene is transiently activated at the onset of mesoderm formation and also possibly the endoderm lineage in the mouse system, and is regulated by Wnt3a [27–29]. Indeed, the expression of the Brachyury gene was significantly increased in the Wnt3a-treated cells as well as in differentiated cells induced by BMP4 and RA (Fig. 5D). Based on both AP⁺CFU formation assays and RT-PCR analysis, we conclude that Wnt3a accelerates hESC differentiation (and cell survival/proliferation) when an antidifferentiation factor is absent.

Direct Examination of Wnt Downstream Effector Molecules Inside hESCs

mRNAs of multiple genes involved in the Wnt signaling pathway were detected in both undifferentiated and differentiated H1 hESCs in our microarray analyses (supplemental online Table 2), similar to data reported for various hESC lines. These include several forms of Wnt antagonist genes such as *sFRP1*, *sFRP3*, *Dkk-1*, *Dkk-2*, *Dkk-3*, and *WIF-1* as well as *Wnt5a*. Genes encoding known downstream molecules of the Wnt canonical signaling pathway such as GSK-3 β , β -catenin, and the family members of TCF/lymphoid enhancer-binding factor (LEF) transcriptional factors are also expressed in both undifferentiated and differentiated hESCs (supplemental online Table 2). In the canonical Wnt signaling pathway, a key event is that tightly regulated β -catenin enters nucleus and transiently activates TCF, which in turn binds its cognate DNA sequence in the regulatory region of dozens of Wnt/ β -catenin target genes. To directly measure the Wnt/ β -catenin canonical signaling in hESCs, we used an improved version of the TOPflash (TOP) reporter system [22], in which the luciferase reporter gene activity is controlled by the β -catenin/TCF transcriptional activation. After transfection, the luciferase activity in undifferentiated or differentiating hESCs is measured, reflecting the internal status of the Wnt/ β -catenin activation.

We detected a low level of β -catenin/TCF-mediated activation in undifferentiated H1 and H9 hESCs maintained by pMEF-CM (Fig. 6). Adding Wnt3a into pMEF-CM significantly increased the TOP activity, consistent with previous data that hESCs are responsive to Wnt stimulation. In differentiating hESCs after BMP4 or RA induction, or Wnt3a alone (without pMEF-CM), the TOP activity was also significantly increased (Fig. 6A). The late-passage H1 hESCs (p79–85) showed a higher level of basal TOP activity (Fig. 6B) than the earlier-passage H1 (p41–45; Fig. 6A) or H9 cells (p29–34; Fig. 6C). In addition, both basal (if any) and Wnt3a stimulatory TOP activities in H1 (Fig. 6B) and H9 (Fig. 6C) hESCs can be fully blocked by the Dkk-1 inhibitor. Taken together with the AP⁺CFU and RT-PCR analyses (Fig. 5), our data indicate that the β -catenin/TCF-mediated activation in undifferentiated hESCs is minimal under the standard culture

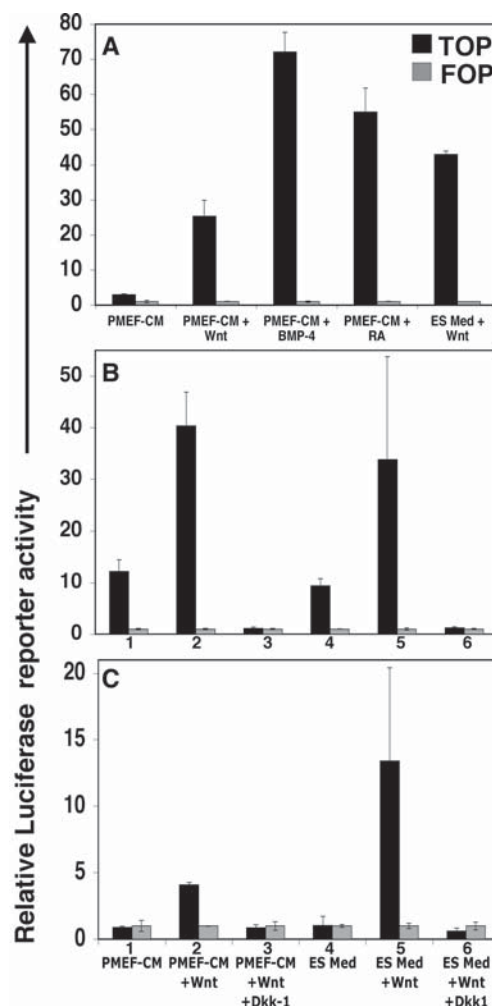


Figure 6. The Wnt/ β -catenin-mediated transcriptional activation is minimal in H1 and H9 hESCs and significantly elevated after differentiation induction. The transcriptional activation activity of β -catenin in hESCs is measured after transfection of a luciferase reporter (TOP) containing the TCF binding sites. A control (FOP) containing defective TCF sites was also used in parallel assays. Enriched hESCs were plated on Matrigel and cultured in the optimal medium (pMEF-CM). After transfection, cells were cultured in either pMEF-CM or the plain ESC medium (ES Med) with various factors for 2 days. The mean and SD ($n = 2$) of the luciferase activity from the TOP or FOP reporter were calculated. For a simple comparison, the mean value of the FOP control in the pMEF-CM control group is defined as 1.00, and relative reporter activity is plotted for each condition. Note that the FOP activity is minimal and similar in all the samples. (A): The TOP and FOP activities in H1 hESCs (p41–45) after 2-day treatment with Wnt3a (100 ng/ml), BMP-4 (10 ng/ml), and RA (10^{-6} M) as in Figure 5. (B): The TOP and FOP activities in late-passage H1 hESCs (p79–85) after treatment by Wnt3a and its soluble antagonist *Dkk-1* (250 ng/ml). (C): The TOP and FOP activities in H9 hESCs (p29–34). H1 (B) and H9 (C) hESCs were treated similarly as indicated by sample numbers below the bar graphs: #1–3 in the pMEF-CM, and #4–6 in ES Med, #2 and #4 with Wnt3a alone, #3 and #6 with Wnt3a and Dkk-1, as compared with no addition (#1 and #4). Abbreviations: BMP-4, bone-morphogenetic protein 4; ES Med, embryonic stem cell medium; FOP, superFOPflash; hESC, human embryonic stem cell; pMEF-CM, primary mouse embryonic fibroblast-conditioned medium; RA, retinoic acid; TCF, transcriptional factor; TOP, superTOPflash.

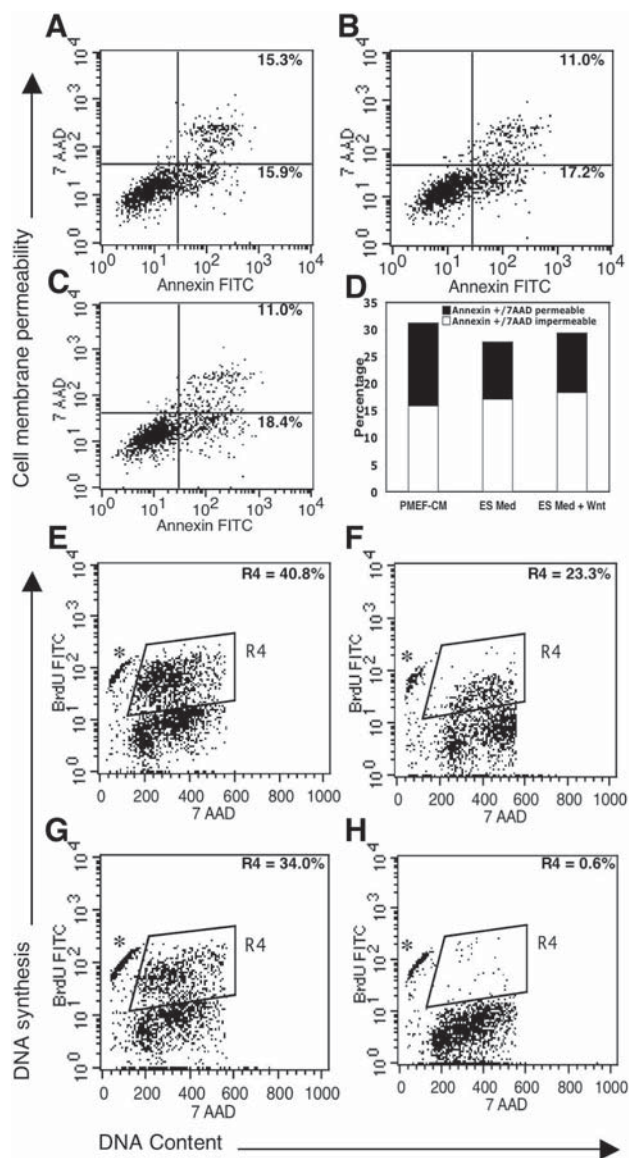


Figure 7. Wnt activation stimulates hESC proliferation and has little effect on apoptosis. (A–D): Flow cytometric analysis of apoptotic cells using Annexin-V surface staining and 7 AAD staining (for DNA in permeable dying cells). Shown are dot plots of the FITC-conjugated Annexin-V (Annexin FITC) versus 7 AAD staining for hESCs cultured in pMEF-CM (A), the basal ESC medium (B), and ESC medium + Wnt3a (C). The percentages of dying cells in the early stage (Annexin⁺/7 AAD nonpermeable) and late stage (Annexin⁺/7 AAD permeable) are indicated in the lower and upper right quadrants, respectively. The relative percentages are summarized in (D). (E–H): Wnt3a activation stimulates hESC proliferation. The percentages of hESCs incorporating BrdU (actively cycling) versus DNA content (stained with 7AAD after permeation step) are shown when cultured with pMEF-CM (E), the basal ESC medium (F), or ESC medium + Wnt3a (G). (H): Cells cultured with pMEF-CM and treated as in (E), but BrdU pulse was omitted to assess nonspecific staining by the anti-BrdU antibody. Apoptotic cells that are stained falsely positive by the anti-BrdU antibody are indicated by an asterisk. Abbreviations: ESC, embryonic stem cell; FITC, fluorescein isothiocyanate; hESC, human embryonic stem cell; pMEF-CM, primary mouse embryonic fibroblast-conditioned medium.

condition using pMEF-CM. However, it is significantly elevated during initial hESC differentiation induced by several different methods. Thus, the Wnt/ β -catenin activation is not indicative of the undifferentiated (and pluripotent) state of hESCs.

Wnt3a Directly Stimulates hESC Proliferation

In the absence of pMEF-CM, we observed more hESCs when Wnt3a was present (ESC medium + Wnt) than when Wnt was absent for 5 days (Fig. 4B). This could be due to either a decrease in apoptosis or an increase in hESC proliferation, or both. To distinguish these two possibilities, we performed Annexin-V staining to detect apoptotic cells and the BrdU incorporation to detect DNA synthesizing/proliferating cells under above culture conditions. The results showed that the Wnt addition did not significantly affect the rate of apoptosis, since percentages of apoptotic cells were similar (Figs. 7A–7D). However, adding Wnt3a increased the percentage of, and level in, cells incorporating BrdU (Fig. 7G), as compared with the basal ESC medium group (Fig. 7F). Therefore, Wnt can directly enhance hESC cycling and proliferation with little effect on preventing apoptosis.

DISCUSSION

To identify the feeder cell-derived factors required for maintaining hESCs in an undifferentiated state, we performed a comparative study using both supportive and nonsupportive feeder cells. This study provides strong evidence that, whereas both feeder types can provide survival/proliferation signals, supportive feeder cells preferentially produce an antidifferentiation soluble factor that is essential to block spontaneous differentiation of hESCs. The paradigm that an antidifferentiation factor is required to block hESC spontaneous differentiation and to achieve self-renewing proliferation appears similar to that in mESCs, but the antidifferentiation soluble factor for hESCs is not LIF (or a related gp130-dependent cytokine), which is important for mESC self-renewal [5, 15]. The nature of such an antidifferentiation factor produced preferentially by human supportive feeder cells remains unknown, although recent data suggest BMP antagonists or activin A may be the major activity produced by pMEFs [30, 31]. In this paper, we also present several lines of evidence demonstrating that Wnt is not sufficient to maintain undifferentiated hESC colonies. Wnt activation can stimulate hESC proliferation but does not suffice to maintain or expand undifferentiated (and pluripotent) H1 and H9 hESCs for more than one passage. The latter is contradictory to a main conclusion of a recent paper, which also used the H1 hESC line [15]. In the previous study [15], the effects on hESCs (and mESCs) were examined after a 5-day treatment by Wnt3a and the synthetic chemical BIO, an inhibitor blocking GSK-3 β kinase activity and stimulating the β -catenin-mediated activation. It remains to be determined whether BIO will be able to expand self-renewing, undifferentiated hESCs using extended culture assays. Mechanistically, it is currently unclear

whether BIO also blocked other kinases at the concentration used [32] and/or whether β -catenin is the sole effector of the GSK-3 β blocking in hESCs. It is also important to sort out why the effect of BIO is different from LiCl, another GSK-3 β inhibitor which apparently induces differentiation ([15], data not shown). In general, we agree with Sato et al. [15] that hESCs are responsive to the Wnt stimulation. However, we believe that Wnt activation is not sufficient to maintain undifferentiated (and pluripotent) ESCs. The following discussion focuses on hESCs because we have not studied the effect of Wnt on mESCs in detail.

Our initial observations after Wnt3a treatment were actually very similar to those described in the previous report [15]: adding Wnt3a to the basal ESC medium improved cell survival/proliferation transiently and resulted in a few compact colonies expressing AP and Oct-4 (Fig. 4E, data not shown). However, subsequent analyses plus functional assays revealed a more complicated picture. We found that it is difficult to distinguish undifferentiated from differentiating hESCs at day 5 based on the reduction in the expression of AP, Oct-4 and additional markers, because hESCs proliferate and differentiate more slowly than mESCs [4]. Instead, we measured the self-renewing property of undifferentiated hESCs by their ability to form colonies of cells expressing the same undifferentiated markers after each successive passage. Specifically, we quantified the ability of a given cell population to form compact colonies of AP⁺ undifferentiated hESCs (AP⁺CFUs) that are high in Oct-4 expression. Similarly, colony-forming assays have also been used in recent studies with mESCs [25, 33] and in numerous historic studies with hematopoietic stem/progenitor cells and MSCs. We believe that a colony-forming assay over time, together with analysis of markers such as AP and Oct-4 associated with undifferentiated hESCs, is better for identifying undifferentiated hESCs with self-renewal ability.

Several conclusions can be drawn from our extended (and colony-forming) culture assays and molecular analyses. First, our data using Wnt antagonists suggest that Wnt is not the critical antidifferentiation factor secreted by the supportive feeder cells (Figs. 3, 4). Wnt antagonists effectively blocked Wnt3a-mediated stimulation of cell survival/proliferation and TOP reporter activities, but only marginally reduced the numbers of AP⁺CFUs when pMEF-CM or Wnt3a was present (Figs. 4B, 6). At this moment, we could not rule out the possibility that the Wnt antagonists may not block all forms of Wnt family members effectively, especially when hESCs are cultured in contact with supportive feeder cells (Fig. 3). However, it is unlikely that the antidifferentiation factor present in pMEF-CM and HAFi-CM is Wnt, especially those Wnt family members capable of activating the β -catenin-mediated TOP reporter activity.

Second, we found that Wnt, at least Wnt3a, which primarily acts through the canonical pathway, is insufficient to substitute for pMEF-CM or HAFi-CM to maintain undifferentiated hESCs. In the absence of pMEF-CM, adding Wnt3a transiently

enhanced cell survival/proliferation of hESCs cultured on Matrigel (Fig. 4B). However, the surviving cells gradually lost their ability to form undifferentiated colonies and showed signs of differentiation (Figs. 4, 5). The observed transient cell survival/proliferation advantage in the Wnt3a-treated group was mainly due to the fact that Wnt directly stimulates hESC proliferation (Fig. 7). This is consistent with numerous reports that Wnt signaling can provide a proliferative signal and that Wnt3a can increase the expression of genes such as *cyclin D* [12, 14, 15]. When the antidifferentiation factor (in pMEF-CM) was absent, however, Wnt accelerated cell proliferation as well as differentiation, because the relative frequency of undifferentiated hESCs after Wnt3a treatment was twofold lower than when only hESC basal medium was used (Fig. 4B).

Third, the Wnt/ β -catenin signaling pathway-mediated activation is low in the undifferentiated hESC population and significantly upregulated in differentiating hESCs induced by several different methods. This is consistent with unpublished observations by Dr. Hyun-Sook Park and colleagues in the Korean Institute of Radiological and Medical Sciences (personal communication). Using two other hESC lines, MizhES and SnuhES, they found that loss of pluripotency and differentiation of hESCs (by LiCl induction and several other methods) is accompanied by the Wnt/ β -catenin pathway activation. Therefore, the Wnt/ β -catenin activation is not associated with the undifferentiated state of hESCs.

It is unlikely that the discrepancy between Sato et al. [15] and our study and the Korean studies is due solely to differences of tissue-culture methods used or hESC lines used (the H1 cell line was used by Sato et al. and us). In fact, the short-term observations are very similar, and differences stem from the long-term outcomes and data interpretations. Similar to our observations with H1 and H9 cell lines, Drs. Ren-He Xu and James Thomson (WiCell Research Institute, Inc.) observed that adding Wnt3a is not sufficient to maintain or expand hESCs for more than one passage (personal communication). Recent data suggest that bFGF (with or without a BMP antagonist) is sufficient to maintain and expand undifferentiated hESCs for many passages [30, 34, 35]. Similar observations have been reported with TGF β and activin A [31, 36]. Our data and these studies collectively and strongly suggest that the external Wnt activation is neither essential nor sufficient to maintain undifferentiated hESCs, despite the fact that they are capable of response to Wnt/ β -catenin activation.

This paper also represents the first study to directly measure Wnt/ β -catenin-mediated activation in hESCs, although previous studies have also demonstrated that the activation is also low in undifferentiated mESCs, using similar TOP reporter assays [15, 37, 38]. It has been observed that the TOP activity is elevated at the onset of mESC differentiation, and that Wnt/ β -catenin signaling is critical to the neural differentiation from mESCs [38]. Although the exact functions of Wnt/ β -catenin signaling and its interplay with other regulators may differ in hESCs and mESCs,

our data suggest that there is an active mechanism to keep Wnt/ β -catenin activation in undifferentiated hESCs at a basal level. However, our current study could not rule out the possibility that noncanonical Wnt signaling pathways independent of β -catenin [39] also play a role in hESCs. Similarly, we could not rule out that β -catenin as a dual-function protein may be critical to other cellular functions in undifferentiated hESCs. Notably, β -catenin is not essential to either undifferentiated mESCs or hematopoietic stem cells in mice, though it is important to differentiation post mesoderm induction [40, 41].

Based on our results as well as previous studies, we favor a new model for the role of Wnt/ β -catenin signaling in hESCs. Wnt, either made endogenously or provided exogenously, acts on hESCs and enhances cell proliferation as on many other cell types. The enhancement of cell proliferation may lead to one of two possible outcomes, dependent on the presence or absence of other signals. In the presence of supportive feeder cells or CM (containing the antidifferentiation factor), endogenous or feeder-derived Wnt works together with other survival/proliferation factors, resulting in self-renewing proliferation of undifferentiated hESCs. In the absence of the antidifferentiation factor, however, excessive Wnt accelerates cell proliferation as well as differentiation (Fig. 4). Thus, our model easily explains why Wnt does not suffice to maintain undifferentiated hESCs although it can act on hESCs and stimulate cell proliferation.

Our data with hESCs support the concept that Wnt signaling on stem cells is also dependent on cell-intrinsic, as well as other extrinsic, signals [14, 42] and help to unify various conclusions from previous studies with other stem cell types. On one hand, Wnt appears to be a stem cell (self-renewing) factor. For example, Wnt/ β -catenin activation can expand murine hematopoietic stem/progenitor cells in vitro when other signals were also present [43, 44]. In vivo, Wnt/ β -catenin activation is commonly observed in “activated” stem cells and subsequently derived transiently amplifying progenitor cell populations [45–47]. This is consistent with the notion that cell proliferation is required for stem cell self-renewal, to maintain the stem cell pool while generating committed progenitor cells. However, careful examination also reveals that the Wnt/ β -catenin activation is transient and normally suppressed in quiescent stem cells [45, 46]. This is likely important to maintain the stem cell pool and homeostasis in vivo. After the transient stem cell activation and amplification (proliferation) of

stem cell–derived, committed progenitor cells, the Wnt/ β -catenin activation is again suppressed in downstream progeny cells that have ceased cell proliferation and become terminally differentiated [43, 46, 47]. When the antidifferentiation signal is absent or an inducing cue is present in a regenerative environment, however, Wnt activation was found to enhance stem cell–fate specification, i.e., differentiation [19, 42, 47]. The cell-fate specification is likely dependent on the activation of quiescent stem cells in vivo by Wnt and other proliferative signals. Therefore, this “activation” model is better in explaining the diverse roles of Wnt in “stem cell–fate determination”, whether the term is used to describe the expansion of undifferentiated stem cells or cellular differentiation (“specification”). Thus, we favor this “activation” model, in which the Wnt/ β -catenin activation is a key event in stem cell–fate determination (self-renewal or differentiation), depending on other extrinsic signals and cell-intrinsic factors.

The regulation of the Wnt/ β -catenin signaling pathway is important to the self-renewal of hESCs, which is poorly understood as compared with mESCs and other stem cell systems. Better understanding of the regulation of Wnt/ β -catenin activation and its interplay with other key signals, such as FGF and TGF β /BMP families, will improve our ability to culture undifferentiated hESCs under defined conditions. It will in turn help us to understand mechanisms governing stem cell self-renewal and differentiation.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Supplemental Table 1. Feeder-cell expression of genes encoding extracellular Wnt **agonists** and **antagonists**

Probe set ID	GenBank accession ID	Gene	Normalized value			
			hMSC (supporter)	HAFi (supporter)	Hs27	WI-38
205648_at	NM_003391	<i>WNT2</i>	0.71(A)	2.20	6.60	1.00(A)
205990_s_at	NM_003392	<i>WNT5A</i>	0.55	1.00	2.10	1.60
213425_at	AI968085	<i>WNT5A</i>	0.75	0.90	3.60	1.38
221029_s_at	NM_030775	<i>WNT5B</i>	1.49(A)	1.00(A)	0.86(A)	1.30
71933_at	AI218134	<i>WNT6</i>	1.00	1.00	1.00	0.80
202037_s_at	NM_003012	secreted frizzled-related protein 1 (<i>sFRP1</i>)	0.91(A)	0.11(A)	11.76	20.77
202036_s_at	AF017987	secreted frizzled-related protein 1 (<i>sFRP1</i>)	0.79(A)	0.31(A)	7.85	13.27
202035_s_at	AI332407	secreted frizzled-related protein 1 (<i>sFRP1</i>)	0.84(A)	1.00(A)	3.27	4.86
204602_at	NM_012242	dickkopf homologue 1 (<i>Dkk-1</i>)	1.31	1.00	1.57	0.21
202196_s_at	NM_013253	dickkopf homologue 3 (<i>Dkk-3</i>)	0.46	1.00	1.14	3.56
214247_s_at	AU148057	dickkopf homologue 3 (<i>Dkk-3</i>)	0.40	1.00	1.50	3.15
219908_at	NM_014421	dickkopf homologue 2 (<i>Dkk-2</i>)	0.86(A)	1.57(A)	0.45(A)	2.08
Beta-actin	X00351	beta-actin (<i>ACTB</i>), <i>n</i> = 3	1.35 1.46	0.92–1.01	0.90–1.00	1.00–1.08

Genes encoding Wnt **agonists** (*WNT*) and **antagonists** (*sFRP* and *Dkk*) and their expression in supportive feeder cells (hMSC and HAFi) and non- or weak-supportive feeder cells (Hs27 and WI-38) were analyzed using Affymetrix GeneChips (U133A) (Affymetrix, Santa Clara, CA, <http://www.affymetrix.com>). Note that some Wnt agonists (such as Wnt3a) and antagonists (such as *sFRP2*) are not included in the U133A chip. Image data were quantified with Microarray Suit version 5.0 (Affymetrix) and further analyzed using GeneSpring version 6.0 software (Silica Genetics, Redwood City, CA). Samples were normalized for expression levels; values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. The value for each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 10, then each measurement for that gene was divided by 1.

A indicates gene whose expression is called “Absent.”

Abbreviations: HAFi, Human Adult Fibroblast, immortalized; hMSC, human marrow stromal cell.

Supplemental Table 2. Expression of genes encoding Wnt signaling pathway proteins in undifferentiated and differentiated H1 hESCs

Probe set ID	GenBank accession ID	Gene	Normalized value		Undifferentiated/differentiated (ratio)
			Undifferentiated	Differentiated	
205648_at	NM_003391	<i>WNT2</i>	0.03(A)	2.63	<0.01
205990_s_at	NM_003392	<i>WNT5A</i>	0.30	1.60	0.19
202037_s_at	NM_003012	secreted frizzled-related protein 1 (<i>sFRP1</i>)	0.76	2.08	0.37
202036_s_at	AF017987	secreted frizzled-related protein 1 (<i>sFRP1</i>)	0.84	1.88	0.45
202035_s_at	A1332407	secreted frizzled-related protein 1 (<i>sFRP1</i>)	0.79	1.79	0.44
203698_s_at	NM_001463	frizzled-related protein 3 (<i>sFRP3</i>)	0.80	9.20	0.09
204602_at	NM_012242	dickkopf homologue 1 (<i>DKK1</i>)	0.18	2.40	0.08
219908_at	NM_014421	dickkopf homologue 2 (<i>DKK2</i>)	0.26(A)	1.53	<0.17
202196_s_at	NM_013253	dickkopf homologue 3 (<i>DKK3</i>)	0.59	1.42	0.42
214247_s_at	AU148057	dickkopf homologue 3 (<i>DKK3</i>)	0.70	1.46	0.48
204712_at	NM_007191	WNT inhibitory factor 1 (<i>WIF-1</i>)	2.00	0.33(A)	>4.74
210220_at	L37882	frizzled homology 2 (<i>FZD2</i>)	0.15(A)	2.08	<0.07
219683_at	NM_0174112.1	frizzled homology 3 (<i>FZD3</i>)	1.80	0.62	2.9
206136_at	NM_003468.1	frizzled homology 5 (<i>FZD5</i>)	1.80	0.30	6.00
203987_at	NM_003506.1	frizzled homology 6 (<i>FZD6</i>)	1.46	0.67	2.18
203705_s_at	AI333651	frizzled homology 7 (<i>FZD7</i>)	1.90	0.21	9.05
203706_s_at	NM_003507.2	frizzled homology 7 (<i>FZD7</i>)	1.84	0.24	7.54
203697_at	U91903	frizzled-related protein (<i>FRZB</i>)	1.00	12.10	0.08
203698_s_at	NM_001463	frizzled-related protein (<i>FRZB</i>)	0.52	4.70	0.11
34697_at	AF074264	low density lipoprotein receptor-related protein 6 (<i>LRP6</i>)	1.46	0.67	2.18
203230_at	AF006011.1	disheveled, dsh homologue 1 (<i>DVLI</i>) ^a	1.30	0.80	1.63
201908_at	NM_00423.2	disheveled, dsh homologue 3 (<i>DVL3</i>) ^a	0.58	1.38	0.42
632_at	L40027	glycogen synthase kinase 3 alpha (<i>GSK-3A</i>)	1.01	0.91	1.11
209945_s_at	BC000251.1	glycogen synthase kinase 3 beta (<i>GSK-3B</i>)	1.00	1.03	0.97
209864_at	AB045118.1	<i>FRAT2</i>	1.68	0.34	4.94
219889_at	NM_005479.1	<i>FRAT1</i>	1.05	0.98	1.07

Probe set ID	GenBank accession ID	Gene	Normalized value		Undifferentiated/differentiated (ratio)
			Undifferentiated	Differentiated	
201533_at	NM_001904.1	Catenin (cadherin-associated protein), beta 1 (<i>CTNNB1</i>)	0.93	1.09	0.85
210948_s_at	AF294627	lymphoid enhancer-binding factor 1 (<i>LEF1</i>)	0.23(A)	2.48	<0.09
221558_s_at	AF288571	lymphoid enhancer-binding factor 1 (<i>LEF1</i>)	0.05(A)	2.06	<0.02
221016_s_at	NM_031283.1	transcription factor 7-like 1 (similar to mouse Tcf3)	1.40	0.70	2.00
203753_at	NM_003199	transcription factor 4 (<i>TCF4</i>), isoform b	0.66	1.54	0.43
212382_at	AK021980	transcription factor 4 (<i>TCF4</i>)	0.68	1.49	0.46
213891_s_at	AI927067	transcription factor 4 (<i>TCF4</i>)	0.73	1.47	0.50
203753_at	NM_003199.1	transcription factor 4 (<i>TCF4</i>)	0.66	1.54	0.43
206796_at	NM_003882	WNT1 inducible signaling pathway protein 1 (<i>WISP1</i>)	0.05(A)	2.21	<0.02
211312_s_at	AB034725	WNT1 inducible signaling pathway protein 1 (<i>WISP1</i>)	0.02(A)	2.21	<0. 01
Controls					
Beta-actin	X00351	Beta-actin (<i>ACTB</i>), $n = 3$	0.96–1.00	1.00	0.98 ± 0.02
214532_x_at	AF268615	POU domain, class 5, transcription factor 1 (<i>POU5F1</i>)	2.31	0.01(A)	>231.00
208286_x_at	NM_002701	POU domain, class 5, transcription factor 1 (<i>POU5F1</i>)	2.36	0.01(A)	>236.00
220184_at	NM_024865	Nanog homeobox (<i>Nanog</i>)	2.25	0.01(A)	>225.00

Green signifies genes encoding positively acting components.

Red signifies genes encoding negatively acting components.

Expression profile of genes involved in WNT signaling pathway was identified using the Affymetrix (U133A) GeneChip analysis. The mean values from duplicate samples of undifferentiated (SSEA-4⁺) and differentiated (SSEA-4⁻) hESC populations are shown. In this chip, multiple specific and control probes for greater than 22,000 transcripts of known and substantiated ESTs are included. The probe from 5 µg total RNA in each sample was made and hybridized according to the manufacturer. Image data were quantified with Microarray Suit version 5.0 (Affymetrix) and further analyzed using GeneSpring version 6.0 software (Silica Genetics). Samples were normalized for expression levels; values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements of that gene. The value of each gene was divided by the median of its measurements in all samples.

A indicates gene whose expression is called “Absent.”

Abbreviations: EST, expressed sequence tag; hESC, human embryonic stem cell.