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Analysis of Oct4-Dependent Transcriptional Networks Regulating Self-Renewal and Pluripotency in Human Embryonic Stem Cells

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ABSTRACT

The POU domain transcription factor OCT4 is a key regulator of pluripotency in the early mammalian embryo and is highly expressed in the inner cell mass of the blastocyst. Consistent with its essential role in maintaining pluripotency, Oct4 expression is rapidly downregulated during formation of the trophoblast lineage. To enhance our understanding of the molecular basis of this differentiation event in humans, we used a functional genomics approach involving RNA interference-mediated suppression of OCT4 function in a human ESC line and analysis of the resulting transcriptional profiles to identify OCT4-dependent genes in human cells. We detected altered expression of >1,000 genes, including targets regulated directly by OCT4 either positively (*NANOG*, *SOX2*, *REX1*, *LEFTB*, *LEFTA/EBAF DPPA4*, *THY1*, and *TDGF1*) or negatively (*CDX2*, *EOMES*, *BMP4*, *TBX18*, *Brachyury [T]*, *DKK1*, *HLX1*, *GATA6*, *ID2*,

and *DLX5*), as well as targets for the OCT4-associated stem cell regulators SOX2 and NANOG. Our data set includes regulators of ACTIVIN, BMP, fibroblast growth factor, and WNT signaling. These pathways are implicated in regulating human ESC differentiation and therefore further validate the results of our analysis. In addition, we identified a number of differentially expressed genes that are involved in epigenetics, chromatin remodeling, apoptosis, and metabolism that may point to underlying molecular mechanisms that regulate pluripotency and trophoblast differentiation in humans. Significant concordance between this data set and previous comparisons between inner cell mass and trophectoderm in human embryos indicates that the study of human ESC differentiation in vitro represents a useful model of early embryonic differentiation in humans. STEM CELLS 2007;25:500–510

INTRODUCTION

The earliest differentiation event in the mammalian embryo occurs during formation of the blastocyst, when trophoblast cells delaminate away from a residual cluster of undifferentiated cells, the inner cell mass, containing the pluripotent embryonic founder cells [1]. Little is known about regulation at this stage of development in humans because of the ethical considerations associated with experimental use of this scarce tissue. However, with the establishment of pluripotent embryonic cell lines from human blastocysts, it is now possible to consider investigating molecular mechanisms that regulate the first stages in human development in vitro [2, 3].

The POU domain transcription factor Oct4/OCT4 is a critical regulator of pluripotency in the mammalian embryo and is expressed in unfertilized oocytes, the ICM and epiblasts of pregastrulation embryos, and in primordial germ cells [4–6]. Downregulation of *OCT4* expression during trophoblast differentiation and the conversion of mutant embryos lacking *Oct4* into exclusively trophoblast-like cells indicates that the transcription factor is required to either establish or maintain pluripotency in the embryo [7]. Suppression of *Oct4/OCT4* in ESCs also induces trophoblast-like differentiation, confirming its essential role in maintaining pluripotency and leading to the

proposal that an early function of this transcription factor is as a “gatekeeper,” preventing differentiation along the trophoblast lineage [8–10]. Studies in mouse and human cells indicate that OCT4 is a component of a network of transcription factors, including the homeobox protein NANOG and HMG-box transcription factor SOX2, that cooperatively maintain pluripotency in ESCs [8, 11–15]. In addition, ESCs also require inputs from extrinsic factors to suppress differentiation. However, the apparently distinct growth factor requirements of mouse and human embryonic stem (ES) [16–18] point to likely variations in how these signals connect to the basic conserved transcriptional regulatory circuits controlling differentiation of pluripotent stem cells [19].

As an extension to our previous work [9, 20], we modeled the first differentiation event in human embryos in vitro by acutely depleting *OCT4* from human ESCs using RNA interference (RNAi)-mediated gene knockdown and examined the resulting changes in global gene expression. Transcriptional changes induced by *OCT4* knockdown are expected to include direct targets of the transcription factor, indirectly associated genes linked with pluripotency, and genes activated upon differentiation along the trophoblast lineage. Combining this information with other data sets on core transcriptional regulatory networks controlling pluripotency and trophoblast differentiation [14, 20] should provide additional insights into the molec-

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ular events that underlie the earliest differentiation event in the human embryo.

MATERIALS AND METHODS

Clone Selection and Microarray Fabrication

The selection of the 15,529 cDNA clones and fabrication of the arrays was as previously described [20].

Cell Culture

Human ESCs (H1 clone) were grown under feeder-free conditions as previously described [9]. Cells were adjusted to dissociation with 0.5 mM EDTA in phosphate-buffered saline (PBS), applied to the cells at 37°C for 5–10 minutes, and routinely passaged in this way. Cells were seeded 24 hours before transfections. For microarray analysis, small interfering RNA (siRNA) transfections were carried out in 75-cm² flasks in triplicate for each siRNA duplex. Cells were seeded at approximately 5×10^6 cells per flask for analysis at 24 hours after siRNA transfection or approximately 2×10^6 cells per flask for analysis at 72 hours after siRNA transfection. For immunohistochemistry, transfections were carried out in six-well plates, and cells were seeded at 2×10^5 cells per well.

Transfections with siRNA

siRNA duplexes were obtained from Dharmacon RNA Technologies (Lafayette, CO, <http://www.dharmacon.com>) and reconstituted following the manufacturer's guidelines. Sense strand sequences for the siRNA duplexes were as follows: enhanced green fluorescent protein (*EGFP*), AAG AAC GGC AUC AAG GUG AAC; *OCT4-1*, AAG GAU GUG GUC CGA GUG UGG; and *OCT4-2*, Dharmacon siGENOME duplex 1 (D-019591-05-0010).

Triplicate ESC cultures were transfected individually with 80 nM siRNA duplexes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) lipofection reagent prepared in OptiMEM (Invitrogen) following the manufacturer's instructions. The siRNA/Lipofectamine 2000 complex was added to the cells in the appropriate volume of ESC culture medium. Cells were fed with fresh culture medium 1 hour prior to transfection and following the removal of the transfection reagent. Cells were transfected once only for RNAi analysis after 24 hours. For RNAi analysis at 72 hours, cells were transfected twice; the second transfection was carried out 24 hours after the first transfection.

Immunoblotting

Cells were lysed and sonicated in 1% SDS buffer. Lysates were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels and immunoblotted as described previously [9]. SHP-2 (1:1,000; sc-280; Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>), *OCT4* (1:1,000; sc-5279; Santa Cruz Biotechnology), SOX2 (1:1,000; sc-17320 X; Santa Cruz Biotechnology), NANOG (1:250; 1997; R&D Systems Inc., Minneapolis, <http://www.rndsystems.com>), and glyceraldehyde-3-phosphate dehydrogenase (1:5,000; 4300; Ambion, Austin, TX, <http://www.ambion.com>) antibodies were used. Secondary horseradish peroxidase-conjugated antibodies (Amersham Biosciences, Buckinghamshire, U.K., <http://www.amersham.com>) were used at a 1:5,000 dilution and detected with the enhanced chemiluminescence reagent (Amersham Biosciences).

Immunohistochemistry

Cells were fixed with 100% methanol at –20°C for 10 minutes, washed once in PBS, and permeabilized in 100% ethanol at room temperature for 1 minute. Thereafter, the cells were washed twice in PBS and blocked in PBS containing 10% goat serum (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) for 1 hour at room temperature. Primary antibodies for *OCT4* (sc-5279) and cytokeratin-18 (sc-6259) were purchased from Santa Cruz Biotechnology and used at 1:100 dilutions in PBS containing 1% goat serum. Alexa Fluor 568 anti-mouse IgG (Invitrogen) secondary

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antibody was used at a dilution of 1:400 to detect antibody binding. Coverslips were mounted in Mowial (Calbiochem, San Diego, <http://www.calbiochem.com>) solution with antifade supplemented with 4,6-diamidino-2-phenylindole nuclear stain. All light and fluorescence microscopy was carried out using a Nikon Microphot SA (Tokyo, <http://www.nikon.com>) microscope and camera.

RNA Isolation and In Vitro Transcription

Total RNA was isolated using TRIzol reagent (Invitrogen) treated with DNase I (Promega GmbH, Mannheim, Germany, <http://www.promega.com>). Messenger RNA amplifications were carried out using the MegaScript T7 High Yield Transcription kit (Ambion) using 3 μ g of total RNA as template. The purity, integrity, and concentrations of generated amplified RNA were evaluated using the Nanodrop Bioanalyzer (Nanodrop Technologies, Wilmington, DE, <http://www.nanodrop.com/>).

Direct Labeling of RNA and Hybridizations

MIAME (Minimum Information About Microarray Experiments) guidelines were adhered to in our experimental design. Three independent labeling (dye swaps, Cy3, and Cy5) reactions per antisense RNA sample pertaining to each biological replicate were carried out using 3 μ g of aRNA per reaction. Full details of labeling and hybridization reactions, slide washing, and scanning have been described previously [20].

Global Data Analysis

Data were normalized in two steps. The first step accounted for the dye effects caused by the difference in Cy3/Cy5 fluorescence labeling in each experimental sample. Here we used the LOWESS method [21]. For each cDNA, we performed statistical tests based on the replicate signals in experiments with *EGFP*- and *OCT4*-depleted samples. Three standard tests were used in parallel, Student's *t* test, the Welch test, and Wilcoxon's rank-sum test [22]. A full description of the normalization process is detailed in the supplemental online Materials and Methods.

Pathway Analysis

Array data were used to test whether entire groups of genes associated with specific pathways show differential expression. Pathways were taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (version KEGG2, 01.06.2005, <http://www.genome.ad.jp/kegg/>). The procedure has been described previously [20].

Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

Confirmations were carried out on a set of genes using RNA derived from the two independent *OCT4* siRNA transfections. The list of primers used, annealing temperatures, genes under investigation, and experimental details are shown in supplemental online Table 5 and supplemental online Materials and Methods.

Identification of Conserved Downstream Targets of *OCT4* in Mouse and Human ESCs

Genes differentially expressed 72 hours after *OCT4* knockdown were selected from the raw microarray data set on the basis of four independent statistical tests (*p* value cutoff, .05) yielding sets of approximately 1,104 up- and downregulated genes. HUGO gene symbol IDs for these genes were checked for occurrence in the *OCT4*-specific target lists generated by Boyer et al. [14] and Loh et al. [15]. The complete lists of direct and indirect targets are given in supplemental online Tables 6 and 7.

Online Database

To enable a global overview of altered gene expression at 24 and 72 hours post-transfection that can be interrogated, we have presented the expression data as a database for searching for expression levels of specific genes and their related gene ontologies (<http://goblet.molgen.mpg.de/cgi-bin/stemcell/pluripotency.cgi>). We used gene on-

tology terminology taken from the Gene Ontology website (<http://www.geneontology.org>). This was imported into the SQLite database (<http://www.sqlite.org>). Data analysis was carried out using R statistics software (<http://www.r-project.org>).

RESULTS

Depletion of OCT4 in Human Embryonic Stem H1 Cells Using siRNA Transfection

To identify factors associated with early stages in human ESC and trophoblast differentiation, we have combined RNAi and microarray based expression profiling to analyze global changes in gene expression induced by suppression of OCT4 function. Triplicate H1 ESC cultures were transfected with either *OCT4* or control *EGFP* siRNA oligonucleotides, and RNA samples were collected at 24 hours, when the very earliest changes in cell morphology resulting from Oct4 knockdown are first apparent, and at 72 hours, when the appearance of enlarged nuclei and flattening of cells predominates within the cultures (Fig. 1A, 1C, 1D). These morphological changes were accompanied by a reduction in the level of the protein as determined by Western blotting (Fig. 1B) and immunocytochemistry (Fig. 1C). In line with previous experiments, *OCT4* siRNAs reduced OCT4 protein levels to less than 30% of the *EGFP* siRNA controls, consistent with transfection efficiency of more than 70% [9]. *OCT4* knockdown was also accompanied by a marked increase in expression of the trophoblast marker cytokeratin 18 and incorporation of the protein into 10-nm filamentous structures typical of an epithelial cell type (Fig. 1D) [20, 23].

Global Expression Analysis

For each of three transfections harvested at 24 and 72 hours, triplicate RNA samples were labeled with either Cy3 or Cy5, including dye swap. Transcription profiles were then generated by using a cDNA microarray (Ensembl Chip) consisting of 15,529 resequenced and annotated clones. The overall correlations between the *OCT4* and *EGFP* RNAi expression data were 0.993 (24 hours) and 0.943 (72 hours), indicating that the expression levels of the vast majority of genes remain unaltered by the procedures. To judge whether a gene is expressed in the respective cells, we computed a background (BG) tag for its signal. This number reflects the proportion of background signal lower than the actual signal [20]. Typically, a BG tag of 0.9 indicates detectable probe signals (Fig. 2B, bottom panel). Using this criterion, we found that 1,038 (6.63%) of genes represented on the chip (probes) were expressed solely at the 24-hour stage, whereas 518 (3.31%) were expressed exclusively at the 72-hour stage, consistent with a restriction of gene expression and therefore developmental competence upon stem cell differentiation [24]. The vast majority of genes were either expressed at both time points (5,923 genes; 37.83%) or not expressed at all (8,178 genes; 52.23%). The full lists of genes expressed solely at 24 and 72 hours after transfection and also at both time points, together with the corresponding ratios, are presented in supplemental online Tables 1, 2, and 3, respectively. For a global overview of the transcriptional changes resulting from the loss of *OCT4* function and insights into the physiological state of human ESCs (hESCs) resulting from *OCT4* depletion, we combined the expression data at the 24- and 72-hour time points in an online database for interrogating the expression levels of specific genes and their related gene ontologies (<http://goblet.molgen.mpg.de/cgi-bin/stemcell/pluripotency.cgi>).

To identify genes with altered expression levels as a result of *OCT4* knockdown, we compared the 24- and 72-hour replicates, with *EGFP* knockdown at the same time points using

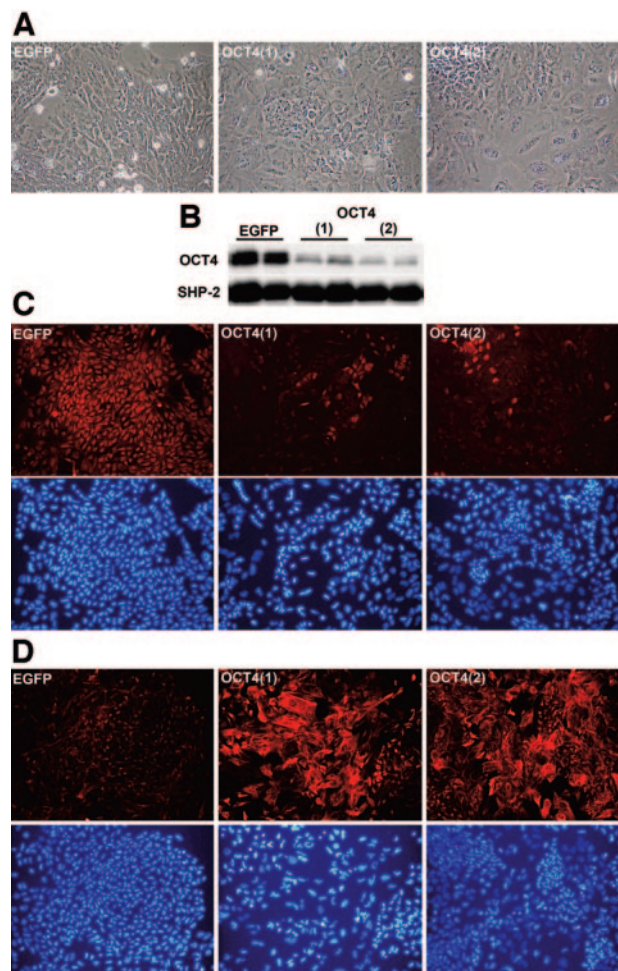


Figure 1. Downregulation of *OCT4* mRNA and protein in H1 human ESCs (hESCs) by small interfering RNA (siRNA) transfection. (A): Morphology of hESCs 72 hours after transfection with *EGFP*, *OCT4* (1), and *OCT4* (2) siRNA. (B): Western blot analysis of OCT4 protein levels in siRNA-transfected ESCs. Cell lysates prepared from duplicate siRNA transfections were fractionated by SDS-polyacrylamide gel electrophoresis, immunoblotted, and probed with OCT4 and SHP-2 (control) antibodies. The reduction in OCT4 protein in *OCT4* (1) and *OCT4* (2) transfections was approximately 30% of the control *EGFP* transfection. (C): OCT4 expression in siRNA-transfected cells. Cells were fixed 72 hours after transfection, immunostained for OCT4, and counterstained with 4,6-diamidino-2-phenylindole (DAPI). (D): Cytokeratin 18 expression in siRNA-transfected hESCs. Cells were fixed 72 hours after transfection, immunostained for cytokeratin 18, and counterstained with DAPI. Abbreviation: EGFP, enhanced green fluorescent protein.

statistical tests for differential expression (described in Materials and Methods). Three distinct tests (Student's *t* test, the Welch test, and Wilcoxon's rank sum test) were used to minimize individual bias [22]. By adopting this approach, we identified a subset of 1,104 marker genes from the 72-hour time point. Of these marker genes, 399 (36%) were downregulated, and another 705 were upregulated. Both sets of marker genes were at a significance level of .05. For example, among these differentially regulated marker genes are previously characterized downstream targets of OCT4, such as the pluripotency markers *NANOG* and *SOX2* and markers of undifferentiated stem cells *ZFP42*, *LEFTY1*, *LEFTY2*, *DPPA4*, *THY1*, *FLJ10884*, and *TDGF1*. The negatively regulated genes included *EOMES*, *BMP4*, *fibroblast growth factor 8 (FGF8)*,

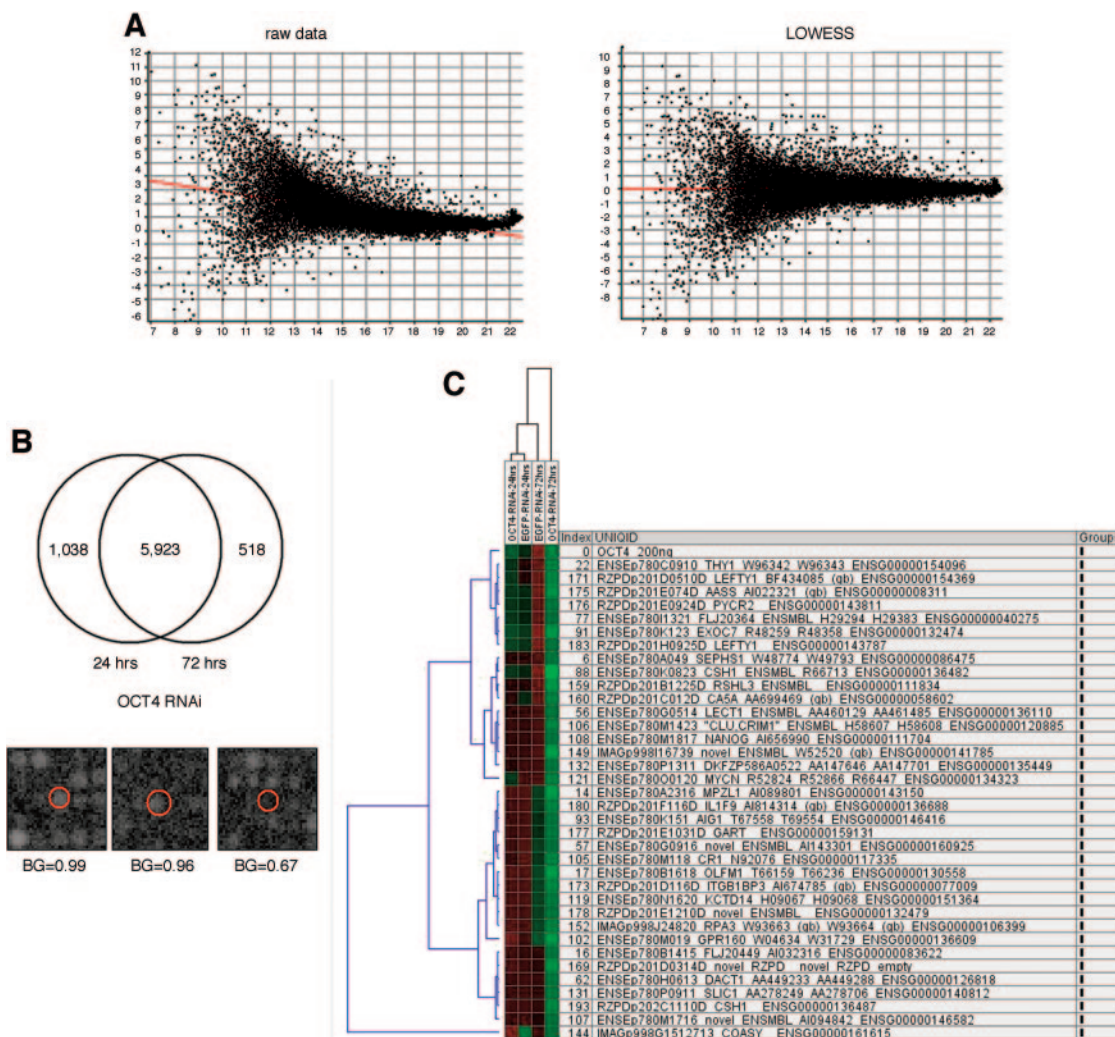


Figure 2. Global data analysis. (A): Effect of LOWESS normalization. Plotted are the ratios of the red and green signals for each spot (log scale, y-axis) and the signal range (log scale, x-axis) of a typical experiment. Whereas the raw data show a nonlinear bias in particular at the extremes of the signal area (top graph), after LOWESS normalization, this bias was eliminated (bottom graph). (B): Venn diagram of genes expressed at the different time points of *OCT4* knockdown. Gene expression was judged by a numerical value (BG tag) computed from a negative control sample (bottom panel). (C): Cluster of genes that show expression patterns most similar to that of *OCT4* across the experimental conditions. Colors correspond to normalized signals. For each gene, signals were divided by the average gene signal across all conditions (log scale). Red boxes indicate that the signal in the particular condition is higher than the average signal, whereas green boxes indicate the opposite. Hierarchical clustering was performed on 199 genes that showed high variation across the four conditions and a significant difference in the *OCT4* and *EGFP* RNAis using Pearson correlation as a pairwise similarity measure and average linkage as an update rule. The analysis was done using J-Express Pro 2.6 software (MolMine AS, Bergen, Norway <http://www.molmine.com>). Abbreviations: BG, background; EGFP, enhanced green fluorescent protein; hrs, hours; RNAi, RNA interference.

DKK1, *HLX1*, *GATA2*, *GATA6*, *ID2*, and *DLX5*, which are implicated in differentiation processes [14, 15], as well as a large number of novel genes. These differentially regulated genes are listed in supplemental online Table 4. Furthermore, we computed Q values for each of the genes to assess statistical significance by the false discovery rate (FDR) [25]. Using an FDR level of 0.05 identifies 721 of the more than 1,104 genes as significant. However, it should be stressed that FDR assessment can also increase the false-negative rate. For example, *ID2*, a differentiation marker directly regulated by *OCT4* (supplemental online Table 6; Fig. 3) and verified as significant by *p* value computation, was rejected by FDR assessment. At an FDR level of 0.1, all genes were marked significant.

By comparing gene expression profiles from 24- and 72-hour-post-transfection samples, we selected genes that show changes in expression similar to those of the selected key genes

OCT4, *EOMES*, and *BMP4*. The *OCT4* hierarchical cluster is shown in Figure 2C and includes genes already implicated in the maintenance of pluripotency and self-renewal, such as *NANOG*; markers of undifferentiated stem cells *LEFTY1*, *LEFTY2*, *DPPA4*, and *THY1*; and novel genes.

To have an overview of negatively regulated downstream targets of *OCT4*, we repeated the profile analysis for *EOMES* and *BMP4*, respectively (supplemental online Figs. 1 and 2). *EOMES* encodes a T-box containing transcription factor expressed in the trophoblast of human and mouse blastocyst and has been shown to be required for mouse trophoblast development and mesoderm formation [20, 26, 27]. The gene encoding bone morphogenetic protein 4 (*BMP4*) is also highly expressed in the human trophoblast [20] and has been shown to promote human embryonic stem cell differentiation into trophoblast [17]. The critical trophoblast stem cell regulator

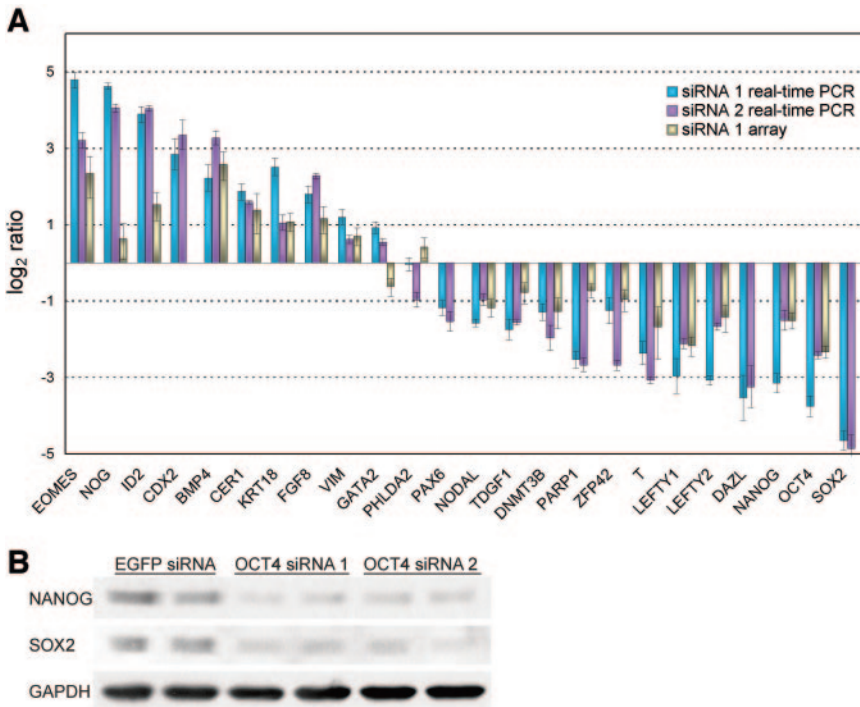


Figure 3. Confirmation of gene expression changes of selected genes by real-time PCR (A) and Western blotting (B). (A): SYBR green real-time reverse transcription-PCRs were carried out on RNA samples harvested 72 hours after *OCT4* knockdown using two independent siRNA molecules in separate transfection experiments (designated siRNA 1 and siRNA 2). Error bars refer to technical variation. Ratios are represented as log ratio (base 2), with values above 0 denoting overexpression and values below 0 denoting repression of gene expression. (B): Western blots using duplicate samples of protein extract of the same set of transfection experiments. The specificities of the primary antibodies used are indicated on the left. Abbreviations: EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; siRNA, small interfering RNA.

CDX2 could not be included in this analysis because the clone is not represented on our array. Nonetheless, real-time polymerase chain reaction (PCR) analysis showed that its expression is induced upon depletion of *OCT4* (Fig. 3).

Included in this set are genes implicated in ESC differentiation, such as (a) the transcription factors *ID3*, *TBX18*, *GATA6*, and *HLX1* [14]; (b) signal transduction pathways crucial for the maintenance of pluripotency, such as WNT (*DKK1*, *FRZB*, *FZD2*), transforming growth factor β (TGF β) (*TGF β 2*), and FGF (*FGF8*); and (c) cytoskeletal and extracellular matrix-associated genes, such as *MFAP5*, *COL4A1*, *KRT14*, *LOXL4*, and *CLDN4*, which encodes a tight junction protein crucial for trophoblast formation. These changes in gene expression overlap with those identified in BMP4-treated hESCs [17] and may reflect the morphological changes seen upon differentiation of hESCs per se and adoption of trophoblast fate (Fig. 1A, 1D).

Expression patterns of a selection of the differentially expressed genes were verified independently using real-time PCR and Western blotting of samples prepared from cells transfected with either of two different *OCT4* siRNAs (Fig. 3). The results shown in Figure 3A confirmed the downregulation of key pluripotency-controlling genes, *OCT4*, *SOX2*, and *NANOG*, and ES-associated genes *LEFTY1*, *LEFTY2*, *TDGF1*, *DNMT3B*, and *ZFP42*.

Expression of mesodermal (*T/Brachyury*) and ectodermal (*PAX6*) markers was also downregulated, re-emphasizing the lineage-restricted differentiation of *OCT4*-depleted cells. The induction of *CDX2* is in agreement with results obtained in mouse and human ESCs [8, 10], but it differs from our previous results with hESCs grown in mouse embryonic fibroblast (MEF)-conditioned medium, presumably reflecting the improved sensitivity of the current reverse transcription (RT)-PCR assays and inherent variability in MEF-conditioned medium preparations [9]. Confirmation of gene expression characteristic of trophoblast lineage is supported by the upregulated expression of key markers, such as *EOMES*, *CDX2*, and *BMP4* [9, 10, 17, 20]. The list of primer sequences used for this assay is given in supplemental online Table 5. Western blot analysis (Fig. 3B) shows downregulation of the transcription factors *NANOG* and

SOX2 proteins upon *OCT4* knockdown, consistent with their cooperation with *OCT4* as part of an interdependent core hES transcriptional regulatory circuit [14, 15].

Downstream Targets of *OCT4*

Our experimental approach will identify both direct and indirect targets downstream of *OCT4*. To distinguish between these possibilities, we cross-checked the *OCT4*-regulated genes with the recently compiled set of transcription targets for *OCT4*, *SOX2*, and *NANOG* identified in hESCs using chromatin immunoprecipitation (ChIP) coupled to promoter microarrays and ChIP-pair end diTag (PET) using mouse ESCs [14, 15]. For the analysis, we defined direct targets as those regulated by *OCT4* alone or in combination with *NANOG* and/or *SOX2*. We found poor overlap between the three data sets, with 60 genes common to our data set and that of Boyer et al. [14], 49 common to ours and the mouse data set [15], and nine common to all three data sets (supplemental online Fig. 3). Included in the nine genes are *OCT4*, *NANOG*, and *SOX2* and the trophoblast inducer *EOMES*. Interestingly, *CDX2* was a conserved target in human [14] and not mouse [15].

The list of direct targets of *OCT4* in ESCs is given in supplemental online Table 6. An additional 162 are bound by *NANOG* and/or *SOX2* but not by *OCT4*, indicating that these are regulated indirectly by *OCT4* through its regulation of these downstream effectors (supplemental online Table 7).

In line with the finding that *OCT4* regulates its own expression and that of other core hES transcription factors [14, 28, 29], it was not surprising that the subset of directly downregulated genes included *NANOG*, *OCT4*, *SOX2*, *HMGB2*, and *NR2F2* (supplemental online Table 6). Interestingly, the promoters for *HMGB2*, a coactivator of *OCT4* activity, and *NR2F2*, a regulator of *OCT4* expression [30, 31], are positively and negatively regulated, respectively, by *OCT4* alone. Additional markers of undifferentiated stem cells identified as positively regulated direct targets were *LEFTY2*, *DPPA4*, and *TDGF1*, whereas *ZFP42*, *LEFTY1*, and *FLJ10884* were classified as indirect targets. As anticipated, components of signal transduction path-

Table 1. Pathways of which gene components show significant expression changes between the OCT4 and EGFP knockdowns at 72 hours after transfection

KEGGID	Pathway description	Genes ^a	Z score ^b	p value ^c	OCT4-RNAi-UP ^d	EGFP-RNAi-UP ^e
hsa04610	Complement and coagulation cascades	25	2.973214	0.001473563	18	7
hsa00230	Purine metabolism	58	2.914994	0.001778544	22	36
hsa04510	Focal adhesion	64	2.708439	0.003380077	43	21
hsa04810	Regulation of actin cytoskeleton	69	2.6696	0.003797128	45	24
hsa04080	Neuroactive ligand-receptor interaction	49	2.660901	0.003896637	30	19
hsa03050	Proteasome	18	2.591246	0.004781487	4	14
hsa00190	Oxidative phosphorylation	58	2.582073	0.004910471	23	35
hsa04010	MAPK signaling pathway	92	2.476518	0.006633556	55	37
hsa04512	ECM-receptor interaction	27	2.25835	0.011961886	18	9
hsa00590	Prostaglandin and leukotriene metabolism	8	2.240448	0.012530885	7	1
hsa04520	Adherens junction	27	2.234325	0.012730806	19	8
hsa00251	Glutamate metabolism	16	2.171768	0.01493653	4	12
hsa00252	Alanine and aspartate metabolism	7	2.02837	0.021261181	1	6
hsa05110	Cholera: infection	13	1.991741	0.023199671	9	4
hsa00710	Carbon fixation	8	1.820364	0.034351738	2	6
hsa00400	Phenylalanine, tyrosine, and tryptophan biosynthesis	5	1.75292	0.039807826	1	4
hsa04630	Jak-STAT signaling pathway	40	1.693603	0.045170345	26	14
hsa00970	Aminoacyl-tRNA biosynthesis	9	1.599342	0.054872314	2	7
hsa04310	Wnt signaling pathway	52	1.575497	0.05757089	31	21
hsa04350	TGF- β signaling pathway	28	1.525685	0.063544186	18	10
hsa00440	Aminophosphonate metabolism	7	1.521278	0.064095066	2	5
hsa00052	Galactose metabolism	10	1.477977	0.069707001	8	2
hsa00260	Glycine, serine, and threonine metabolism	17	1.443812	0.074395975	5	12
hsa04110	Cell cycle	45	1.439163	0.075052216	21	24
hsa00640	Propanoate metabolism	15	1.306312	0.095723304	9	6
hsa04210	Apoptosis	30	1.30609	0.095761041	18	12

Pathways were taken from the KEGG database. Gene expression was compared in the *OCT4* RNAi and *EGFP* RNAi at 72 hours, and the differences of array signals were used for computing Wilcoxon's paired signed rank test. Genes that were judged as nondetectable by the background value criterion were excluded from analysis. Shown are pathways with gene numbers >4, but it should be noted that the normal approximation is valid for sample sizes >25. The complete list of the pathway results is given in supplemental online Table 8.

^a Number of genes that were taken into account for computing the statistical test.

^b Standard normal approximation of the test statistic.

^c *p* value of the standard normal distribution for the respective *Z* score.

^d Number of genes that have higher expression in *OCT4* knockdown than in *EGFP* knockdown.

^e Number of genes that have higher expression in the *EGFP* knockdown than in the *OCT4* knockdown.

Abbreviations: ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; KEGGID, KEGG pathway identifier (*Homo sapiens*); MAPK, mitogen-activated protein kinase; RNAi, RNA interference.

ways implicated in the maintenance of pluripotency, such as WNT (*DKK1*, *FZD2*), TGF β (*NODAL*, *LEFTY1*, *LEFTY2*, *MADH3*, *ID2* and *PITX2*), FGF (*FGF8* and *FGF2*), and Hedgehog (*PTCH*), are regulated by OCT4.

The remaining genes that are either up- or downregulated upon OCT4 knockdown but do not appear in the data sets of Boyer et al. [14] and Loh [15] may represent either previously undiscovered novel OCT4/SOX2/NANOG targets or genes regulated by downstream targets of OCT4 other than SOX2 and NANOG or simply not included in the promoter analysis (supplemental online Table 4). Moreover, this set of genes would also be expected to include a large number of genes involved in trophoblast differentiation.

Signaling and Metabolic Pathways Crucial for the Maintenance of Pluripotency

ESC self-renewal and pluripotency requires inputs from extrinsic factors and their downstream effectors [32]. We therefore analyzed the 24- and 72-hour data set for components of signaling pathways by assigning *p* values using Wilcoxon's matched pair signed rank test to compare groups of genes associated with particular pathways instead of conventional gene-wise analysis [20]. The analysis, summarized in Table 1, identifies changes in key components of the WNT, transforming growth factor (TGF) β , fibroblast growth factor (EGF), mitogen-activated protein kinase, NOTCH, Hedgehog, JAK/STAT, and extracellular matrix signaling pathways, as well as regulators of the cytoskeleton, apoptosis, cell cycle, and metabolic processes,

such as oxidative phosphorylation, methionine metabolism, and folate biosynthesis. The list of complete KEGG annotated pathways identified as operative in ESCs is given in supplemental online Table 8.

TGF β and BMP Signaling

Consistent with recent reports identifying TGF β /ACTIVIN/NODAL signaling as critical for maintaining hESC pluripotency [33–35], expression of *NODAL* ligand, its coreceptor *TDGF1*, and the antagonists *LEFTY1* and *LEFTY2* is reduced following *OCT4* knockdown (Fig. 4C). Downregulation of *LEFTY1*, *LEFTY2*, and *NODAL* could result from them being SOX2/NANOG targets (supplemental online Tables 6 and 7) or through loss of autocrine NODAL signaling as hESCs differentiate [36]. In contrast, expression of *FST* (FOLLISTATIN), a potent ACTIVIN and weak BMP antagonist is significantly upregulated on *OCT4* knockdown (Fig. 4B). Since *FST* transcription can be induced by ACTIVIN A, this might point to increased ACTIVIN signaling triggered by OCT4 depletion, coincident with downregulation of the ACTIVIN A antagonist *TDGF1*. OCT4 knockdown increases expression of *BMP4* and its downstream target gene *ID2*, as well as reducing its antagonist *CHORDIN* (Fig. 4A), in line with the observation that BMP4 induces trophoblast differentiation in hESCs [17].

FGF Signaling

FGF signaling is known to play an essential role in preventing hESC differentiation [35, 37, 38], and reduced expression of the

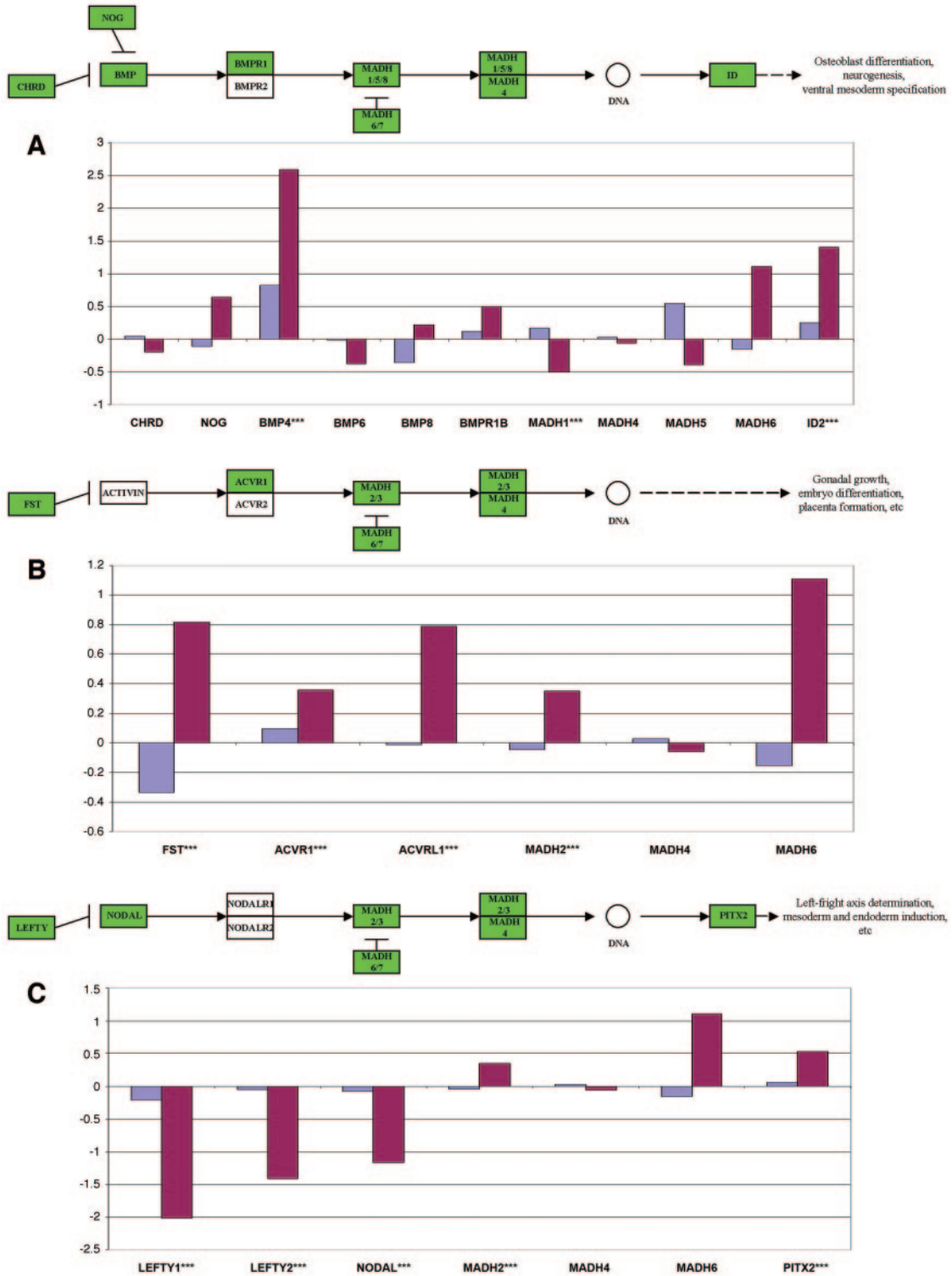


Figure 4. Comparative expression patterns of genes involved in the transforming growth factor- β signaling pathway. The BMP and ACTIVIN/NODAL axes of the pathway are presented in (A) and (B, C), respectively. A sequential illustration of the pathways (adapted from the KEGG database) is given above each histogram. Genes within open boxes are those with background tags >0.9, and therefore their expression was deemed as not detected. Abbreviation: BMP, bone morphogenetic protein.

hESC autocrine factor *FGF2* and *FGF12* was observed on OCT4 knockdown (Figs. 3, 5A). In contrast, however, expression of *FGF8* and the novel FGF-like receptor *FGFRL1* was upregulated, indicating that hESC differentiation along the tro-

phoblast lineage may involve complex modulation of FGF signaling. It may be significant that a potential NANOG binding site is identifiable within the *FGF8* promoter (supplemental online Table 7).

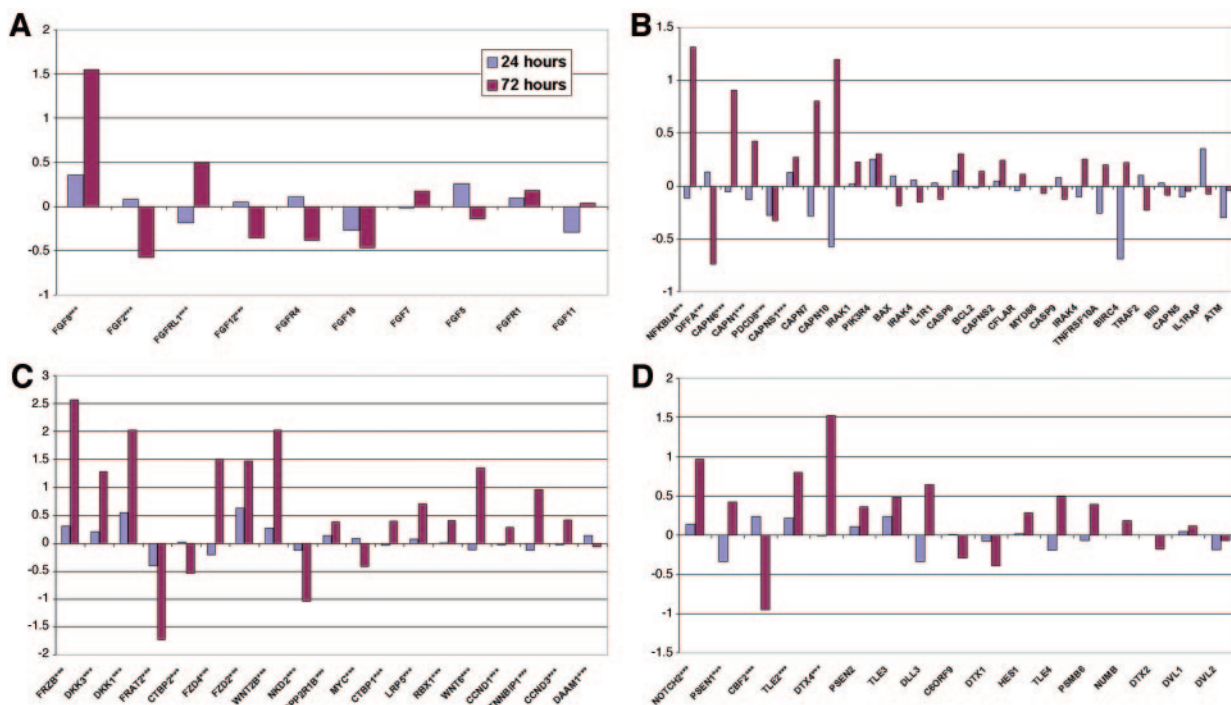


Figure 5. Comparative expression patterns of genes involved in FGF (A), apoptosis (B), WNT (C), and NOTCH (D) signaling pathways. Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor.

WNT Signaling

The role of WNT signaling in human ESCs is controversial because of conflicting reports as to its role in ESC self-renewal [39, 40]. Our analysis of this pathway shows that although expression of WNT antagonists *DKK1*, *DKK3*, and *FRZB* increases upon OCT4 knockdown, *FRAT2*, a GSK3 β -interacting positive effector of WNT signaling [41], is downregulated (Fig. 5C). The presence of OCT4, NANOG, and SOX2 binding sites within the *DKK1*, *FZD2*, and *FRAT2* promoters indicates that these WNT components are influenced directly by these key stem cell regulators (supplemental online Table 6).

NOTCH and Hedgehog Signaling

Although both these pathways regulate the fate of many types of stem cells, there is currently little evidence for their involvement in controlling the fate of embryonic stem cells [42]. Examination of NOTCH pathway components revealed downregulation of *CBF2* and upregulation of *NOTCH2*, *TLE2*, *DTX4*, and *HES1* upon OCT4 knockdown (Fig. 5D). *TLE2*, a transcriptional repressor [43], is regulated by NANOG and SOX2 (supplemental online Tables 6 and 7). Analysis of Hedgehog (Hh) signaling components showed reduced levels of both *PTCH1*, a Hh receptor and target for SOX2 and NANOG (supplemental online Table 7), and the Hh-regulated transcriptional repressor *GLI3*.

Apoptosis

Efficient growth of undifferentiated ESCs (self-renewal) requires the inhibition of apoptosis, as well as differentiation [44]; we therefore screened the data set for differentially expressed apoptosis-related genes (Fig. 5B). Although expression levels of the pro- and antiapoptotic genes *BCL2* and *BAX* did not change, genes encoding the DNA fragmentation factor (*DFFA*), the apoptosis-inducing factor (*PDCD8*), and *CASPASE 3* were significantly downregulated, whereas *NFKBIA* and the calpains *CAPN6*, *CAPN1*, and *CAPNS1* were upregulated on OCT4 knockdown.

Epigenetic Control of Pluripotency and Trophoblast Lineage Specification

Mutant mouse ESCs lacking DNA methyltransferase activity [45, 46], methyl DNA binding protein function [47], or histone acetylase activity [48] exhibit impaired differentiation, highlighting the critical role chromatin modification plays in regulating embryonic differentiation. To investigate whether the expression of chromatin and epigenetic modifiers is affected by OCT4 depletion, we identified differentially regulated genes involved in methyl/folate cycle, DNA methylation, methyl DNA binding, and histone modification communication (supplemental online Fig. 4). Downregulation of *MAT2A* (methionine adenosyltransferase II α) and *MTRR* (5-methyltetrahydrofolate-homocysteine methyltransferase) was observed at 72 hours of OCT4 depletion (supplemental online Fig. 4A), supporting the involvement of folic acid metabolism in the maintenance of pluripotency [49]. The de novo methyltransferase *DNMT3B* was downregulated upon OCT4 depletion, contrasting with the maintenance methylase *DNMT1*, which showed no significant change. The histone lysine methyltransferase (H3-K4-HMTase) *SET7* was dramatically upregulated upon OCT4 knockdown, contrasting with downregulation of the histone lysine methyltransferase *EZH2* (H3-K27-HMTase) (supplemental online Fig. 4B). The acetylases *H2AFY* and *H2AFY2* and the deacetylase *HDAC6* also show significant upregulation on OCT4 knockdown (supplemental online Fig. 4C, 4D). These expression patterns perhaps indicate that the HES-to-trophoblast transition is accompanied by significant changes in histone acetylation and methylation patterns.

The nonhistone chromatin-associated proteins *HMGB1*, *HMGB3*, *DPPA4*, *NASP*, chromatin assembly factor 1 (*CHAF1A*), *PHF17*, *PHF5A*, *POLE3*, and retinoblastoma binding protein 7 (*RBBP7*) are all significantly downregulated as a consequence of OCT4 depletion (supplemental online Fig. 4B). Of these, *HMGB1*, *DPPA4*, and *HMGB3* have previously been shown to be highly enriched in undifferentiated stem cells and

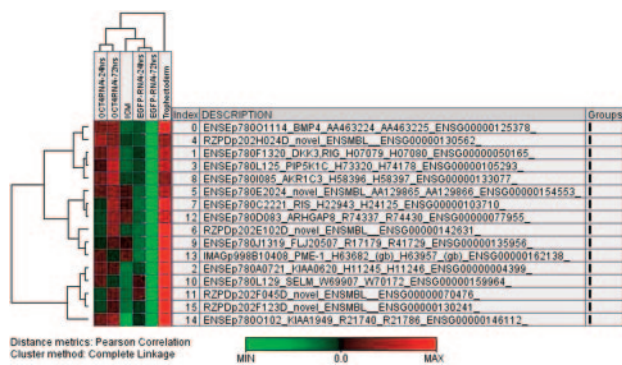


Figure 6. Cluster analysis of 15 genes bearing expression profiles the most similar to that of the trophoblast marker *BMP4*. The comparison was made between this study and the previously published expression data on human ICM and trophoblast cells [20]. Colors correspond to normalized signals. For each gene, signals were divided by the average gene signal across all conditions (log scale). Red boxes indicate that the signal in the particular condition is higher than the average signal, whereas green boxes indicate the opposite. Hierarchical clustering displays subgroups using Pearson correlation as a pairwise similarity measure and complete linkage as an update rule. The analysis was done using J-Express Pro 2.6 software (MolMine AS). Abbreviations: EGFP, enhanced green fluorescent protein; ICM, inner cell mass; MAX, maximum; MIN, minimum; RNAi, RNA interference.

isolated ICM cells [20]. The RBBP7-encoded protein has been shown to interact with MBD3 and may have a role in the regulation of cell proliferation and differentiation [50].

Interestingly, we also observed downregulated expression of *PARP1* (Fig. 3), which encodes a chromatin-associated enzyme, poly(ADP-ribosyl) transferase, capable of modifying nuclear proteins. This DNA-dependent ribosylation has been shown to regulate cell proliferation, transformation, and differentiation [51]. Indeed, *Parp1*-deficient mouse embryonic stem cells differentiate more readily into trophoblast-like cells, implying that one of the functions of this protein is to restrict differentiation in this lineage [52].

Altered expression of imprinted genes was also observed (upregulation of *CALCR* and *GNAS* and downregulation of *KIP2* and *UBE3A*; supplemental online Fig. 4E). *KIP2* (a cyclin-dependent kinase inhibitor) acts as a key regulator of embryogenesis through regulation of cell cycle by blocking the activity of G1 cyclin/Cdk complexes and the regulation of actin dynamics through binding to LIMK-1 [53, 54]. Differential gene expression of other cell cycle-related genes was also observed (supplemental online Fig. 5), including the upregulation of *CDKN2B*, a cyclin-dependent kinase inhibitor, and downregulation of *CDC25A*, which is required for progression from G1 to the S-phase of the cell cycle, consistent with the limited G1 phase and rapid cell cycle characteristic of undifferentiated ESCs [55, 56].

RNAi-Mediated Suppression of OCT4 Function in Human ESCs Recapitulates Primary Differentiation at the Blastocyst Stage of Development

To examine how closely the transcriptomes of an ICM and trophoblast (TE) cell matches that of an undifferentiated ESC and *OCT4* RNAi-mediated trophoblast cell, we compared our current data set with that derived from the blastocyst [20]. We identified potential candidate genes that were overexpressed in the TE- and *OCT4*-deficient ESCs compared with the ICM and undifferentiated ESCs when a cluster analysis of genes coregulated in the same manner as *BMP4* was performed (Fig. 6). Genes upregulated in the TE- as well as the *OCT4*-depleted hESCs are involved in the organization of the extracellular

matrix (*PDLIM3*), cell growth and differentiation (*AKR1C3* and *PLXND1*), transcriptional regulation (*PME-1*, *MGC11349/ZXDC*, and *KIAA1245/COASI*), signal transduction processes (*ARL7*, *PIP5K1C*, *RASL12*, *ARHGAP8*, *SELM*, and *DKK3*, an inhibitor of the WNT pathway), and novel genes (*KIAA1949*, *C14orf173*, and *FLJ20507/TMEM127*). We do not present detailed analysis of this data set here for the simple reason that the vast amount of data is beyond the scope of this study. In summary, these results would imply that these TE marker genes could serve as additional factors required for inducing trophoblast differentiation and further propagation of these cells.

DISCUSSION

The first differentiation event in mammalian development is the formation of the trophoblast; this event is controlled by antagonism between *OCT4* and the trophoblast transcription factors *CDX2* and *EOMES* [27]. To begin dissecting the complex molecular events that underpin this event in humans, we adopted a functional genomics approach using RNAi to suppress *OCT4* function in a human ESC line and microarray-based gene expression profiling. The quality of the data set was rigorously tested in several ways, including numerous statistical tests, achieving consistent reproducibility between replicates as measured by the coefficient of variations of intensity for each gene. The data set is therefore qualitative, quantitative, and comprehensive.

Our microarray analysis shows that *OCT4* knockdown in hESCs is accompanied by a reduction in overall complexity of gene expression and differential regulation of more than 1,000 genes, many of which are consistent with a loss of pluripotency and specification of the trophoblast lineage. Among the differentially expressed genes, 60 correspond to direct *OCT4* targets identified in human ESCs [14], 49 to targets in mouse ESCs [15], and nine (including *OCT4*, *SOX2*, *NANOG*, and *EOMES*) to targets identified as conserved in human and mouse ESCs. An additional set of 162 consists of targets for *SOX2* and *NANOG*, but not *OCT4* [14, 15]. This pattern is compatible with *OCT4*, *SOX2*, and *NANOG* constituting an interdependent network, where loss of expression of one factor ultimately leads to the extinction of the others. A differentiation pathway may depend on factor-specific interactions (for example, between *OCT4*, *EOMES*, and *CDX2* [27]) or other genes. The overall low overlap may be attributed to the differences in the platforms used (RNAi, ChIP-chip, and ChIP-PET) and also the transcription factor binding sites being mapped. In addition, off-target indirect effects induced by the *OCT4*-RNAi manipulation in our study and that in the mouse [15] may have contributed to the disparity between our list of *OCT4* targets. Nonetheless, we cannot exclude the possibility of species differences in the mode of regulation of pluripotency and self-renewal of ESCs. Indeed, our analysis of changes in gene expression and gene ontologies highlights the involvement of cell signaling interactions, epigenetic modifications, chromatin remodeling, and metabolic processes in the ES-to-trophoblast transition.

Examination of differentially expressed components of signal transduction pathways supports previous findings demonstrating the importance of TGF β , BMP, and FGF signaling in regulating hESC differentiation [18, 35]. *NODAL*, its coreceptor *TDGF1*, and transcriptional targets, *LEFTY1/2* were downregulated upon *OCT4* knockdown, whereas the ACTIVIN-regulated antagonist Follistatin was upregulated. This might reflect a switch from *NODAL*/*TDGF1* to ACTIVIN signaling during trophoblast differentiation and would be compatible with the recently reported involvement of ACTIVIN in trophoblast pro-

liferation in mouse [57, 58]. BMP4, an inducer and marker of trophoblast, was upregulated upon OCT4 knockdown within 24 hours. Given the importance of this factor in regulating ESCs from both mouse and human ESCs, it is notable that the BMP4 promoter was not identified as a target of OCT4, nor of SOX2 or NANOG, indicating that the induction could be mediated indirectly [14]. This could entail a cascade involving GATA6, a target of OCT4 and NANOG in hESCs and regulator of BMP4 expression [59] or NANOG [60]. FGF2 expression is downregulated upon OCT4 knockdown, consistent with FGF signaling blocking hESC differentiation. However, the induction of FGF8 also points to a likely role for this pathway in trophoblast differentiation. Indeed, induction of components of NOTCH signaling upon OCT4 knockdown could mediate aspects of FGF signaling to promote the generation of trophoblast progenitors [61], in a manner related to that described for neural differentiation in ESCs and somatic progenitor cells [62, 63]. Many signaling pathways regulate cell survival, as well as differentiation, and it was therefore interesting to find that some proapoptotic genes, including *CASPASE 3*, were downregulated on OCT4 knockdown, supporting the notion that ESCs may be inherently poised for apoptosis because of conflict between signals maintaining the balance between self-renewal and differentiation [16, 44].

OCT4 downregulation also had significant effects on expression of epigenetic and chromatin modifiers. The reciprocal expression patterns of the histone lysine methyltransferases *SET7* (up) and *EZH2* (down), as well as downregulation of the DNA methyltransferase *DNMT3B* and a number of histone and nonhistone chromatin-associated proteins, suggest that loss of pluripotency is associated with global changes in chromatin organization [64]. These changes may be actively involved in ESC differentiation per se, but they may, in addition, specifically reflect the generation of an extraembryonic cell type.

As anticipated, the list of OCT4-dependent genes includes as yet uncharacterized novel genes that may represent candidates for further investigation into pluripotency and trophoblast

differentiation. Our data provide a reference for combining RNAi and functional genomics using human ESCs to study some of the earliest differentiation events of human postimplantation development. This is supported by the finding that the trophoblast marker BMP4 is enriched in both the TE- and the OCT4-depleted cells at both the 24- and 72-hour time points. Furthermore, the global analysis (data not shown) revealed distinct and overlapping expression patterns between the ICM and undifferentiated ESCs, thus implying that the ICM consist of a transit population of pluripotent cells and that cultured ESCs are an in vitro adaptation of these. Alternatively, the differences between the two data sets may be explained by experimental limitations associated with the scarce human embryo material [20]. Detailed analysis and confirmation of these differences are under way and therefore not included here, as they are beyond the scope of the current study. This type of undertaking should also contribute to the construction of a molecular framework that facilitates robust and predicable control of ESC differentiation and their successful and safe application in stem cell-based therapy in the future.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Analysis of Oct4-Dependent Transcriptional Networks Regulating Self-Renewal and Pluripotency in Human Embryonic Stem Cells

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