Manipulation of *OCT4* Levels in Human Embryonic Stem Cells Results in Induction of Differential Cell Types

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To fully understand self-renewal and pluripotency and their regulation in human embryonic stem cells (hESCs), it is necessary to generate genetically modified cells and analyze the consequences of elevated and reduced expression of genes. Genes expressed in hESCs using plasmid vectors, however, are subject to silencing. Moreover, hESCs have a low plating efficiency when dissociated to single cells, making creation of subcloned lines inefficient. In addition to overexpression experiments, it is important to perform loss-of-function studies, which can be achieved rapidly using RNA interference (RNAi). We report stable long-term expression of enhanced green fluorescent protein (eGFP) in hESCs using a lentiviral vector, and establishment of an eGFP-expressing subline (RG6) using manual dissection. To demonstrate the efficacy of RNAi in hESCs, an RNAi expression vector was used to achieve reduced expression of eGFP in hESCs. To evaluate the role of *OCT4* in the regulation of hESC self-renewal and differentiation, a vector expressing a hairpin RNA targeting endogenous expression of *OCT4* was constructed. In a novel experiment in hESCs, the *OCT4* cDNA sequence was cloned into an expression vector to allow for the transient upregulation of *OCT4* in hESCs. The ability to manipulate levels of *OCT4* above and below enodogenous levels allows the determination of *OCT4* function in hESCs. Specifically, reduced expression of *OCT4* in hESCs promoted upregulation of markers indicative of mesoderm and endoderm differentiation, and elevated levels of *OCT4* in hESCs promoted upregulation of markers indicative of endoderm derivatives. Thus, both upregulation and downregulation of Oct4 in hESCs results in differentiation, but with patterns distinct from parallel experiments in mice. Exp Biol Med 232:1368–1380, 2007

Key words: stem cell; self-renewal; differentiation; knockdown; overexpression

Introduction

Identification of the program regulating the maintenance of pluripotency for human embryonic stem cells (hESCs) is vital to the use of hESCs as a model of human development and the evolution of hESC-based cellular therapies. The POU family transcription factor *Oct4* (*Pou5f1*) is essential for early mammalian development, and it is likely central to the regulation of self-renewal in hESCs. In the murine embryo, expression of *Oct4* is necessary for development beyond the blastocyst stage, and *Oct4* is known to regulate pluripotent cell growth in both the mouse embryo and mouse embryonic stem cells (mESCs; Refs. 1–3). *Oct4* expression can be detected in all blastomeres of the murine embryo, and it later becomes restricted to the inner cell mass (ICM) cells of the blastocyst (3). Similar expression patterns also exist in human blastocysts (4). A population of cells that migrate along the inner trophoblast layer in the mouse embryo also transiently upregulate *Oct4* expression. The transient

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expression of *Oct4* subsides, and subsequently these cells differentiate into primitive endoderm (3). These expression patterns have been elegantly defined *in vitro* in mESCs (2). Murine embryos lacking *Oct4* develop to blastocysts, but ICM cells differentiate to trophectoderm and are no longer pluripotent (1). Inducible *Oct4* expression has been used to define levels necessary for maintenance of pluripotency in mESCs (2). Evidence suggests that a less than 2-fold increase over endogenous levels of *Oct4* results in differentiation of mESCs to primitive endoderm and mesoderm (2). In contrast, cells that have reduced levels of the *Oct4* protein express markers indicative of trophectoderm (2). Similarly, recent experiments have shown that RNA interference (RNAi) technology targeting *Oct4* can recapitulate trophectoderm differentiation in mESCs (5, 6), and hESCs (6, 7).

Given the inherent differences among species, it is valuable to consider similarities and differences between *Oct4* gene expression in mESCs and hESCs. Although genetic modification of hESCs is necessary to fully explore the factors that regulate self-renewal, unique properties of hESCs make these studies difficult. For overexpression studies, stable transgene expression is useful. Previous studies in our lab and other labs have established that expression of genes in plasmid vectors is subject to significant silencing in hESCs. The drastic reduction in viability of hESCs that have been dissociated to single cells also renders traditional subcloning of genetically modified hESCs difficult (8). Moreover, hESCs have a low plating efficiency when dissociated to single cells (9). The problem of transgene silencing in hESCs can be addressed using lentiviral vectors, which are resistant to silencing, unlike plasmid or retroviral vectors (10–13). We and others (14) chose to use lentiviral vectors for stable expression of eGFP in hESCs because of their ability to efficiently transduce human cells and evade silencing, as has been shown in experiments with hematopoietic precursors (15). By fluorescently marking cells transduced with lentivirus, sublines can then be established by manual manipulation of hESC colonies. Although homologous recombination studies are not possible when studying human development *in vivo*, RNAi technology can be used with hESCs to gather information about gene function in hESCs as a model of the human embryo. Synthetic siRNAs offer an alternative to traditional targeted mutagenesis (knockout mutations) for testing gene function in mammalian cells (16, 17), as well as oocytes and preimplantation embryos (18). Recently, expression vectors have been used to deliver transgenic expression of short hairpin RNA (shRNA) molecules to mESC-derived embryos and effectively recapitulate a deletion phenotype (19). Further, a method of fluorescently marking cells expressing RNAi constructs was used that allowed monitoring of the effects of interference in the expression of genes in mESCs (5). Finally, RNAi has been used in hESCs to effectively diminish gene function (7, 20, 21).

In this study, lentiviral vectors, RNAi technology, and transient transgene overexpression are used in combination to explore the consequences of manipulating *OCT4* gene expression in hESCs. We demonstrate that the manipulation of *OCT4* levels in hESCs results in widespread differentiation of these cells, indicating that *OCT4* has a similar but not identical regulatory role in hESCs. Since interference with self-renewal would prevent the subcloning of undifferentiated hESCs, short-term expression studies were performed. RNAi was then used to evaluate the role of *OCT4* in the self-renewal of hESCs in short-term assays. We have constructed a plasmid vector containing a sequence encoding a hairpin RNA to target endogenous *OCT4* mRNA and demonstrated that decreased levels of *OCT4* in these cells leads to induction of endoderm and mesoderm differentiation. Furthermore, differentiation patterns appear divergent from the early differentiation pathways of the mouse embryo and mESCs. In addition, we demonstrate that novel transient upregulation of *OCT4* in hESCs leads to an induction of endodermal precursors. These data, in contrast to the OCT4 downregulation data, are consistent with similar experiments in mESCs. We conclude that enforced expression in combination with RNAi can be used to elucidate and confirm pathways in hESCs that parallel the regulation of self-renewal and differentiation in mESCs and early embryonic development.

Materials and Methods

Derivation and Maintenance of the HSF-6 Cell Line. The HSF-6 cell line (National Institutes of Health Registry designation UC06) was derived from an eight-cell embryo donated at Day 3 of culture by patients undergoing fertility treatments at University of California San Francisco (UCSF). Although it contained many cell fragments and the blastomeres varied in size, the embryo progressed to the blastocyst stage and hatched from the zona pellucida during 3 further days of culture in G2.2 medium (Vitrolife Inc., Englewood, CO). The ICM from the collapsed blastocyst was cultured on irradiated CF-1 mouse embryonic fibroblasts (MEFs) in hESC medium: knockout–Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA), 20% knockout serum replacer (Gibco), 4 μg/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 100 μ*M* nonessential amino acids (UCSF Cell Culture Facility, San Francisco, CA), 0.1 m*M* β-mercaptoethanol (Sigma, St. Louis, MO), and 1 m*M* glutamine (Gibco). The blastocyst attached and formed a colony of cells that resembled established human ESC lines within 1 week. The initial ICM outgrowth was passaged manually with a drawn Pasteur pipet and placement on fresh feeder layers. Subsequent passages were made by incubation with 1 μg/ml collagenase IV (Gibco) for 20 mins in hES medium at 37°C, vigorous pipetting to break colonies into clumps of approximately 100 cells, and two washes with ES medium. The HSF-6 line has been shown to express the stem cell–associated transcription factor *OCT4* and cell surface markers SSEA-3 and SSEA-4. HSF-6 differentiated *in vivo*

in teratoma assays and *in vitro* to the three germ lineages. Characterization of the HSF-6 line can be found at http://escells.ucsf.edu. The HSF-6 line has been maintained with a similar morphology and differentiation potential with a normal human XX karyotype for more than 70 passages and multiple freeze-thaw cycles.

Lentiviral Transduction of HSF-6 Cells with eGFP Transgene. Prior to infection, HSF-6 cells were maintained in six-well plates on irradiated feeders in ESC culture medium. After 3 days in culture, viral supernatant of SMPU-MND-EGFP was added to 2 ml ESC medium at a final concentration of 6.25×10^6 IFU/ml. The infection was carried out for 24 hrs, after which time cells were washed and cultured for several passages. Expression of eGFP was monitored by fluorescence microscopy (Nikon TS100; Technical Instruments, Burlingame, CA) and flow cytometry using a FACSCalibur flow cytometer (Beckton Dickinson, Mountain View, CA). A strategy for isolating eGFP-expressing sublines was employed in which areas of homogeneous expression within individual colonies were manually excised using a pulled Pasteur pipette and transferred to new plate of feeders containing hESC medium. These HSF-6/eGFP sublines were then cultured in hESC media on irradiated MEFs and were passaged manually until enough colonies exhibiting homogeneous GFP expression were present to passage using collagenase. The process of locating and isolating homogeneous eGFP-expressing colonies or portions of colonies was repeated until an expressing line was established. The cells were then assayed for eGFP expression by flow cytometric analysis and reverse transcription polymerase chain reaction (RT-PCR; Fig. 1B and C). The eGFP-expressing subline used for further studies was designated RG6.

Vector Construction and Electroporation. The pRed and pCH-eGFPi vectors have been described previously (5). To construct pCHOCTi, the target siRNA sequence corresponding to nucleotides 670–688 of the human OCT4 mRNA were chosen and cloned into the *Bgl*I and *Not*I sites of pCH-eGFPi after annealing the sense and antisense strand oligonulceotides 5′-GATCTGGGGCAAGCGATCAAGCAGCGATTCAAGAGACGTTCGCTAGTTCGTCGCTTTTTTGGGC-3′ and 5′-GGCCGCCCAAAAAAGCGACGAACTAGCGAACGTCTCTTGAATCGCTGCTTGATCGCTTCGCCCA-3′. To construct pOCT4-IRES-eGFP, total RNA was extracted from undifferentiated HSF-6 cells using RNeasy kit (Qiagen, Valencia, CA), 500 ng total RNA was used in reverse transcription reaction, and cDNA was generated with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Full-length *POU5F1* (NM_002701) cDNA was amplified in a reaction with Platinum Taq DNA polymerase (Invitrogen) using the following primers: forward 5′-GGCGCCTTCCTTCCCCATGG-3′ (nucleotides 41–60) and reverse 5′-CTCCCCCTGTCCCCCATTCCTAGA-3′ (nucleotides 1152–1175). Full-length cDNA was confirmed by agarose gel electrophoresis, and the fragment was

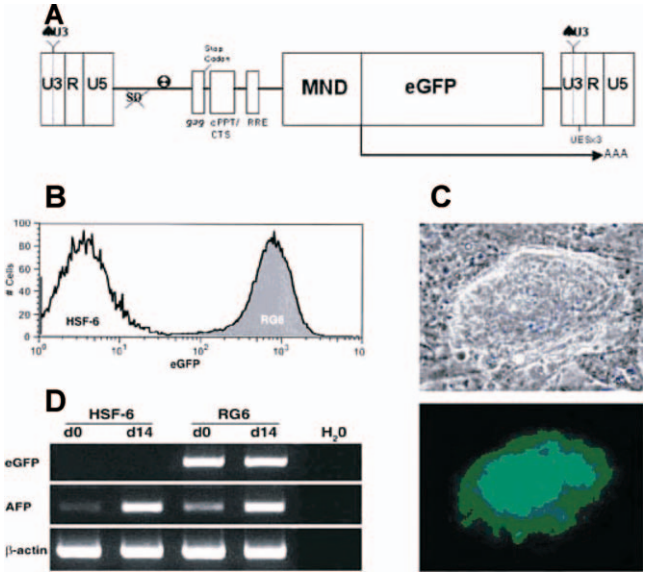

Figure 1. Introduction of the eGFP transgene into hESCs. (A) SMPU-MND-eGFP lentiviral transfer vector used to transduce HSF-6 cells with the eGFP transgene. The 5′ splice donor site has been eliminated (SD), and the central polypurine tract and central termination sequence (cPPT/CTS) have been reinserted to increase the rate and extent of proviral integration. Expression of the eGFP gene is driven off of the MND promoter. The 3′ LTR contains UE3 sequences from SV40 to augment polyadenylation and prevent read through of downstream cellular genes. (B) Flow cytometric analysis comparing RG6 and HSF-6 cells. The shaded peak shows RG6 cell counts, whereas the other peak shows HSF-6 cells. (C) Bright-field and fluorescent images of RG6 cells. Cells exhibit characteristic hESC morphology, and expression of eGFP can be seen throughout the colony. (D) RT-PCR of RG6 and HSF-6 cells. Amplifications were performed on cDNA synthesized from undifferentiated (d0) cells and embryoid bodies left in differentiation culture for 14 days (d14) from both cell lines. Differentiation patterns of RG6 and HSF-6 are similar, as indicated by AFP expression levels. Enhanced GFP expression by RG6 hESCs remains constant at d0 and d14. Beta-actin was included as a control.

subcloned into pCR-XL-TOPO (Invitrogen) to generate pCR-OCT4. To clone the OCT4 cDNA into pIRES2-eGFP (Clontech, Palo Alto, CA), pCR-OCT4 was digested with *Eco*RI, the digested fragments were separated by agarose gel electrophoresis, and a 1175-bp fragment was isolated. This fragment was then cloned into the *Eco*RI site of pIRES2-eGFP. The OCT4 cDNA sequence confirmed in sequencing reactions using the following primers: forward 5′-CGGTGGGAGGTCTATATAAGCA-3′; and reverse 5′-GGTACCGTCGACTGCAGAAT-3′.

RG6 and HSF-6 cells were cultured in six-well plates on irradiated CF-1 embryonic feeder fibroblasts, harvested with collagenase, and washed once with hESC medium and twice with phosphate-buffered saline (PBS). The resulting clumps of 50–100 hESCs were resuspended in 500 μl electroporation medium (hES medium containing 10% fetal bovine serum [FBS]; Hyclone, Logan, UT), 25 μg of the appropriate plasmid DNA was added, and the volume was equilibrated to 300 ml in PBS. The DNA and cells were combined and transferred to a 0.4-cm cuvette. The cells were then electroporated (Gene Pulser; BioRad, Hercules,

CA) at 280 V and 875 μF, and 1×10^6 cells were seeded in a six-well plate on irradiated CF-1 feeders and grown in electroporation medium. The medium was changed after 12 hrs of culture to hESC medium without fetal calf serum, and cells were grown for 7 days.

OCT4 Western Blot Electroporated HSF-6 cells were grown on MEF cells for 3, 4, and 7 days. At teach time point, cells were washed with PBS, and cell lysates were harvested by applying 300 μl lysis buffer (20 m*M* HEPES, 1.5 m*M* $MgCl_2$, 420 m*M* KCl, 0.2 m*M* EDTA, 25% glycerol, 20 m*M* phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 100 μg/ml leupeptin) directly to the cultures. Lysates were spun at 10,000 *g* for 5 mins. Protein concentration was determined using Bradford assay, and equal amounts of protein from each lysate were run on gradient 4%–15% Tris-HCL gel (BioRad). Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad), and the membrane was treated with both anti-OCT4 antibody (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH antibody (diluted 1:300; Chemicon International Inc., Temecula, CA) overnight. Secondary antibodies conjugated with horseradish peroxidase (Zymed, South San Francisco, CA) were then applied for 1 hr. The blot was visualized using ECL reagent (Amersham Biosciences, Piscataway, NJ). Intensity of OCT4 bands was determined for each lane and normalized to the corresponding intensity of GAPDH for each lane. The mean normalized intensity was calculated. Relative intensities of each OCT4 band are expressed as a ratio of normalized intensity to the mean normalized intensity.

OCT4 Immunohistochemistry. After electroporation, hESCs were grown on feeder cells on top of cover slips for 72 hrs. The cells were then fixed with 2% paraformaldehyde and permeabalized with 0.1% Triton X-100. Donkey serum (1%) in PBS was then applied to block any nonspecific binding. Primary antibody, goat anti-human OCT4 (diluted 1:100 in 1% donkey serum, 0.5% Triton X-100, in PBS; Santa Cruz Biotechnology) or goat Ig (diluted 1:200 in 1% donkey serum, 0.5% Triton X-100, in PBS) were applied for 1 hr. Cells were washed with PBS and treated with secondary antibody (donkey anti-goat Ig fluorescein isothiocyanate [FITC]; diluted 1:100 in 1% donkey serum, 0.5% Triton X-100). Cells were washed a final time with PBS and viewed with fluorescence microscopy.

RT-PCR. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and mRNA was extracted from the total RNA using the Oligotex mRNA Mini Kit (Qiagen) protocol. Messenger RNA was reverse transcribed into cDNA (Superscript II Reverse Transcriptase; Invitrogen) and subsequently subjected to PCR for the evaluation of the expression of eGFP mRNA. Amplification was performed using Taq DNA Polymerase (Invitrogen) under the following conditions: 93°C for 3 mins; 1 cycle; 93°C for 1 min, 55°C for 90 secs; 72°C for 1 min; for cycle numbers see Table 1; 72°C for 10 min; 1 cycle. RT-PCR primer information is in Table 1.

Semiquantitative RT-PCR. Total RNA was extracted from cells using the Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions, and mRNA was extracted from the total RNA using the Oligotex mRNA Mini Kit (Qiagen) protocol. RT-PCR was performed using the Superscript II Reverse Transcriptase (Invitrogen), and mRNA was reverse transcribed into cDNA and subsequently subjected to multiplex PCR for the semiquantitative evaluation of the mRNA expression of genes of interest. Amplification of genes of interest was performed with Taq DNA Polymerase (Invitrogen), and the expression of β-actin was used as an internal control. PCR products were resolved by electrophoresis on 1.5% agarose gel containing ethidium bromide. Gels were scanned (Nucleotech Inc., San Mateo, CA), and the bands were quantified using Gel Expert analysis software (Nucleotech Inc.). The level of gene expression was determined as a percentage of the β-actin expression. RT-PCR conditions 93°C for 3 mins: 1 cycle; 93°C for 1 min; 55°C for 90 secs; 72°C for 1 min: cycle number is dependent on gene-specific primer (Table 1); and 72°C for 10 mins: 1 cycle. RT-PCR primer information contained in Table 1.

Results

Creation of RG6 Subline. To establish stable genetically modified hESC lines, lentiviral vectors developed for the ability to evade silencing in human stem cells were used. Established HSF-6 cells cultured on CF-1–irradiated mouse fibroblast feeders were exposed to 6.25×10^5 IU of SMPU-MND-EGFP virus (Fig. 1A) for 24 hrs, followed by culture in hESC medium without selection. Cells expressing eGFP were visible in the culture 48 hrs after infection. At Day 6, flow cytometric analysis for eGFP expression showed that 6% of hESCs in the culture expressed eGFP.[1] Portions of colonies showing homogeneous expression of eGFP were manually dissected using a pulled Pasteur pipette over several passages until a subline consistently expressing eGFP was established, which we have named RG6. Analysis by flow cytometry revealed that 99.5% of the cells of the RG6 line were positive for eGFP 20 passages after viral transduction (Fig. 1B). RG6 cells expressed the transcription factor OCT4, a marker of undifferentiated hESCs (22, 23), at levels comparable to those of undifferentiated HSF-6 cells, and they exhibited characteristic ES cell morphology. Upon differentiation of RG6 cells in embryoid body (EB) cultures, RG6 cells exhibited expression profiles of differentiation markers similar to the HSF-6 parental line (Fig. 1C). Expression of eGFP in RG6 remained constant during differentiation (Fig. 1C). When viewed by fluorescence microscopy, the RG6 cells were visible as homogeneous green colonies (Figs. 1D and 2A). RG6 has been cultured for more than 2 years

[1]Rodriguez RT, Firpo MT. Unpublished data.

Table 1. RT-PCR Primer Information

Gene	Accession no.	Product size	No. of PCR Cycles	3′ Primer sequence (5′–3′)	5′ Primer sequence (5′–3′)
AFP	NM001134	192	22	ACTGCAATTGAGAAACCCACTGGAGATG	CGATGCTGGAGTGGGCTTTTTGTGT
b-ACTIN	NM001101	685	26	CACCCTGAAGTACCCCATCGAGCA	CAGGTCTTTGCGGATGTCCACGTCAC
BRACHYURY	NM003181.2	124	38	CCCGCGCACTACACACCCCTCACC	CCTTGGGCTGCGGCGTCGTACTG
EBAF	NM003240	195	34	CTAGGATCTCTAACAAACGCAGAAG	TGACAAGTTCACTAAGACAGTGTGG
eGFP	AF323988	406	26	GGCAAGCTGACCCTGAAGTTCATCTG	CCGTCCTCGATGTTGTGGCGGATCT
EOMES	NM005442	225	38	CGGCGGGATCTTGCGGAGGACT	CCGCCAGCACCACCTCTACGAACAC
FOXD3	NM012183	130	30	GAAGCCGCCTTACTCGTACATC	GGGGAACTTCTCCTGTAGTAGG
hCG	NM000737	193	26	GTCAACACCACCATCTGTGC	AGAGTGCACATTGACAGCTGAG
NODAL	NM018055	143	34	GAGACATATGTGCATGTATTTTTGGA	TGAGATTGACGGACTCTTTTTAATC
OCT4	NM002701	169	38	TGTTCTTACAAGTCTTCTGCCTTTT	GCTGAATACCTTCCCAAATAGAAC
OSTEOPONTIN	NM000582	196	34	CATACCAGTTAAACAGGCTGATTCT	GTCATCATCATCTTCATCATCCATA

without loss of eGFP expression, supporting the conclusion that the stable expression of introduced genes required for gain-of-function genetic analyses can be achieved in hESCs using lentiviral vectors.

RNA Interference of eGFP in hESCs. To asses the ability of RNAi to target and diminish gene function in hESCs, an shRNA expression vector was employed, pCH-eGFPi (5), containing the mouse H1 RNA polymerase III promoter driving expression of an shRNA transcript targeting eGFP (Fig. 2K). When introduced into hESCs, this construct is predicted to express a hairpin RNA transcript containing a 19-mer antisense eGFP RNA at the 3′ end of the hairpin, a 9-nucleotide spacer, and a 19-mer of complimentary sense eGFP RNA on the 5′ end of the hairpin. Simultaneously, the vector drives the constitutive expression of a red fluorescent protein, DsRed2, as a reporter under control of the cytomegalovirus (CMV) promoter in the opposite orientation. A control plasmid, pRed (5), that contained only the DsRed2 element without the eGFP RNAi component was used. RG6 hESCs were electroporated with 25 μg of either pRed (5) or pCH-eGFPi and were assayed 7 days after electroporation. Cells electroporated with pRed (5) expressed both eGFP and DsRed2 when viewed by fluorescence microscopy. In contrast, cells receiving the pCH-eGFPi (5) vector also expressed the DsRed2 reporter, but they exhibited a clear reduction in eGFP fluorescence (Fig. 2I). Expression of eGFP was also monitored by RT-PCR. Cells containing the RNAi construct expressed eGFP at lower levels compared with cells transfected with either the control plasmid or untransfected cells (Fig. 2J). None of the RNAi-transfected cells expressing DsRed2 maintained expression of eGFP (Fig. 2), demonstrating that the introduction of the construct resulted in the knockdown of the eGFP gene in RG6 cells.

Interference with OCT4 Expression in hESC Results in Differentiation to Endoderm and Mesoderm. After establishing that RNAi was an effective system for gene expression interference in hESCs, we tested its ability to target endogenous genes in hESCs; specifically, the OCT4 gene and its protein product. In this case, the pCHeGFPi vector was modified so that the sequence coding for the eGFP shRNA was replaced with a sequence to target endogenous OCT4 mRNA. The shRNA expression vector was introduced into HSF-6 hESCs, and gene expression patterns were assayed 3, 4, and 7 days after electroporation. Gene expression in cells electroporated with a control construct (pRed) containing the CMV/DsRed2 cassette without an shRNA sequence were also assayed and function as basal levels for comparison. Levels of OCT4 mRNA were seen to decrease throughout the first 4 days of assay. At 3 days after electroporation, cell cultures transfected with the pCH-OCTi vector expressed OCT4 at around 55% of the levels of those cells containing the control pRed vector (Fig. 3), and by 4 days after electroporation, the OCT4 levels had decreased to 35% of the control group (Fig. 3). This decrease in mRNA was

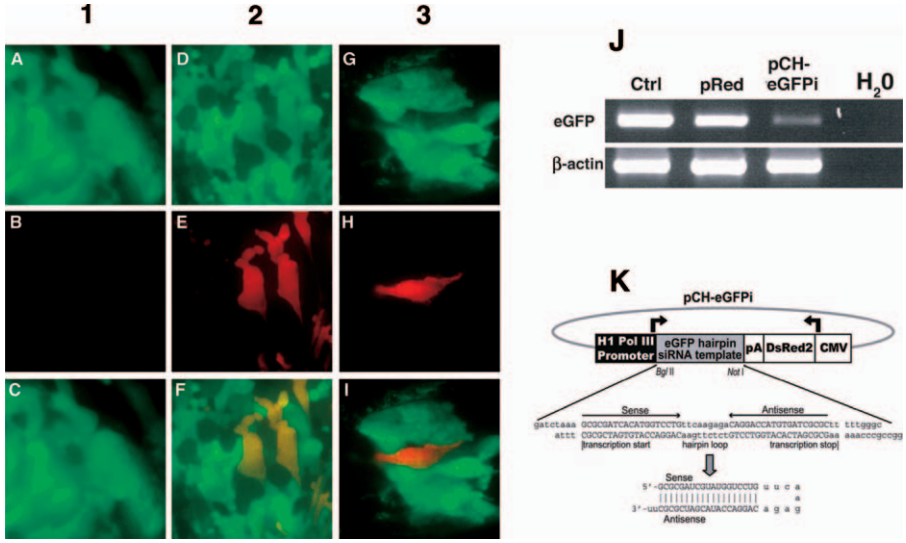

Figure 2. Knockdown of eGFP expression in RG6 cells. (A–I) Knockdown of eGFP expression in RG6 cells examined using confocal microscopy. Untransfected cells expressed high levels of eGFP (A) and no DsRed (B), as shown in a merged image (C). RG6 cells transfected with the control pRed plasmid express high levels of eGFP (D) and DsRed (E). A merged image (F) indicates co-expression of both DsRed and eGFP (orange cells) in the cells transfected with pRed. When RG6 cells were electroporated with the pCHreGFPi plasmid, untransfected cells continued to express high levels of eGFP (G), whereas cells that contained the plasmid expressed high levels of DsRed (H). The merged image (I) illustrates the lack of overlap between DsRed-expressing cells and eGFP-expressing cells. (J) Gene expression analysis in RG6 cells. There is a dramatic reduction in expression of eGFP when the knockdown plasmid (K) was added. Columns: 1) untreated RG6 cells; 2) cells electroporated with the pRed control plasmid; and 3) cells electroporated with the pCHreGFPi knockdown plasmid. Beta-actin expression was included as a positive control.

correlated with a loss of OCT4 protein detected by immunostaining in the cells transfected with the pCH-OCTi construct (Fig. 4D). In contrast, expression of the OCT4 protein was readily detected in cells transfected with pRed (Fig. 4A). In addition to expressing decreased levels of OCT4, cells electroporated with pCH-OCTi exhibited morphologic characteristics of differentiation, including enlarged nuclei, and cytoplasm (Fig. 4D–F).

Detection of elevated expression of downstream endoderm markers was observed in cultures containing the pCH-OCTi vector as early as 72 hrs (Fig. 3). This induction of endoderm upon *OCT4* knockdown is consistent with other studies (6). These data suggest that decreased levels of *OCT4* in the culture result in a reduction in the amount of OCT4 protein available for the OCT4/FOXD3 heterodimer. It has been previously determined in that OCT4 completely inhibits the binding of FOXD3 to the promoter regions of the early endoderm-specific transcription factors *HNF3*α and *HNF3*β (24). Predictably, levels of *FOXD3* mRNA in all hESC cultures containing either pRed or pCH-OCTi throughout all time points remained relatively unchanged, suggesting that *OCT4* does not have a role in transcriptional control of *FOXD3* expression, but *FOXD3* regulation of hESC differentiation status is altered by OCT4 levels. Increased levels of free FOXD3 protein may, however, affect downstream targets and promote transcription of early endoderm-specific genes *HNF3*α and *HNF3*β, in accordance with mouse models of endoderm differentiation (25, 26). To test this hypothesis, we assayed levels of *AFP* mRNA, an endoderm-specific gene that is a downstream target of *HNF3*α and *HNF3*β (26). Expression of *AFP* was maintained at low levels (~20%–25% β-*ACTIN* levels) in the cultures transfected with pRed (Fig. 3). In contrast, the cultures transfected with pCH-OCTi expressed elevated levels of *AFP* throughout, with a maximum 2-fold increase over control levels at Day 4 (Fig. 3). These data support the conclusion that endogenous levels of *OCT4* act to prevent transcription of endoderm-specific genes in undifferentiated hESC (Fig. 5A).

Changes in a second pathway regulated by *OCT4* levels were also observed. An *Oct3/4*-dependent enhancer element has been identified by Niwa *et al*. (27) to be located upstream of the *Ebaf*/*Lefty1* coding region in mouse, and *Ebaf* was confirmed to be a direct target of *Oct4*. This predicts a correlation between the decrease in levels of *OCT4* and the levels of *EBAF* in the cells with reduced expression of *OCT4*. When *EBAF* mRNA levels were assayed 72 hrs after electroporation, an approximate 40% reduction was observed compared with cultures electroporated with the control pRed construct (Fig. 3). Zebrafish and mouse studies have demonstrated that *Lefty* genes acts as antagonists to *Nodal* signaling (28, 29). Mouse mutants lacking *Lefty2* expression exhibit excess mesoderm as a direct result of increased *Nodal* signaling (30). Recent biochemical analyses have also demonstrated that Lefty inhibition of Nodal activity may be due to competitive binding and faster diffusion of Lefty (31). Although detectable increases in the amount of *NODAL* mRNA were not found between our knockdown and control cultures, there was a detectable increase in NODAL activity. Using

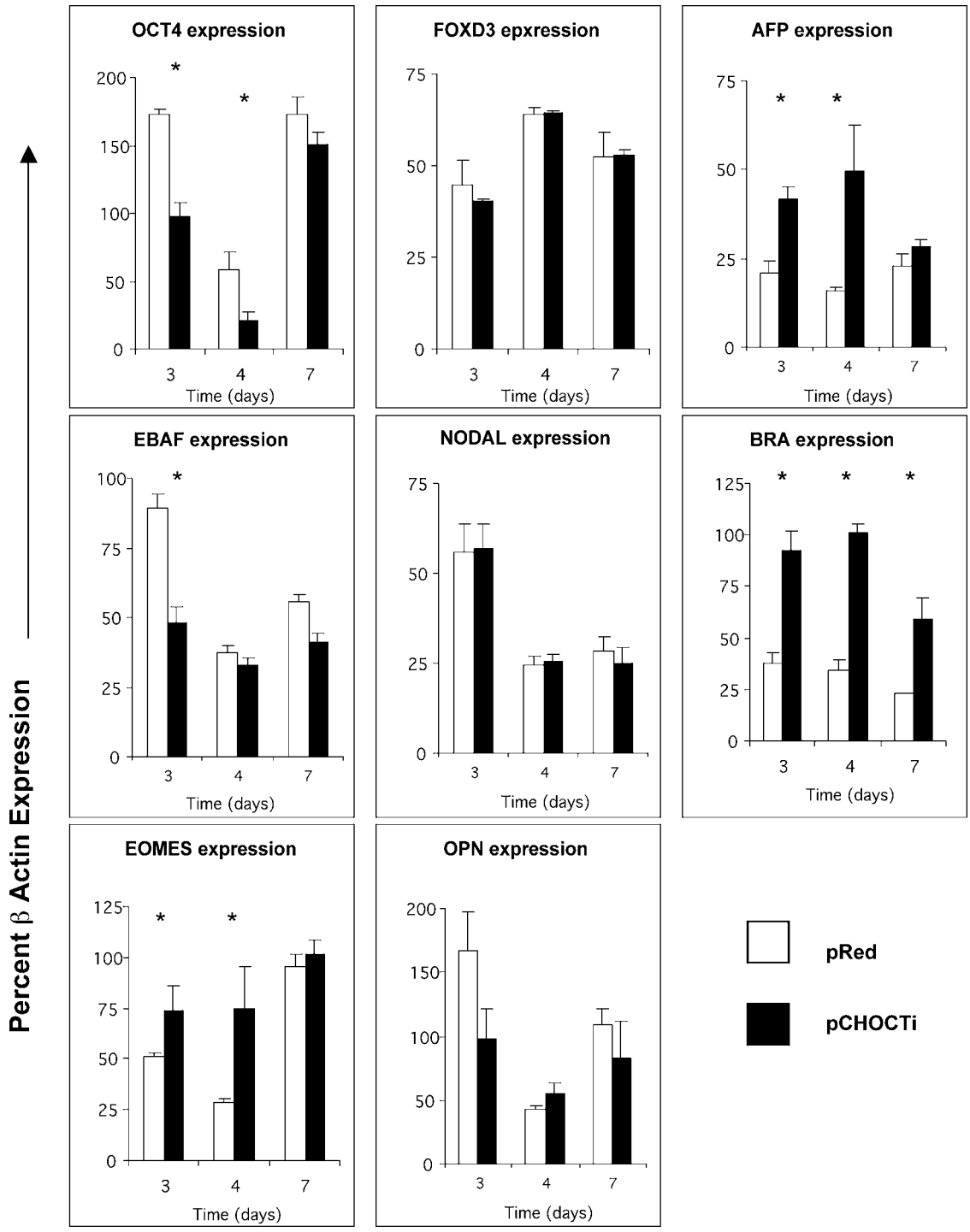

Figure 3. Decreased expression of OCT4 leads to differentiation in hESCs. Reverse transcriptase PCR expression analysis of lysates extracted from HSF-6 hESCs after electroporation with either the control pRed construct or the pCHOCTi expression vector targeting endogenous OCT4 mRNA. Messenger RNA from cell lysates was reverse transcribed, and the resulting cDNA was amplified by PCR with primers specific for OCT4, FOXD3, AFP, EBAF (LEFTYA), NODAL, BRA, OPN, and EOMES. Each reaction was a duplex reaction in which β-actin expression was used as an internal control. Expression of each gene is quantified and expressed as a percentage of β-actin in the reaction. Cells were harvested 3, 4, and 7 days after electroporation. Reactions were run in triplicate. Error bars denote SD. Asterisk denotes $P < 0.05$ using the Student's *t* test.

BRACHYURY (*BRA*) expression as an assay of mesoderm differentiation, the cultures containing the pCH-OCTi construct exhibited dramatic increases in levels of *BRA* mRNA throughout the course of the experiment (Fig. 3). These increases correlated with decreases in *EBAF* expression (Fig. 3). Increased *BRA* expression suggests an induction of mesoderm differentiation in these cultures. Increased expression of *EOMESODERMIN* (*EOMES*), a

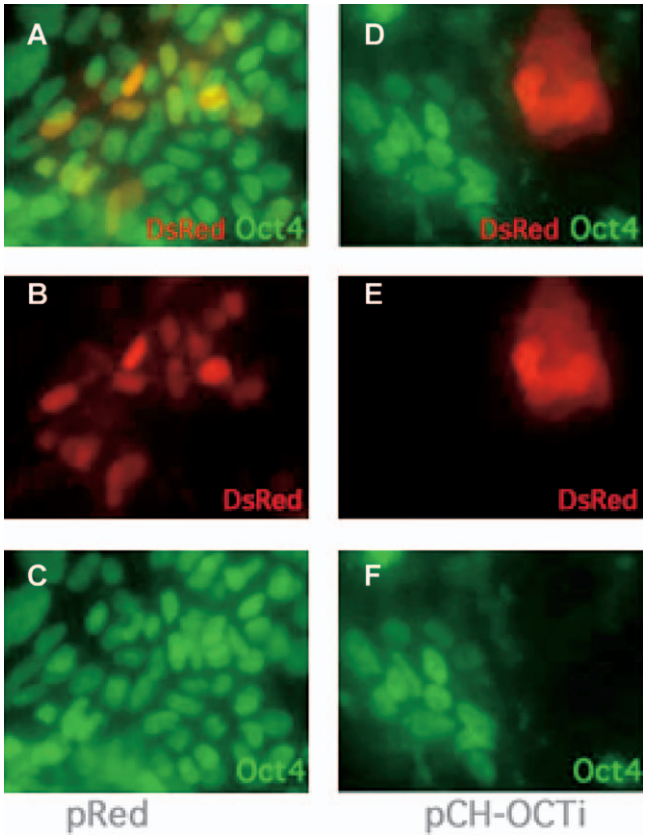

Figure 4. OCT4 protein is undetectable in HSF6 hESC cells that have been transfected with the pCHOCTi vector. Cells were incubated sequentially with anti-Oct4 antibody and an FITC-conjugated secondary antibody. Cells were then visualized by fluorescent microscopy. Cells transfected with the pRed vector express high levels of OCT4 protein (C), as well high levels of DsRed reporter (B). The cells express the two proteins together, as shown in a merged image (A), and maintain characteristic hESC morphology. Nuclei remain small, and there is a small amount of cytoplasmic space between the OCT4-positive nuclei. In contrast, cells that contain the pCHOCTi construct do not express OCT4 protein (F), even when they are in the proximity of undifferentiated HSF6 hESCs, shown in a merged image (D). In addition, cells expressing the DsRed reporter are larger than undifferentiated hESCs, with excess cytoplasm and an enlarged nucleus (D, E).

gene necessary for mesoderm induction and trophoblast development (32), was also detected in cultures with decreased amounts of *OCT4* mRNA (Fig. 3). However, there was no detectable expression of mRNA for human chorionic gonadotropin (hCG), another more specific trophoblast marker, in these cultures. These data support the conclusion that *OCT4* acts to support self-renewal in hESCs by actively antagonizing genes that affect early lineage formation, in the case of *FOXD3*, and by activating transcription of genes that protect against inducers of early lineage formation, in the case of *EBAF* (Fig. 3).

In addition to *AFP* and *BRA*, changes in expression of *OSTEOPONTIN* (*OPN*) were observed in cell populations with reduced levels of *OCT4* expression. No changes in ectoderm marker expression was detected (data not shown). The OCT4 protein binds the PORE element contained within an intron of the *OPN* gene as a homodimer, and it activates transcription (22, 33). *OPN* is expressed in mESCs and at the blastocyst stage in mouse embryos, and it is hypothesized to be important for induction of endoderm and implantation of embryos (22, 33, 34). *OPN* expression was initially downregulated in the knockdown cultures but was slightly upregulated at Day 4 (Fig. 3). This pattern is similar to the variable expression of *Opn* observed by Botquin *et al.* (33) in early mouse development.

Overexpression of OCT4 in hESCs Results in Differentiation to Endoderm. To test the effects of overexpressed *OCT4* in hESCs, a vector was constructed expressing the *OCT4* cDNA and eGFP separated by an internal ribosomal entry site (IRES) sequence, all under the control of the CMV promoter. Gene expression of the cells was assayed at 3, 4, and 7 days after electroporating the cells. Gene expression analysis of cells transfected with the pIRES2eGFP construct containing the IRES and eGFP sequences but omitting the *OCT4* cDNA was included as a control. Elevated *OCT4* mRNA levels were detected in cultures transfected with pOCT4-IRES-eGFP at both 72 and 96 hrs after electroporation compared with pIRES-eGFP–treated cultures (Fig. 6). The elevated levels of *OCT4* dropped dramatically when assayed 7 days after vector introduction (Fig. 6) due to the transient nature of the transduction. To confirm that this elevated expression was being enacted at the protein level, a Western blot for OCT4 was performed on cell lysates from the same experiment. Protein levels were assayed to be higher at every time point throughout the experiment (Fig. 7), with the maximun difference being at Day 4, a 1.7-fold difference in intensity (Fig. 7). *AFP* transcripts are present at low levels in all cultures throughout the first 4 days but become upregulated in the Day 7 cultures containing the pOCT4-IRES-eGFP construct (Fig. 6), when OCT4 protein levels were still elevated over the control (Fig. 7). This is consistent with observations in the murine blastocyst, whereby transient upregulation of Oct4 consequently led to endoderm differentiation (3). Further, the *OCT4* homolog in zebrafish, pou5f1/pou2, has been shown to be essential for endoderm formation (35, 36). No changes in levels of ectoderm markers were detected (data not shown). These data support the conclusion that increased embryonic *OCT4* expression stimulates endoderm formation in early human development as well.

Slight increases in *EBAF* mRNA in the cultures with pOCT4-IRES-eGFP were observed, which did not correlate with increased *BRA* expression compared with the control (Fig. 6). Detectable differences in *NODAL* expression were found in the cultures with increased *OCT4* mRNA. *NODAL* mRNA was detected at elevated levels in the control cultures compared with the cultures overexpressing *OCT4*. This is in contrast to the knockdown data, where no noticeable changes in *NODAL* expression were detected. *OCT4* may therefore act to repress *NODAL* expression through a mechanism that regulates *NODAL* expression,

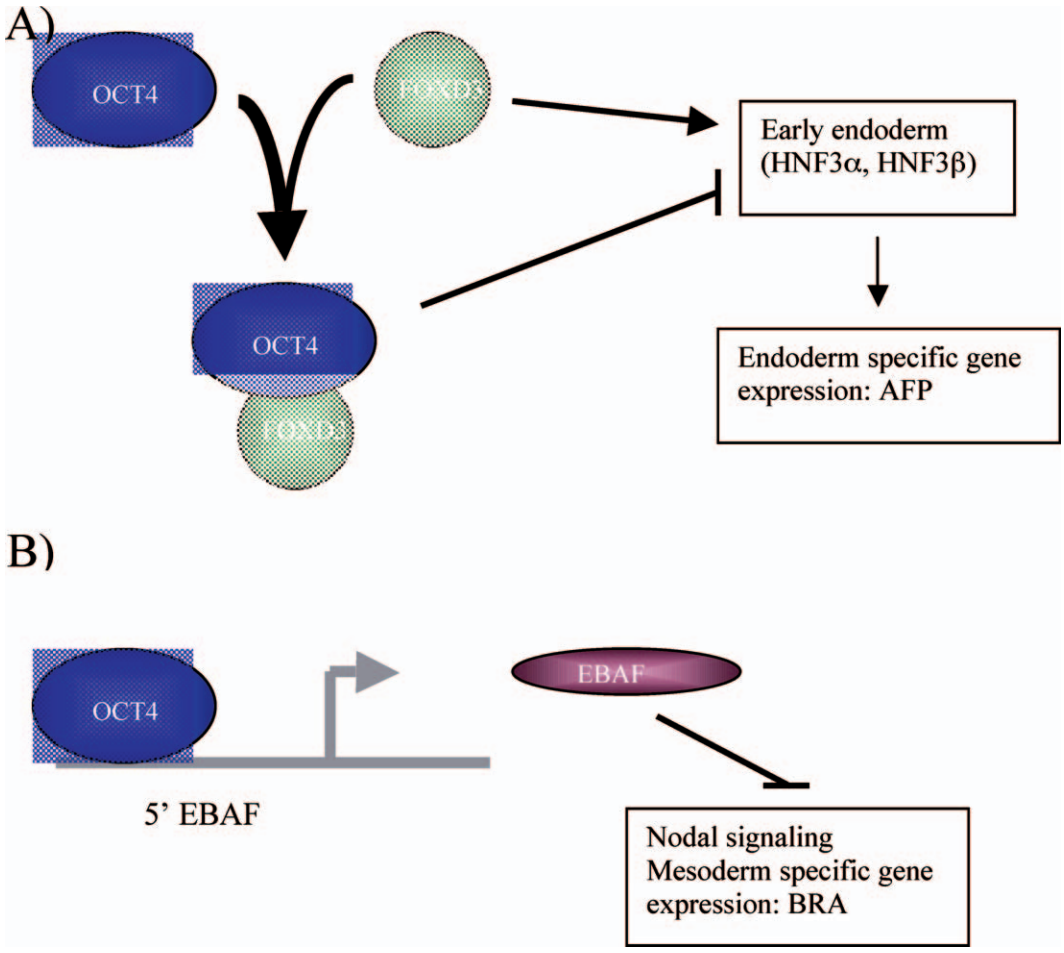

Figure 5. Model for action of OCT4 protein in maintenance of pluripotency in hESCs. (A) OCT4 interacts with the DNA-binding domain of a transcription factor FOXD3, preventing its binding to promoter regions of lineage-specific transcription factors HNF3α/β, effectively preventing their transcription and maintaining an undifferentiated state. (B) The Oct4 protein binds to the promoter regions of genes, including EBAF, and promotes their transcription. The Ebaf protein acts as an antagonist to the lineage-specific signaling molecule Nodal, preventing the differentiation of early mesoderm.

which is maintained at normal levels even when *OCT4* is downregulated. In addition, another mesodermal precursor marker, *EOMES*, was not detected at elevated levels the cultures overexpressing *OCT4* compared with the controls. The trophoblast marker hCG was not detected at all in these cultures. The lack of hCG transcripts combined with the lack of increased EOMES in these cultures precludes trophoblast differentiation when *OCT4* mRNA is increased above endogenous levels. These data, along with a lack of increases in *BRA* expression in these cultures, also suggest that *OCT4* upregulation acts to repress mesoderm development in hESCs.

Variable expression of *OPN* was observed in cultures with increased expression of *OCT4*. Specifically, there is a downregulation at 3 days after electroporation, followed by an upregulation of expression of *OPN* 4 days after vector introduction. These patterns are similar to those observed in the cultures with reduced *OCT4*. Expression of *OPN* has been observed in mESCs and at the blastocyst stage in mouse embryos, and it is thought to be important for induction of endoderm and implantation of embryos *in vivo* (22, 33, 34). Increased expression of *OPN* in response to increased *OCT4* mRNA at Day 4, combined with the increased expression of the gene *AFP* (Fig. 6), suggests that in hESCs, like mESCs, overexpression of *OCT4* induces early endoderm differentiation.

Discussion

Genetic modification of hESCs is a necessary but challenging tool for the full utility of these cells to be realized. The viability of hESCs is reduced by more than 100-fold following dissociation to single cells, making subclones of genetically modified hESCs difficult (8, 9). Adding to the difficulty of genetically altering hESCs is the ability of similar cell types to significantly silence transgene expression (37, 38). We have shown that stably expressing transgenic hESCs can be generated using lentiviral vectors that overcome the propensity of these pluripotent cells to silence ectopically expressed genes (10–13). We have also demonstrated that expression of an shRNA interferes with

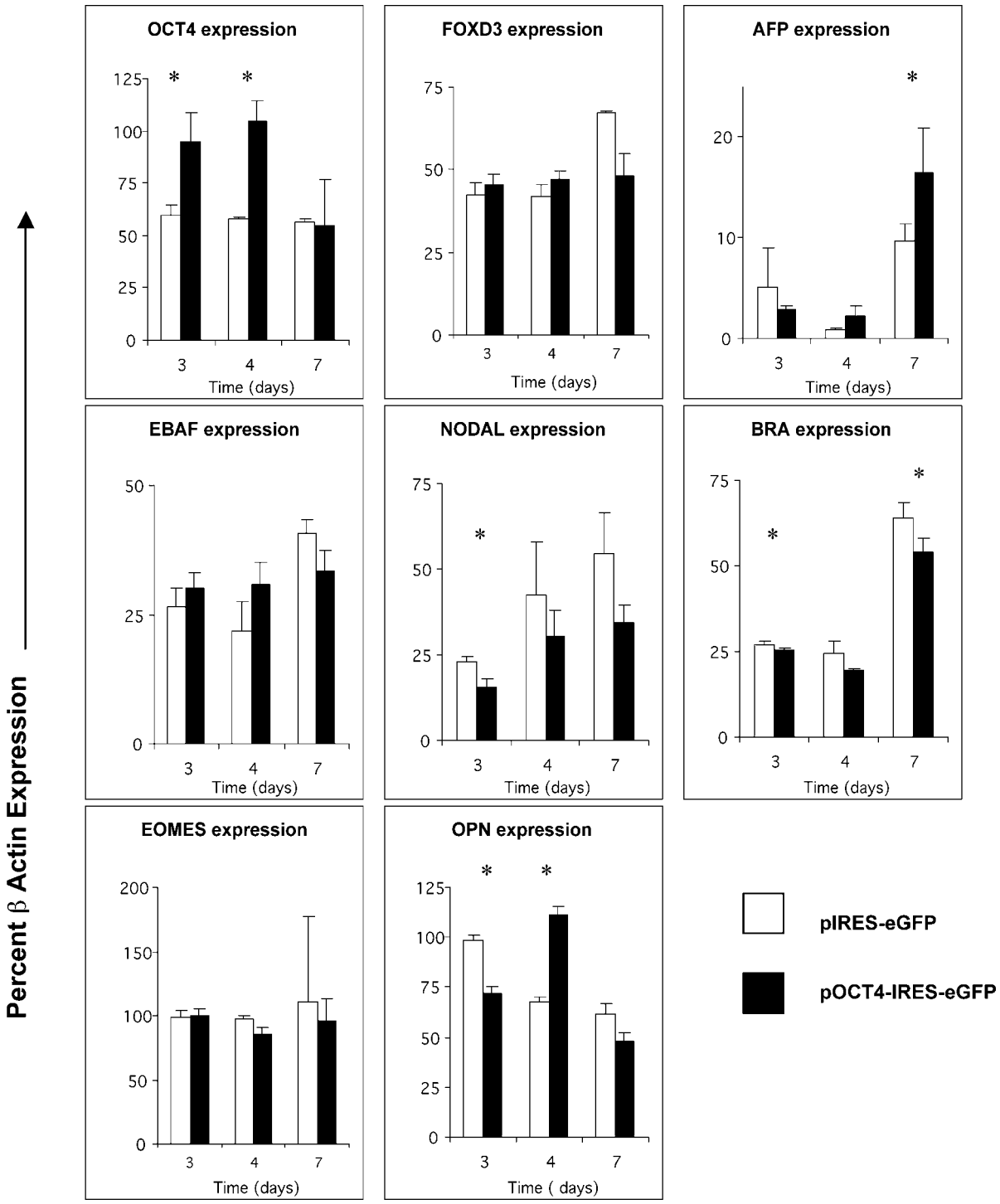

Figure 6. Increased levels of OCT4 mRNA in hESC cultures lead to endoderm differentiation. Analysis of mRNA levels of OCT4 and other genes from forced expression cultures containing the pOCT4-IRES-eGFP vector or control cultures containing the pIRES2-eGFP vector. Messenger RNA from cell lysates was reverse transcribed, and the resulting cDNA was used as template in semiquantitative RT-PCR reactions with the following gene-specific primers: OCT4, FOXD3, AFP, EBAF (LEFTYA), NODAL, BRA, OPN, and EOMES. Expression of each gene is quantified and expressed as a percentage of β-actin in the reaction. Cells were harvested 3, 4, and 7 days after electroporation. Reactions were run in triplicate. Error bars denote standard deviation. Asterisk denotes $P < 0.05$ using the Student's *t* test.

the expression of genes in hESCs. Further, a method of marking cells expressing RNAi constructs was used that allowed for monitoring of the effects of interference in the expression of genes and evaluation of their role in self-renewal of hESCs. Since interfering with genes important for self-renewal in hESCs would most likely prevent the ability to subsequently subclone them, we chose to use transient short-term expression studies. Levels of *OCT4*

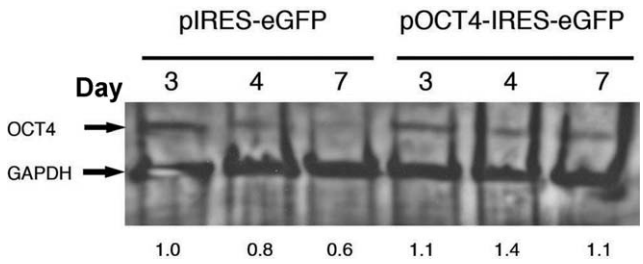

Figure 7. Increased OCT4 protein observed in cultures electroporated with pOCT4-IRES-eGFP determined by Western blot analysis. Cell lysates were blotted onto PVDF membrane and then treated with both anti-OCT4 antibody and anti-GAPDH antibody. The blot was visualized with ECL reagent. Intensity of OCT4 bands was determined for each lane and normalized to the corresponding intensity of GAPDH of the same lane. Intensity of each OCT4 band was expressed as a ratio of normalized intensity to the mean normalized intensity.

mRNA expression were manipulated first by RNAi, and transient upregulation was achieved with an expression vector coding *OCT4* cDNA. These cultures were then evaluated to determine the role of *OCT4* in self-renewal of hESCs in short-term assay. Both increasing and decreasing the levels of *OCT4* in hESCs promoted differentiation of these cells. The differentiation patterns observed in hESCs differ from the established patterns in mESCs (2, 5, 6).

Quantitative studies of *Oct4* levels in mESCs reveal that increased levels of *Oct4* led to endoderm and mesoderm differentiation, whereas decreased levels resulted in the induction of markers indicative of trophectoderm differentiation (2). Similar results for trophectoderm differentiation have been demonstrated using RNAi for *Oct4* in mESCs (5, 6). However, Hay *et al.* also demonstrated induction of GATA6 and AFP, markers indicative of endoderm differentiation, in mESCs and hESCs, but did not demonstrate induction of mesoderm in mESCs or hESCs (6).

These knockdown studies have shown that reduction in *OCT4* expression in hESCs results in the expression of markers characteristic of endoderm and mesoderm differentiation (Fig. 3), but they do not show increased levels of definitive trophectoderm marker expression, as hCG was not detected in any of the cultures assayed in these experiments. However, induction of both trophoblast and early endoderm markers has been demonstrated in hESCs with reduced expression of *OCT4* under culture conditions designed to promote differentiation, and at low levels with RNAi (6, 39). It has been suggested that this may be due to either differentiation of both cell types or an asynchronous differentiation of these cells along a similar pathway (6). The ability to detect elevated levels of mesodermal precursors in cultures with reduced expression of *OCT4* complicates this situation further. The genes *EOMES* and *BRA* are expressed at elevated levels in the knockdown cultures (Fig. 3). *EOMES* expression patterns in the mouse include trophoblast cells, the primitive streak, embryonic mesoderm, and the anterior visceral endoderm (40, 41). In addition, elevated *AFP* transcripts were detected in these cultures, indicating endoderm induction. These data further support the model that reduction of *OCT4* expression in hESCs leads to the induction of primitive cell types, which subsequently gives rise to differentiation to multiple cell lineages. Furthermore, that *OCT4* works to actively repress genes that induce early differentiation and to promote the transcription of genes that repress early lineage formation.

Evidence suggests that increased *Oct4* in mESC cultures leads to the induction of cells expressing markers of both endoderm and mesoderm (2). Induction of genes indicative of endoderm differentiation in cultures with increased *OCT4* expression were observed, but no increases in the expression of the genes *EOMES*, *BRA* (Fig. 6), and *hCG* were found. The lack of mesodermal precursors in these cultures differs from mESC experiments (2). This is not the first instance of the differences in effect of gene function in mESCs and hESCs. The dependence of mESCs on *LIF* and its signaling through *gp130*/*Stat3* to support undifferentiated growth has been well defined (42–45). In contrast, *LIF* as well as *gp130*/*STAT3* signaling has been shown to be unimportant in hESC maintenance (46). Our data support the model that in hESCs, *OCT4* acts to suppress mesoderm differentiation but has a unique effect on endoderm precursors: it appears that manipulation of the levels of *OCT4* above or below endogenous levels stimulates endoderm differentiation.

In future studies, the combined use of RNAi to knock down expression and lentiviral transduction to overexpress genes suspected to be functionally important for stem cell maintenance will facilitate efforts aimed at identifying pathways important in the maintenance of pluripotency, as illustrated by the results of manipulating expression of OCT4 in hESCs. The use of broad expression profiling combined with the ability to quickly diminish gene expression in these cells will lead to the identification of additional genes functionally important for stem cell maintenance, self-renewal, and proliferation. Further, the elucidation of pathways regulating differentiation will be instrumental in the use of hESC differentiation as a model of human development, especially when they diverge from similar experiments in mESCs. Finally, an understanding of hESC differentiation may provide the ability to direct the differentiation of hESCs to specific tissues and cell types, advancing the clinical utility of hESC-derived cells and tissues.

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