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Short Communication

The WNT receptor FZD7 contributes to self-renewal signaling of human embryonic stem cells

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Abstract

A number of recent studies identified nuclear factors that together have the unique ability to induce pluripotency in differentiated cell types. However, little is known about the factors that are needed to maintain human embryonic stem (ES) cells in an undifferentiated state. In a search for such requirements, we performed a comprehensive meta-analysis of publicly available SAGE and microarray data. The rationale for this analysis was to identify genes that are exclusively expressed in human ES cell lines compared to 30 differentiated tissue types. The WNT receptor FZD7 was found among the genes with an ES cell-specific expression profile in both SAGE and microarray analyses. Subsequent validation by quantitative RT-PCR and flow cytometry confirmed that FZD7 mRNA levels in human ES cells are up to 200-fold higher compared to differentiated cell types. ShRNA-mediated knockdown of FZD7 in human ES cells induced dramatic changes in the morphology of ES cell colonies, perturbation of expression levels of germ layer-specific marker genes, and a rapid loss of expression of the ES cell-specific transcription factor OCT4. These findings identify the WNT receptor FZD7 as a novel ES cell-specific surface antigen with a likely important role in the maintenance of ES cell self-renewal capacity.

Keywords: Frizzled receptors; germ layers; OCT4; SAGE; WNT signaling.

During the last year, a number of groundbreaking studies identified a set of nuclear factors including OCT4, SOX2, KLF4, MYC, NANOG and LIN28 that have the unique ability to 'reset' an adult differentiated cell type in specific combinations. These nuclear factors act in concert to 'erase' all previous fate decisions and the transcriptional programs that determine phenotype and function of a differentiated cell (Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). As a result, 'reprogrammed' cells assume a phenotype closely resembling embryonic stem (ES) cells and functional studies so far suggest that these cells, termed induced pluripotent stem cells (iPS), also share fundamental functional characteristics with ES cells, including self-renewal capacity and pluripotency. The iPS concept, reprogramming of somatic cells into stem cells (Takahashi and Yamanaka, 2006), and its potential for regenerative medicine (Hanna et al., 2007) has rapidly become the focus of a large number of studies. However, the question of which signaling pathways are required for stem cells to maintain an undifferentiated state and their self-renewal capacity has received relatively little attention and remains to be solved. The aim of this study was to identify elements of transcriptional regulation and signal transduction pathways that are active in stem cell populations to ensure maintenance of inherent stem cell self-renewal capacity.

WNT signaling has been implicated in the regulation of embryonic development (Cadigan and Nusse, 1997) and self-renewal of stem cell populations (Reya et al., 2003; Willert et al., 2003; Otero et al., 2004; Sato et al., 2004). WNT ligands interact with Frizzled (FZD) surface receptors and this interaction induces activation of Dishevelled (DSH), which leads to stabilization and nuclear accumulation of β-catenin (CTNNB1). DSH is a key component of a membrane-associated FZD/WNT receptor complex that, when activated by FZD receptor ligation, inhibits a cytoplasmic complex of proteins that includes Axin, GSK3β, and APC. The Axin/GSK3β/APC complex promotes the ubiquitinylation and subsequent proteasome-mediated degradation of β-catenin. Inhibition of the β-catenin destruction complex by WNT/FZD/DSH interaction stabilizes cytoplasmic β-catenin, induces nuclear translocation of β-catenin and complex formation with TCF4/LEF1 transcription factors to promote transcriptional activation of specific gene expression cassettes that are typically active during early developmental processes. β-Catenin seems to regulate self-renewal signaling and differentiation of ES cells through differential co-activator usage. Depending on whether β-catenin binds to CBP (CREBBP1) or p300 (EP300), β-catenin complexes induce TCF4/LEF1-dependent transcriptional programs that are related to either self-renewal or differentiation processes, respectively (Teo et al., 2005; Miyabayashi et al., 2007).

To identify genes that function to maintain human ES cells in an undifferentiated state, we first performed a meta-analysis of publicly available gene expression data that were generated on the SAGE and Affymetrix U133A2.0 GeneChip platforms. To this end, we analyzed 32 SAGE libraries (11 human ES cells; 21 differentiated tissue types) and 54 Affymetrix U133A2.0 GeneChips (24 human ES cells; 30 differentiated tissue types) and compared gene expression values in human ES cells against the average for differentiated tissue types covering all three germ layers (ectodermal, mesodermal and endodermal tissue types). We sorted SAGE and Affymetrix U133A2.0 GeneChip data based on the ratio of gene expression values in human ES cells vs. differentiated cell types. Given that both technical platforms have an almost genome-wide scope, we focused our analysis on those genes identified as ES cell-specific in both SAGE and Affymetrix U133A2.0 data sets. Based on this SAGE/Affymetrix U133A2.0 GeneChip-'consensus' database, we identified 21 genes that are expressed at >5-fold higher levels in ES cells compared to differentiated cell types. This catalog of ES cell-specific genes includes *LIN28*, *OCT4*, *DNMT3B*, *TDGF1*, *ECAT11*, *DPPA4*, *IMP3*, *CD24*, *DLG7*, *TOP2A*, *PODXL*, *SOX2*, *TMEFF1*, *FZD7*, *MYCN*, *HMGA1*, *LEFTY1*, *NANOG*, *CYP26A1*, *TACSTD1* and *MELK*. Interestingly, *LIN28*, *OCT4*, *SOX2* and *NANOG* are also implicated in reprogramming of iPS cells, whereas expression of two other nuclear factors used for reprogramming of iPS cells, namely *KLF4* and *MYC*, are not ES cell-specific.

Among the 21 genes identified in our SAGE/Affymetrix U133A2.0 GeneChip consensus database, we focused subsequent analysis on FZD7, which encodes the Frizzled 7 receptor for WNT molecules. This choice was made because FZD7 encodes a surface receptor, which is thus easily accessible to flow cytometry analysis and functional testing. In addition, recent studies indicate that WNT signaling may play a critical role in the regulation of self-renewal signaling in human and mouse ES cells (Miyabayashi et al., 2007). Figure 1 shows the gene expression profile of FZD7 in human ES cells compared to endodermal, mesodermal and ectodermal cell types as determined by Affymetrix U133A2.0 GeneChips (A) and SAGE (B) (Table 1). Affymetrix U133A2.0 GeneChip analysis involved two probe sets for FZD7 (203705_s_at and 203706_s_at). Comparison of average gene expression values from 24 ES cell lines compared to 30 differentiated tissue types revealed that FZD7 mRNA levels were 15.9- and 9.4-fold higher in ES cells according to these two probe sets. Likewise, SAGE tag counts for the FZD7-specific SAGE tag (TACAGATCAC; UniGene cluster 173859) were on average approximately 10-fold higher in human ES cells (11 SAGE libraries) compared to differentiated tissue types (21 SAGE libraries).

Although identification of ES cell-specific genes based on two independent technologies (SAGE and microarrays) already eliminated many false-positive candidate genes, we further validated ES cell-specific expression of FZD7 mRNA by quantitative real-time RT-PCR (Figure 2, Table 2). To this end, we amplified FZD7 and GAPDH transcripts from human ES cells (H1, H9) and endodermal, mesodermal and ectodermal tissue types in two independent experiments. This analysis confirmed that FZD7 mRNA levels are between 6- and 200-fold higher in ES cells compared to endodermal, mesodermal and ectodermal tissues (Figure 2). To validate FZD7 protein expression on the surface of human ES cells, we analyzed H1 and H9 cells by flow cytometry for surface expression of both FZD7 and CD24. CD24 was also identified as an ES cell-specific surface antigen in our SAGE/Affymetrix consensus database and subsequently validated by quantitative RT-PCR (data not shown). Although 293T embryonic kidney cells lack surface expression of both FZD7 and CD24, both surface antigens are expressed on the surface of H1 and H9 cells (Figure 3). Together, these findings establish FZD7 as a novel ES cell-specific surface antigen.

To investigate a potential role of FZD7 during embryonic development, we first investigated whether FZD7 mRNA levels are regulated during very early differentia-

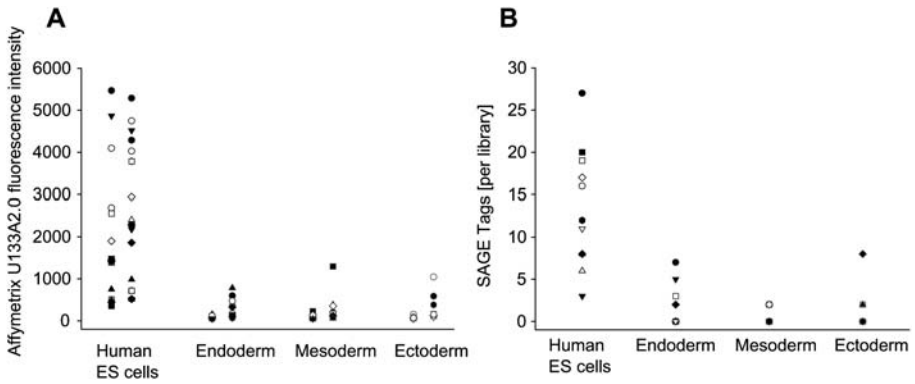

Figure 1 Identification of FZD7 as an ES cell-specific gene by microarrays and SAGE.
Gene expression values for (A) two FZD7 Affymetrix U133A2.0 probe sets (203705_s_at and 203706_s_at) and (B) one FZD7-specific SAGE-tag (TACAGATCAC; UniGene cluster 173859; panel B) are shown as scatter plots. The ratios for Affymetrix U13A2.0 probe sets 203705_s_at and 203706_s_at for human ES cells (n=24) compared to differentiated tissue types (n=30) are 15.9- and 9.4-fold, respectively. For SAGE analysis, data from 11 human ES cell SAGE libraries (average SAGE tag count 14.1) and 21 SAGE libraries from differentiated tissue types (average SAGE tag count 1.4) were analyzed as previously described (Klein et al., 2004). Each individual symbol represents FZD7 gene expression data from one Affymetrix U133A2.0 array or one SAGE library. Affymetrix U133A2.0 arrays and SAGE libraries were arranged in four groups for derivation of the samples investigated: human ES cells or differentiated endodermal, mesodermal and ectodermal cell types. The Affymetrix U133A2.0 arrays and SAGE libraries used are listed in Table 1.

Table 1 Gene expression data used for meta-analysis.

Affymetrix U133A2.0 data for gene expression analysis

Germ layer	Differentiated tissue	GEO accession number
Ectoderm	Normal amygdala	GSM44694
	Normal brain	GSM44690
	Normal fetal brain	GSM44691
	Normal caudate nucleus	GSM44695
	Normal cerebellum	GSM44689
	Normal corpus	GSM44696
	Normal hippocampus	GSM44697
	Normal skin	GSM44686
	Normal spinal cord	GSM44700
	Normal thalamus	GSM44698
Mesoderm	Normal bone marrow	GSM44693
	Normal breast	GSM44683
	Normal heart	GSM44671
	Normal kidney	GSM44675
	Normal skeletal muscle	GSM44676
	Normal spleen	GSM44673
	Normal uterus	GSM44684
Endoderm	Normal bladder	GSM44682
	Normal colon	GSM44680
	Normal liver	GSM44702
	Normal fetal liver	GSM44706
	Normal lung	GSM44704
	Normal fetal lung	GSM44705
	Normal pancreas	GSM44677
	Normal prostata	GSM44678
	Normal small intestine	GSM44679
	Normal stomach	GSM44703
	Normal thymus	GSM44672
	Normal thyroid	GSM44685
	Normal trachea	GSM44688
Mesoderm/ectoderm	Normal adrenal gland	GSM44692
Extraembryonic tissue	Normal placenta	GSM44681
Germ cells	Normal ovary	GSM44674
	Normal testis	GSM44701
ES cell lines	H1a	Thomson et al., 1998
	H1b	Thomson et al., 1998
	H1c	Thomson et al., 1998
	H1	Thomson et al., 1998
	H1	Thomson et al., 1998
	H1	Thomson et al., 1998
	H9-2 A	Thomson et al., 1998
	H9-3 A	Thomson et al., 1998
	H9-4 A	Thomson et al., 1998
	HSF1-1 A	Abeyta et al., 2004
	HSF1-1 A	Abeyta et al., 2004
	HSF1-1 A	Abeyta et al., 2004
	HSF6-1 A	Abeyta et al., 2004
	HSF6-1 A	Abeyta et al., 2004
	HSF6-1 A	Abeyta et al., 2004
	I6	Amit et al., 2002
	I6	Amit et al., 2002
	I6	Amit et al., 2002
	HS181A_133A	Hovatta et al., 2003
	HS181B_133A	Hovatta et al., 2003
	HS235A_133A	Hovatta et al., 2003
	HS235B_133A	Hovatta et al., 2003
	HS237A_133A	Hovatta et al., 2003
	HS237B_133A	Hovatta et al., 2003

SAGE libraries used for gene expression analysis

ES cell line	Reference	CGAP number
BG01	Mitalipova et al., 2003	SHE19
H1	Thomson et al., 1998	SHE15
H1	Thomson et al., 1998	SHE14
H7	Thomson et al., 1998	SHE17
H9	Thomson et al., 1998	SHE16
H9	Thomson et al., 1998	SHE13
H13	Thomson et al., 1998	SHES1
H14	Thomson et al., 1998	SHES2

Table 1 (Continued)

HES3	Developed by ES Cell International	SHE10
HES4	Developed by ES Cell International	SHE11
HES6	Developed by ES Cell International	SHES9
SAGE libraries from differentiated tissues		
Title	**Source**	**GEO accession number**
SAGE_pooled_GBM	Brain, 5 pooled Duke glioblastoma	GSM765
SAGE_BB542_whitematter	Brain, normal, white matter	GSM676
SAGE_95-259	Breast tumor	GSM670
SAGE_96_260	Metastasis from breast tumor	GSM671
SAGE_normal_cerebellum	Normal cerebellum	GSM761
SAGE_Tu_102	Colon, primary tumor	GSM755
SAGE_NC1	Normal colonic epithelium	GSM728
SAGE_normal_gastric	Normal gastric epithelial tissues	GSM784
SAGE_glioma_1150	Glioma	GSM1498
SAGE_293-CTRL	Uninduced 293 cells	GSM668
SAGE_Duke_Kidney	Normal kidney tissue	GSM708
SAGE_Duke_leukocyte	Leukocyte	GSM709
SAGE_Duke_757	Medulloblastoma tumor	GSM693
SAGE_normal_liver	Normal liver tissue	GSM7885
SAGE_normal_lung	Normal lung tissue	GSM762
SAGE_OC14	Ovarian carcinoma	GSM731
SAGE_HOSE_4	Ovary, normal surface epithelium	GSM719
SAGE_H126	Pancreas	GSM716
SAGE_CAPAN1	Pancreas	GSM768
SAGE_Chen_Tumor_Pr	Prostate tumor tissue	GSM686
SAGE_gastric_cancer_G234	Gastric cancer	GSM757

tion steps of mouse ES cells. To this end, we performed a time course analysis of FZD7 mRNA levels during induced mesodermal differentiation of CGR8 mouse ES cells cultured using the hanging drop technique to form embryoid bodies/multicellular aggregates. To this end, gene expression values of two Fzd7-specific probe sets (Affymetrix Mouse Genome 430.2; 1450043_at and 1450044_at) were plotted against the time course of induced differentiation of mouse CGR8 ES cells. Both probe sets showed similar kinetics, with initially high mRNA levels of Fzd7 followed by a steep decrease on day 4 of differentiation. After day 4, Fzd7 mRNA levels remained stable for both probe sets. These findings support the hypothesis that Fzd7 expression levels are high in undifferentiated ES cells but are downregulated at a defined stage of embryonic development (Figure 4).

Next we investigated a possible function of FZD7 in the maintenance of self-renewal capacity of human ES cells. To this end, we tested the consequences of shRNA-mediated knockdown of FZD7 in human ES cells (H1). H1 cells were cultured on mouse embryonic fibroblasts and lentivirally transduced as previously described (Jang et al., 2006). Lentiviral vectors encode short hairpin

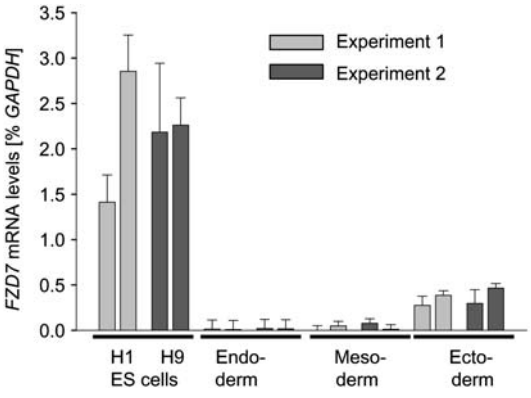

Figure 2 Validation of FZD7 as an ES cell-specific gene by quantitative RT-PCR.
Total RNA was isolated from H1 and H9 cells (human ES cell lines), liver and gut tissue (endoderm), muscle and spleen (mesoderm), and brain and skin tissue (ectoderm). mRNA was reverse-transcribed and cDNAs were used for quantitative RT-PCR as previously described (Feldhahn et al., 2007). Based on the SYBRGreen method, mRNA levels were measured for FZD7 and GAPDH using the RT-PCR primers listed in Table 2. FZD7 mRNA levels are given as a percentage of GAPDH based on mean±SEM for triplicate measurements. Results for two independent experiments are shown.

Table 2 Oligonucleotide sequences.

RT-PCR primer	Sequence
FZD7 forward	5′-GGAAATCTCTCCCTTCATTTACT-3′
FZD7 reverse	5′-CCAGAATCACTTTGAAGTTTACC-3′
AFP forward	5′-ACCACTGCCAATAACAAAATAAC-3′
AFP reverse	5′-AACAGTTATGTCTTCCCTCTTCA-3′
GATA6 forward	5′-CATGACTCCAACTTCCACCT-3′
GATA6 reverse	5′-GAATACTTGAGCTCGCTGTTCT-3′
RUNX forward	5′-CTGGATTGGTCATTTAGAGTTTC-3′
RUNX reverse	5′-TTAAGAGAACACAGGAAAAGGAG-3′
VEGFR2 forward	5′-GATTGATGAAGAATTTTGTAGGC-3′
VEGFR2 reverse	5′-ACTGACTGATTCCTGCTGT-3′
NF68kD forward	5′-ATATTGAGATTGCAGCTTACAGG-3′
NF68kD reverse	5′-TCTTTTGCTTCTTCAGACTCTTC-3′
NCAM forward	5′-GAGATGCCAAAGATAAAGACATC-3′
NCAM reverse	5′-AGTTGTTGGACAGGACTATGAAT-3′
OCT4 forward	5′-GAAAGAGAAAGCGAACCAGTATC-3′
OCT4 reverse	5′-TTTGGGATTAAGTTCTTCATTCA-3′
GAPDH forward	5′-CAGGAGCGAGATCCCTC-3′
GAPDH reverse	5′-AAGATCATCAGCAATGCCTC-3′
shRNAmir target sequences	
FZD7 sense	GTTTCCCGTTGGTTGTTAAT
FZD7 antisense	ATTAACAACCAACGGGAAAC
Scrambled sense	GTTCGCCTAGCTAGATAAT
Scrambled antisense	ATTATCTAGCTAGGCGTAAC

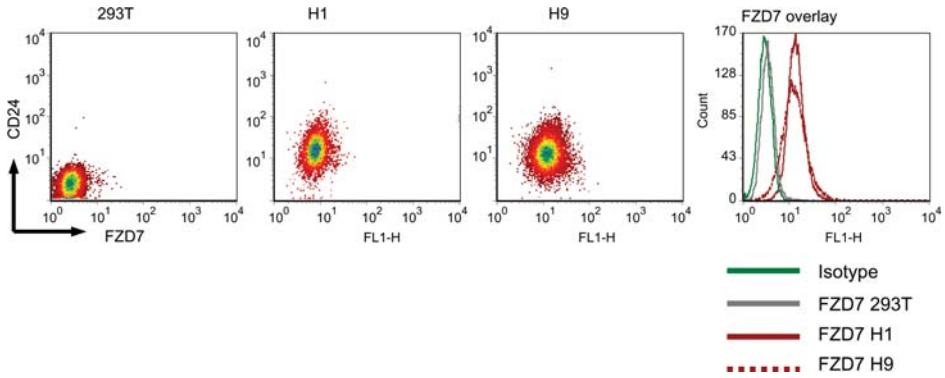

Figure 3 FZD7 receptor surface expression on human ES cells.
Human embryonic kidney (293T) and ES cells (H1 and H9) were stained with antibodies against human CD24 (BD Biosciences, Heidelberg, Germany) and FZD7 (Lifespan Biosciences, Seattle, WA, USA, and R&D Systems, Minneapolis, MN, USA). Adherent cells were mobilized by Accutase treatment (Sigma Aldrich, St. Louis, MO, USA), stained for 15 min at room temperature and then analyzed in a FACscan flow cytometer (BD Biosciences).

RNAs with a microRNA context (shmiR). The microRNA context is derived from miR30 and was introduced to increased processing of shRNA stem loops by Dicer and Drosha to form functional siRNA duplexes. ShmiR constructs were used that either bind FZD7 mRNA or a scrambled non-targeting control, in which nucleotides of the anti-FZD7 shmiR are exchanged at every second position between plus and minus strands. RNA interference lentiviral vectors encode shmiRs and GFP through an internal ribosomal entry site. Therefore, human ES cells transduced with either FZD7- or a non-targeting-shmiR could be identified based on GFP expression. Quality controls to ensure that H1 cells did not undergo malignant transformation or differentiation include karyotyping and flow cytometry using antibodies against SSEA1 and SSEA4 (data not shown, available upon request). Four days after lentiviral transduction, dramatic phenotypic changes in cell colonies were observed for ES cells transduced with FZD7-shmiRs, but not 'scrambled' controls (Figure 5A). Transduction with FZD7-specific shmiRs resulted in differentiation of ES cells predominantly at the external margin of individual ES cell colonies (arrows, Figure 5A). Next, transduced $CD24^{+}GFP^{+}$ ES cells were sorted by FACS and shmiR-mediated knockdown of FZD7 in the transduced cells was verified by quantitative RT-PCR. The ratio of FZD7 mRNA copies in ES cells transduced with FZD7-shmiRs compared to 'scrambled' control was calculated 4 days after transduction (Figure 5B). Compared to morphologically undifferentiated ES cell colonies that were transduced with a non-targeting 'scrambled' shmiR, mRNA levels of FZD7 were approximately 60-fold lower in ES cells transduced with FZD7-specific shmiRs. Differentiation of human ES cells upon FZD7 knockdown became apparent by marked changes in colony morphology and approximately six-fold downregulation of OCT4 mRNA levels (Figure 5B). For quantitation of OCT4 mRNA levels, we used RT-PCR primers specific for the functional OCT4A isoform. RT-PCR primers also avoid amplification of transcripts from non-coding *OCT4* pseudogenes (Lee et al., 2006). From these findings we conclude that FZD7 contributes to maintenance of an undifferentiated state in human ES cells.

We next investigated whether FZD7 globally suppresses differentiation of ES cells or prevents differentiation towards cell types of a specific germ layer. To this end, we studied regulation of germ layer-specific markers (AFP and GATA6 for endoderm, RUNX1 and VEGFR2 for mesoderm, NF68 and NCAM for ectoderm) upon FZD7

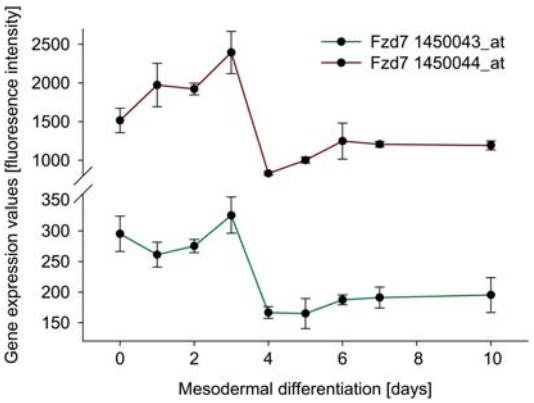

Figure 4 Time course of FZD7 mRNA levels during induced mesodermal differentiation of mouse embryoid bodies.
CGR8 mouse ES cells (ECACC 95011018) were cultured without feeder cells in Glasgow minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml leukemia inhibitory factor, and 50 μM β-mercaptoethanol (β-ME) in 0.2% gelatine-coated flasks. To induce mesodermal differentiation, the hanging drop protocol was used (Doss et al., 2007). Briefly, an ES cell suspension of 2.5×10^{4} cells/ml was prepared in Iscove's modified Dulbecco's medium supplemented with 20% fetal calf serum, 1% non-essential amino acids (vol/vol), 2 mM L-glutamine, and 100 μM β-ME. A 20-μl aliquot of the suspension was spotted on the inside of the upper lid of a 10-cm bacteriologic dish and then placed over the bottom dish containing 5 ml of PBS. On day 2, the multicellular aggregates that formed were transferred to suspension in a new dish with 20 ml of IMDM supplemented with 20% fetal calf serum, 1% non-essential amino acids (vol/vol), 2 mM L-glutamine, and 100 μM β-ME. ES cell aliquots were taken from undifferentiated ES cells (day 0) and from multicellular aggregates/embryoid bodies after various time points (as indicated on the x-axis). From isolated cells, RNA was extracted and subjected to Mouse Genome 430 2.0 GeneChip analysis. For each condition/time point, three different samples were analyzed and the mean-±SEM is indicated. The curves show gene expression values for two FZD7 probe sets (1450043_at and 1450044_at).

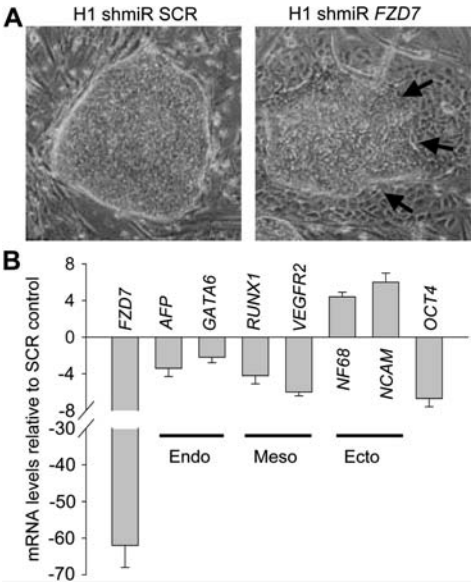

Figure 5 FZD7 contributes to self-renewal signaling of human ES cells.
Human ES cells (H1) were transduced with lentiviral vectors encoding a short hairpin RNA against FZD7 with miR30 context (shmiR FZD7) or a non-targeting 'scrambled' control (shmiR SCR). Four days after transduction, morphological changes in ES cell colonies were documented (A) and transduced $CD24^+$ GFP^+ ES cells were sorted using a FACS Aria cell sorter. Total RNA was isolated from sorted cells, reverse-transcribed and subjected to quantitative RT-PCR for FZD7 and germ layer-specific antigens AFP, GATA6 (endoderm), RUNX1, VEGFR2 (mesoderm) and NF68 and NCAM (ectoderm) and the ES cell-specific transcription factor OCT4. For each gene, the ratio of mRNA levels in the shmiR-FZD7- vs. shmiR-SCR-transduced H1 cells was calculated (y-axis). For instance, mRNA levels for FZD7 were >60-fold reduced upon shmiR-FZD7-mediated knockdown. Consistent with changes in the morphology of ES cell colonies (A), knockdown of FZD7 also affected mRNA levels of endo- and mesodermal genes and induced downregulation of OCT4 (B).

knockdown by quantitative RT-PCR. mRNA levels for the endodermal antigens AFP (4-fold) and GATA6 (2-fold) and the mesodermal antigens RUNX1 (5-fold) and VEGFR2 (6-fold) were consistently downregulated upon FZD7 knockdown. Conversely, mRNA levels of the ectodermal antigens NF68 and NCAM were four- and six-fold upregulated upon FZD7 knockdown, respectively. These finding suggest that knockdown of FZD7 leads to preferential ectodermal development at the expense of the two other germ layers. In this case, FZD7 may be needed to selectively suppress ectodermal differentiation.

To study knockdown of FZD7 and subsequent downregulation of OCT4 in individual pluripotent cells, we studied pluripotent embryonic carcinoma cells (NCCIT) that were transduced with a reporter construct driving expression of GFP under the control of the human OCT4 promoter. NCCIT/OCT4-GFP reporter cells were transduced with retroviral constructs encoding the FZD7-specific shmiR or a 'scrambled' non-targeting shmiR, together with puromycin resistance. From parallel experiments using a GFP-control vector, we estimated a transduction efficiency of 90–95%. The GFP-based lentiviral shmiR constructs described above could not be used in these experiments because their GFP signal would interfere with the OCT4-GFP reporter used in these experiments. NCCIT/OCT4-GFP reporter cells were transduced with retroviral constructs with an FZD7-specific shmiR or a 'scrambled' non-targeting shmiR together with and incubated in the presence of puromycin for 14 days. Knockdown of FZD7 was verified at the protein level by flow cytometry (Figure 6). In parallel, activity of the OCT4 reporter was measured. Transduction of NCCIT/OCT4-GFP reporter cells with an FZD7-specific shmiR construct resulted not only in marked downregulation of FZD7 surface expression, but also in loss of OCT4-expressing subclones (scrambled, 7.5%; FZD7 knockdown, 2.5%; Figure 6).

Taken together, these findings establish FZD7 as a novel surface antigen expressed on human ES cells. Knockdown of FZD7 mRNA levels leads to reduced surface expression of FZD7, loss of OCT4 mRNA expression and loss of the typical morphology of undifferentiated ES cell colonies. FZD7 seems to preferentially block ectodermal differentiation, because knockdown of FZD7 results in higher levels of ectodermal antigens NF68 and NCAM. Further experiments to clarify the potential role of FZD7 in the suppression of ectodermal development are currently under way.

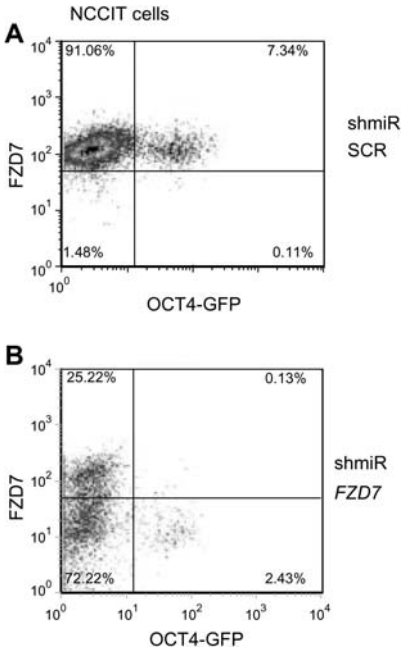

Figure 6 FZD7 contributes to maintenance of OCT4 expression in human embryonic carcinoma cells.
Human embryonic carcinoma cells (NCCIT) are pluripotent, express the ES cell-specific transcription factor OCT4 and were used as a surrogate for human ES cells in this experiment. To measure the potential effect of FZD7 knockdown on regulation of OCT4, we stably transduced NCCIT cells with a reporter construct for OCT4 expression (OCT4-GFP). NCCIT/OCT4-GFP cells were then transduced with a retroviral vector encoding shmiR-FZD7 or shmiR-SCR (non-targeting scrambled control) short hairpin RNAs and puromycin resistance. Transduced cells were selected for 14 days by incubation in puromycin-containing medium. Then the cells were analyzed by flow cytometry for expression of GFP (reflecting transcriptional activation of OCT4) and FZD7 (to measure the degree of FZD7 knockdown by the shmiR-FZD7 construct used).

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