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Rapid Communication

## Specific Knockdown of Oct4 and $\beta$ 2-microglobulin Expression by RNA Interference in Human Embryonic Stem Cells and Embryonic Carcinoma Cells

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**Key Words.** Human embryonic stem cells • Embryonal carcinoma cells • RNA interference  
Oct4 •  $\beta$ -2-microglobulin • GFP • Differentiation • Gene expression

### ABSTRACT

We have used RNA interference (RNAi) to downregulate  $\beta$ 2-microglobulin and Oct4 in human embryonal carcinoma (hEC) cells and embryonic stem (hES) cells, demonstrating that RNAi is an effective tool for regulating specific gene activity in these human stem cells. The knockdown of Oct4 but not  $\beta$ 2-microglobulin expression in both EC and ES cells resulted in their differentiation, as indicated by a marked change in morphology, growth rate, and surface antigen phenotype, with respect to SSEA1, SSEA3, and TRA-1-60 expression. Expression of

*hCG* and *Gcm1* was also induced following knockdown of Oct4 expression, in both 2102Ep hEC cells and in H7 and H14 hES cells, consistent with the conclusion that, as in the mouse, Oct4 is required to maintain the undifferentiated stem cell state, and that differentiation to trophectoderm occurs in its absence. NTERA2 hEC cells also differentiated, but not to trophectoderm, suggesting their equivalence to a later stage of embryogenesis than other hEC and hES cells. *Stem Cells* 2004;22:659–668

### INTRODUCTION

Human embryonic stem (hES) [1] cells offer the opportunity for the in vitro production of multiple cell types for use in regenerative medicine. A key to unlocking this potential is development of methods for controlling gene expression and, consequently, cell differentiation. One tool that might be exploited is RNA interference (RNAi) [2] to manipulate specific signaling pathways in a transient manner and so influence the selection of specific pathways of differentiation by a

pluripotent stem cell. To explore this possibility we have used RNAi to determine whether the transcription factor Oct4 is required to maintain the undifferentiated state of hES cells, as well as human embryonal carcinoma (hEC) cells, their malignant equivalent from teratocarcinomas, and whether forced knockdown of Oct4 expression results in differentiation toward trophectoderm. Murine ES cells have been shown to depend on the correct levels of Oct4 expression for their maintenance of an undifferentiated stem cell phenotype, and they

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differentiate to trophectoderm in its absence [3]. However, various differences exist between mouse and human ES cells (e.g., the nonresponsiveness of hES cells to leukemia inhibitory factor [LIF] [1] and their different surface antigen phenotypes [4]), so that one cannot assume, a priori, that a regulatory factor active in mouse ES cells necessarily exhibits the same function in their human counterparts. Here we show that RNAi can be used effectively to downregulate genes in a specific manner in hEC and hES cells. Further, our results confirm the hypothesis that, indeed, Oct4 expression is required to maintain the undifferentiated state of human EC and ES cells.

## MATERIALS AND METHODS

### Cell Culture and Stable Transfection

NTERA2 cl.D1 and 2102Ep cl.2A6 hEC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), as previously described [5, 6]. The hES cell lines, H7 and H14 [1] were cultured in knockout-DMEM (Invitrogen, Carlsbad, CA) supplemented with 20% serum replacement (Invitrogen) and 4 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), on feeder layers of mouse embryonic fibroblasts that had been mitotically inactivated with mitomycin C [7]. For stable transfection with an enhanced green fluorescent protein (eGFP) transgene, the cells were seeded at  $3 \times 10^4$  per  $\text{cm}^2$  and transfected one day later with plasmid pCAG-eGFP using ExGen 500 (Fermentas, Inc., Hanover, MD) following the manufacturer's protocol. Puromycin (1  $\mu\text{g}/\text{ml}$ ) was added to the cultures, 24 hours after transfection, and puromycin-resistant green-fluorescent colonies were selected and expanded for further experiments.

### RNA Interference

Double-stranded, short (21-mer) interfering RNA (siRNA) corresponding to eGFP,  $\beta 2$ -microglobulin ( $\beta 2M$ ), and *Oct4* were designed with the following sense and antisense sequences and were synthesized by Xeragon Inc. (now Qiagen, Valencia, CA):

#### *eGFP*:

(sense) 5'-CGUAAACGGCCACAAGUUCdTdT-3'  
(antisense) 5'-GAACUUGUGGCCGUUUACGdTdT-3', starting from nucleotide 66 of the *eGFP* coding sequence

#### $\beta 2M$ :

(sense) 5'-GAUUCAGGUUUACUCACGUdTdT-3'  
(antisense) 5'-ACGUGAGUAAACCUGAAUCdTdT-3', starting from nucleotide 91 of  $\beta 2M$  sequence (accession number AB021288)

#### *Oct4-A*:

(sense) 5'-AGCAGCUUGGGCUCGAGAAAdTdT-3'  
(antisense) 5'-UUCUCGAGCCCAAGCUGCdTdT-3', starting from nucleotide 610 of *Oct4* sequence (accession number NM\_203289)

#### *Oct4-B*:

(sense) 5'-CAUGUGUAAGCUCGCGGCCdTdT-3'  
(antisense) 5'-GGGCCGCAGCUUACACAUGdTdT-3', starting from nucleotide 404 of *Oct4* sequence (accession number NM\_203289)

Cells were harvested with 0.25% trypsin, 1 mM EDTA in phosphate buffered saline (PBS) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (for EC cells) or 0.05% trypsin, 1 mM EDTA in PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (for ES cells) and plated in six-well plates at  $2 \times 10^4$  per  $\text{cm}^2$ . The next day, when the cultures were 30%–50% confluent, siRNA was introduced into the cells using the Oligofectamine transfection reagent (Invitrogen). In brief, 10  $\mu\text{l}$  siRNA (20- $\mu\text{M}$  solution) was incubated with 4  $\mu\text{l}$  Oligofectamine in 190  $\mu\text{l}$  Optimem (Invitrogen) for 20 minutes; the mixture was then added to the cells in a final volume of 1.2 ml. The transfected cells were cultured and were fed daily with fresh medium until they were assayed.

### Flow Cytometry

Antigen expression was assayed by immunofluorescence and flow cytometry as previously described [8], using the following monoclonal antibodies: MC631, anti-stage-specific embryonic antigen-3 (SSEA3) [9]; MC813-70, anti-stage-specific embryonic antigen-4 (SSEA4) [10]; MC480, anti-stage-specific embryonic antigen-1 (SSEA1) [11]; TRA-1-60 [12]; TRA-2-54, anti-liver/kidney/bone alkaline phosphatase [13]; BBM1, anti- $\beta 2$ -microglobulin [14]; W6/32, anti-HLA-A,B,C [15]. In all cases, immunofluorescence with the specific antibodies was compared with that from a negative control antibody obtained from the parent myeloma cell line P3X63Ag8, as previously described [8].

### Immunofluorescent Localization of Surface Antigens on Cells In Situ

The medium was removed from cultures of the hEC and ES cells growing in six-well tissue culture plates and replaced with primary antibody diluted in culture medium. Antibody from myeloma line P3X63Ag8 was used to provide a negative control. The cells were then incubated for 30 minutes at 37°C, after which the cells were washed three times with PBS (with  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ). A secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgM (1:20), was added to the cells and incubated for 30 minutes at 37°C. The cells were then washed three times with PBS and visualized under a UV microscope.

### Western Blotting

Cells were harvested using trypsin: EDTA and lysed in radioimmunoprecipitation (RIPA) buffer (1×PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) at 10<sup>7</sup> cells/ml. Lysates containing the equivalent of 1.5 × 10<sup>4</sup> cells per lane were electrophoresed using SDS-PAGE (10% polyacrylamide gel) and blotted overnight onto polyvinylidene difluoride (PVDF) membrane. Membranes were stained with Ponceau-S to check loading and transfer, and then blocked for 1 hour with 5% fat-free milk solution. Samples were probed with a goat polyclonal antibody against Oct4 (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) at 0.25 μg/ml and a 1:4000 dilution of anti-goat IgG-peroxidase conjugate (Sigma Chemical Corp., St. Louis, MO). Staining was visualized using an electrogenerated chemiluminescence (ECL) kit (Amersham Biosciences Corp., Piscataway, NJ).

### Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Total RNA (2 μg) was reverse transcribed using 1 μg oligo-dT primer with MMLV Reverse-Transcriptase (Promega U.S., Madison, WI) in a 40-μl reaction volume containing 1.25 mM deoxyribonucleoside triphosphate (dNTP) at 37°C. Oligonucleotide primers for PCR detection of human chorionic gonadotropin (*hCG*), *Cdx2*, *Gcm1*, and *β-actin* were designed using the PrimerSelect program from the DNASTAR software package (DNASTAR Inc., Madison, WI). PCR was performed using 1 μl of cDNA in 25 μl PCR containing 15 pmol of each primer, 0.1 mM dNTP, and 0.3 units Taq polymerase (Promega). Primer sequences used and conditions of these reactions were as follows:

hCG-f: 5'-ATGGGCGGGACATGGGCATCCA-3'  
 hCG-r: 5'-GGCCCCGGGAGTCGGGATGG-3' (70°C annealing, ×35 cycles)  
 Cdx2-f: 5'-CCTCCGCTGGGCTTCATTC-3'  
 Cdx2-r: 5'-TGGGGTTCTGCAGTCTTTGGTC-3' (60°C annealing, ×30 cycles)  
 Gcm1-f: 2 5'-GTGGACCCCATGAAGCTCTA-3'  
 Gcm1-r: 2 5'-GCAGTGATCCAAACCCAAGT-3' (65°C annealing, ×35 cycles)  
 β-actin-f:  
 5'-ATCTGGCACCACACCTTCTACAATGAGTGCG-3'  
 β-actin-r:  
 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (70°C annealing, ×25 cycles)

### RESULTS

The hES lines H7 and H14 are pluripotent cell lines derived from early human embryos. Both 2102Ep and NTERA2 are

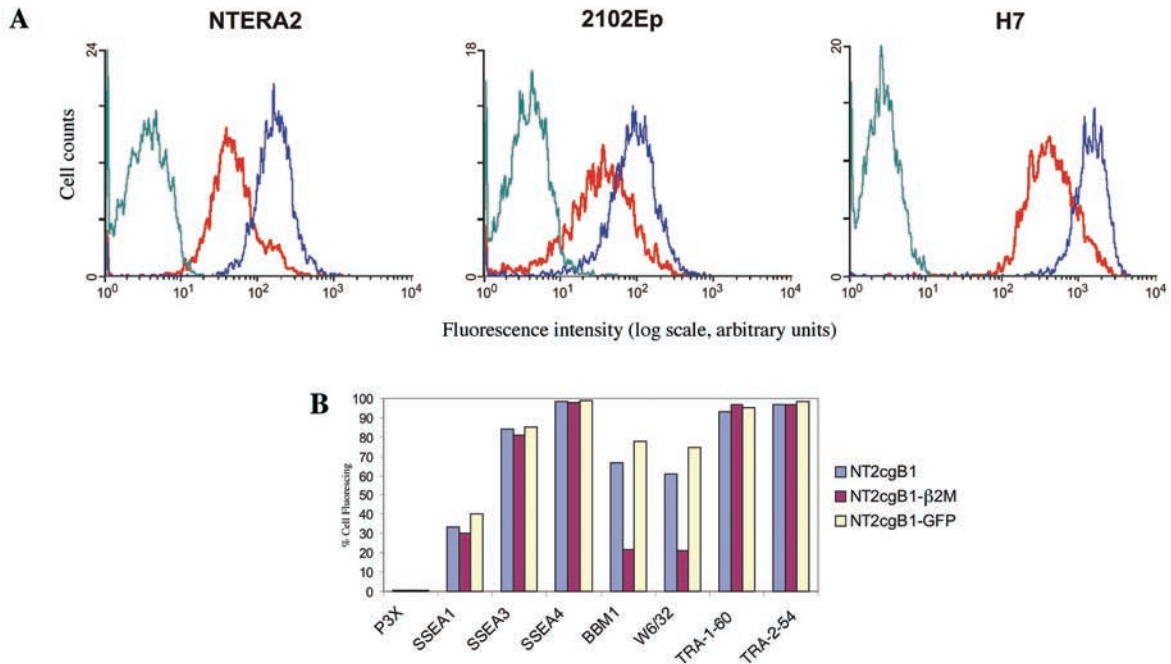
hEC cell lines derived from teratocarcinomas [5, 6]; they closely resemble hES cells and the inner cell mass of human blastocyst stage embryos [4, 16]. Although 2102Ep cells differentiate slightly when passaged at low density, NTERA2 but not 2102Ep cells differentiate, most notably in a neural direction, when exposed to retinoic acid [8]. By contrast, the hES cultures always contained a significant level of spontaneously differentiated cells [4].

To establish conditions for the use of RNAi with these cells, and to ascertain whether the RNAi technique itself might induce nonspecific effects, we first used RNAi to knock down expression of eGFP and β2M in stably transfected, eGFP-expressing NTERA2, 2102Ep, and H7 cells. After 3–5 days, the expression of eGFP and β2M was specifically downregulated, 5- to 10-fold, only by their corresponding siRNA (Fig. 1a). At the same time, the expression of several developmentally regulated surface antigens characteristic of undifferentiated hEC and hES cells SSEA3, SSEA4, TRA-1-60, and TRA-2-54 [4] was unaffected, while expression of SSEA1, which is not expressed by the undifferentiated cells but is expressed by some of their differentiated derivatives, was not induced. Thus, siRNA treatments do not appear to induce hEC or hES differentiation in a nonspecific way (Fig. 1b). However, expression of cell surface β2M and the heavy chain of class 1 HLA (which is dependent on β2M expression [17]), was specifically downregulated after treatment with siRNA to β2M. RNAi efficiency varied with cell density (2 × 10<sup>4</sup> cells per cm<sup>2</sup> was optimal) and different transfection reagents (Oligofectamine was the most efficient tested).

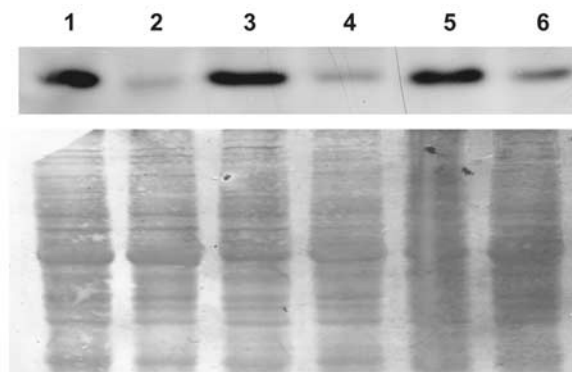
Using the same protocol, we then treated NTERA2 and 2102Ep hEC cells and H7 hES cells in parallel with *Oct4* or β2M siRNA. Initially, we designed two siRNAs, corresponding to different regions of the *Oct4* gene to target Oct4 expression (Oct4-A and Oct4-B; see Materials and Methods). Both did cause downregulation of *Oct4* mRNA and protein expression. However, Oct4-A siRNA proved more efficient than Oct4-B, as judged by western blotting (data not shown). Therefore, Oct4-A was used in the further studies.

To determine the optimal time for RNA interference of Oct4 expression, 2102Ep cells were treated with *Oct4* and β2M siRNAs, and the expression of Oct4 was assayed by western blotting 3, 5, and 7 days after RNAi. The level of Oct4 protein expression remained constant in the cultures treated with siRNA to β2M. By contrast, it was substantially downregulated 3 and 5 days after treatment with *Oct4* siRNA, while levels began to recover by 7 days (Fig. 2).

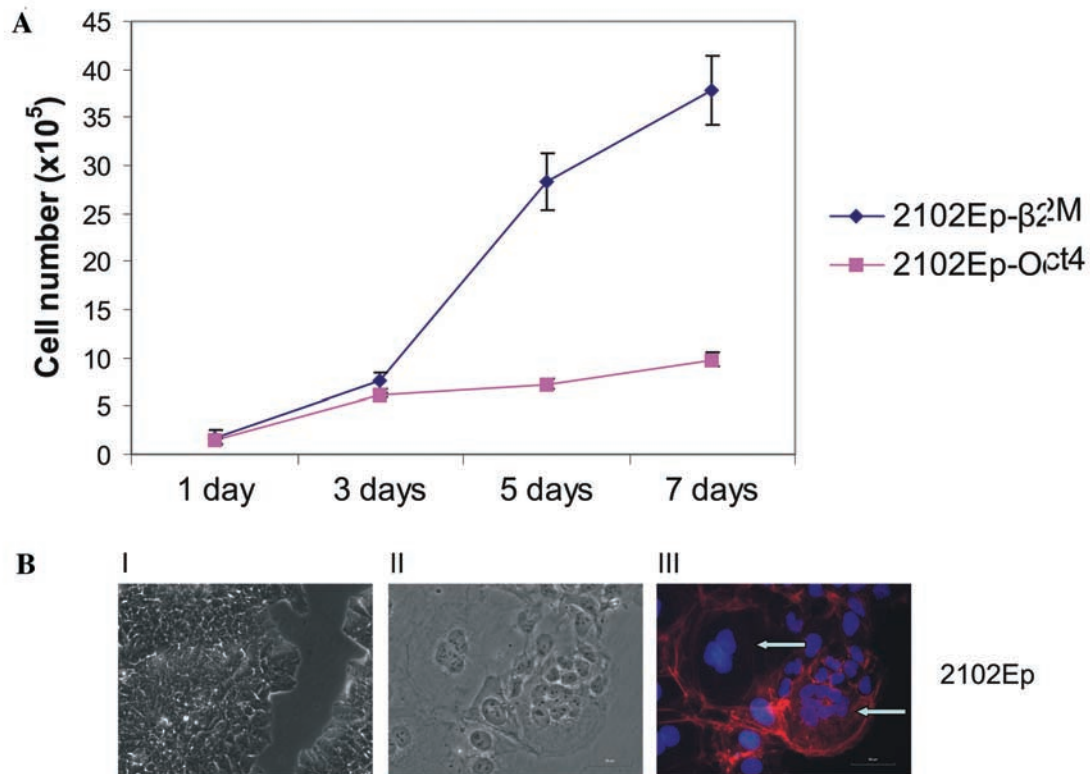
The knockdown of Oct4 expression was accompanied by a marked reduction in cell growth, in comparison with cells treated with β2M siRNA (Fig. 3). The cells exhibited a markedly different morphology and, when left for over 7



**Figure 1.** Short interfering RNA (siRNA) can specifically downregulate targeted gene expression in human embryonal carcinoma (EC) and embryonic stem (ES) cells without affecting their undifferentiated state. NTERA2 and 2102Ep human EC and H7 human ES cells, stably transfected with an enhanced green fluorescent protein (eGFP) expression vector, were treated with siRNA, targeting either *eGFP* or  $\beta$ 2-microglobulin ( $\beta$ 2M), using several different transfection reagents: Lipofectin (Invitrogen), Superfect (Qiagen), Fugene 6 (Roche Diagnostics), ExGen500 (MBI Fermentas), GeneJuice (Novagen), and Oligofectamine (Invitrogen). Of these, Oligofectamine was judged the most effective for RNA interference. **(A):** Fluorescence-activated cell sorter (FACS) analysis of eGFP expression in cells treated with eGFP siRNA (red histograms) or  $\beta$ 2M siRNA (blue histograms) or untreated (green histograms). The analysis was carried out 3 days after RNAi in NTERA2 and H7 cells and 5 days after RNAi in 2102Ep cells. The fluorescence intensity is recorded on a log scale, and there is a 5- to 10-fold reduction in eGFP expression after eGFP siRNA. **(B):** Surface marker antigen expression by green fluorescent NTERA2 EC cells, either untreated (NT2cgB1) or 6 days after exposure to siRNA to eGFP (NT2cgB1-GFP) or  $\beta$ 2M (NT2cgB1- $\beta$ 2M). No effect on surface antigens characteristic of human EC cells was seen; however,  $\beta$ 2M siRNA caused a marked downregulation of cell surface expression of  $\beta$ 2-microglobulin (detected by BBM1) and HLA-A,B,C heavy chain (detected by W6/32), as expected.



**Figure 2.** siRNA directed to *Oct4* causes downregulation of Oct4 protein expression. Western blot for Oct4 expression in 2102Ep cells treated with siRNA targeting either  $\beta$ 2M (Lanes 1, 3, and 5) or *Oct4* (Lanes 2, 4, and 6) after 3 days (Lanes 1, 2), 5 days (Lanes 3, 4), and 7 days (Lanes 5, 6). Note the substantial reduction in Oct4, 3 and 5 days after treatment with Oct4 siRNA and then its partial recovery after 7 days, due to unaffected cells beginning to overgrow the culture. The lanes were each loaded with lysate containing the equivalent of  $1.5 \times 10^5$  cells. Following electrophoresis and blotting, membranes were stained with Ponceau-S to determine equal loading and transfer (lower panel).



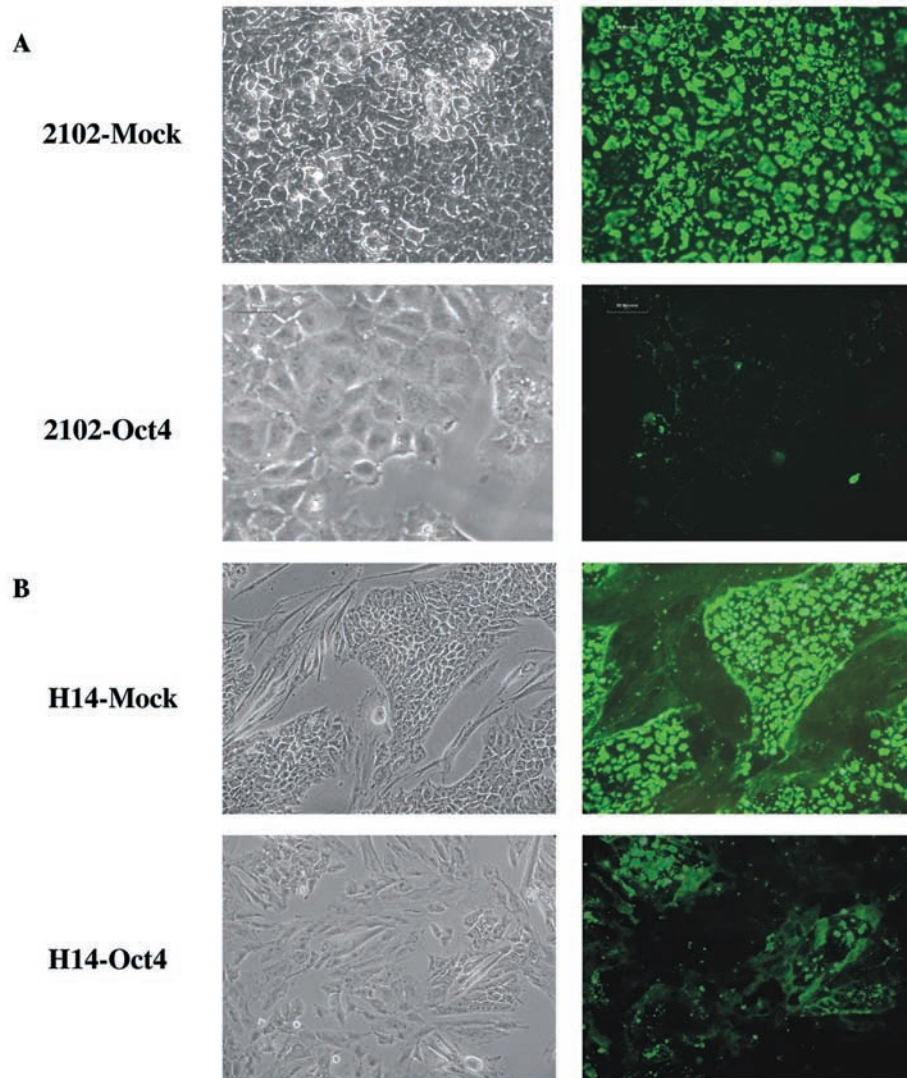
**Figure 3.** Effect of *Oct4* RNAi on growth rate and the appearance of multinucleated cells in 2102Ep human EC cells. **(A):** Growth of 2102Ep cells after treatment with  $\beta$ 2M and *Oct4* siRNAs ( $n = 3$ ;  $\pm$  standard deviation). **(B):** The morphology of (I) untreated 2102Ep cells or of multinucleated cells appearing (II) after *Oct4* RNAi phase contrast and (III) after staining with DAPI and rhodamine-phalloidin, 7 days after RNAi.

days, syncytial giant cells could be found in the *Oct4* siRNA-treated cultures. These treated cells had lost expression of the characteristic human EC and ES cell marker antigen, TRA-1-60 (Fig. 4). Many of these large flat cells, including syncytial cells, could be found in treated cultures several weeks later, although the remaining, initially small, population of unaffected cells began to overgrow the culture and mask the apparently differentiated cells that did not proliferate. Similar effects on morphology and expression of cell surface TRA-1-60 were seen in ES cultures (data not shown).

To investigate further the effects of *Oct4* knockdown on the phenotype of human EC and ES cells, the expression patterns of several developmentally regulated antigens and genes were analyzed in NTERA2 and 2102Ep EC cells and H7 ES cells 5 days after siRNA knockdown of *Oct4* expression (Fig. 5; Table 1). In each case, *Oct4* protein levels were substantially reduced by siRNA for *Oct4*, in comparison with cells treated with siRNA targeting  $\beta$ 2M. Reciprocally, cell surface  $\beta$ 2M expression was reduced by  $\beta$ 2M siRNA, in comparison with cells treated with *Oct4* siRNA. At the same time, in cultures treated with *Oct4* siRNA, significant populations of cells appeared with substantially reduced expres-

sion of the stem cell marker antigens SSEA3 and TRA-1-60, compared with those treated with  $\beta$ 2M siRNA. Cells positive for SSEA1, which is not expressed by human EC and ES cells, also appeared in cultures of 2102Ep and H7, though not significantly in NTERA2, after treatment with *Oct4* siRNA. These observations were quite clear in the case of the EC cell lines but were masked to some extent in the H7 hES line because of the initial heterogeneity of these cultures that results from uncontrolled spontaneous differentiation. Nevertheless, the results were consistent and were also observed, for example, in a second hES line, H14, in a separate experiment (Fig. 6a). In the latter case, the effect of *Oct4* siRNA was compared with “mock-treated” control cultures, rather than in comparison with a  $\beta$ 2M siRNA control.

These results indicate that the expression of *Oct4* is required for the maintenance of hEC and hES cells in an undifferentiated state and that knockdown of *Oct4* expression in human EC and ES cells results in their differentiation, as in the case of mouse ES cells [3]. Further, at least for the 2102Ep human EC cells and the H7 and H14 human ES cells, the induction of SSEA1 would be consistent with differentiation toward trophectoderm, as in mouse ES cells, since

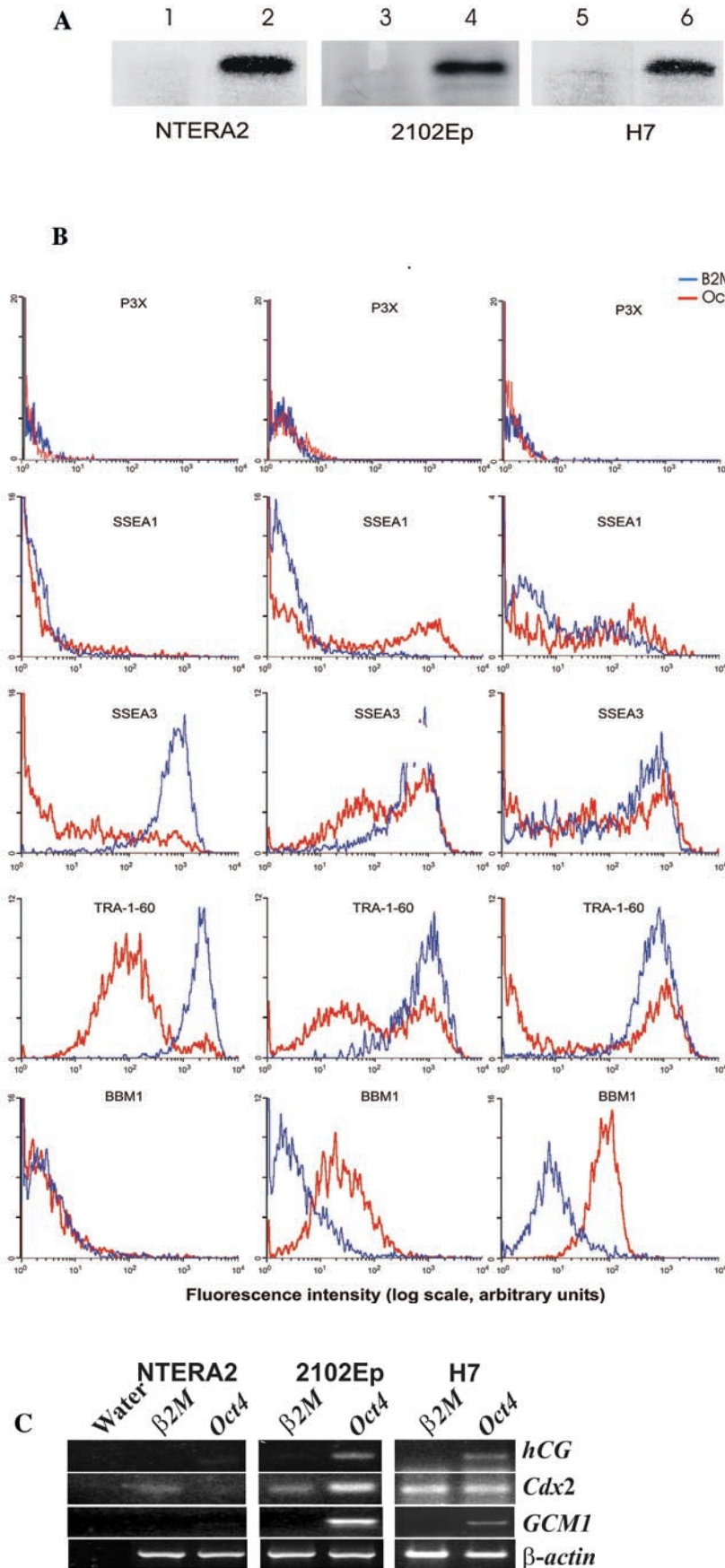


**Figure 4.** Morphology and TRA-1-60 expression of human EC and ES cells after Oct4 RNAi. (**A**): Phase contrast (left) and TRA-1-60 staining (right) of 2102Ep cells 5 days after mock (top) and Oct4 RNAi (bottom) treatments. (**B**): Phase contrast (left) and TRA-1-60 staining (right) of H14 cells 3 days after mock (top) and *Oct4* siRNA (bottom) treatments.

SSEA1 is strongly expressed by human trophectoderm [16]. To test this hypothesis, the expression of *Cdx2*, *hCG*, and *Gcm-1* was assayed by RT-PCR (Fig. 5c). The transcription factor *Cdx2* has been reported to mark trophectodermal stem cells in the mouse [18]. In the case of the hEC and hES cells, we found that untreated or control cells treated with  $\beta 2M$  siRNA expressed low levels of *Cdx2*, which was upregulated in 2102Ep and downregulated in NTERA2 but remained unchanged in H7 cells after *Oct4* siRNA treatment. At the same time, *hCG* and *Gcm1*, also markers of trophectoderm, were upregulated after *Oct4* siRNA in 2102Ep and H7 cells, though they were not detected in NTERA2 cultures. In a separate experiment, *hCG* was also upregulated in H14 hES cells after *Oct4* siRNA (Fig. 6b).

## DISCUSSION

Human ES cells and their malignant counterparts, EC cells, which are the stem cells of teratocarcinomas, closely resemble one another, as well as cells from the inner cell mass of human blastocysts [4, 16]. For example, they share expression of several widely used surface antigen markers that are characteristic of the undifferentiated cells. Indeed, most of those in current use were first developed in studies of EC cell lines. The extensive pluripotency of human ES cells offers great potential for their use in understanding the mechanisms that guide early human embryogenesis, as well as in providing a source of normal functional differentiated cells for a range of applications from drug discovery and toxicology to regenerative medicine. However, investigations of these



**Figure 5.** The effect of *Oct4* knockdown by siRNA on the expression of surface antigens, and *hCG*, *Gcm1*, and *Cdx2* by NTERA2 and 2102Ep hEC cells and H7 hES cells 5 days after treatment with *Oct4* or  $\beta$ 2M siRNA. **(A):** Western blot for Oct4 in NTERA2 (lanes 1, 2), 2102Ep (lanes 3, 4), and H7 (lanes 5, 6); siRNA to *Oct4* (lanes 1, 3, and 5) and siRNA to  $\beta$ 2M (lanes 2, 4, and 6). Lysates containing the equivalence of  $1.5 \times 10^5$  cells were loaded in each lane. Ponceau-S staining confirmed similar loading and transfer for lysates (not shown). **(B):** Surface antigen expression after treatment with *Oct4* siRNA (red histograms) or  $\beta$ 2M (blue histograms). P3X63Ag8 is used as a negative first antibody control. Note the disappearance of markers of the undifferentiated state, SSEA3 and TRA-1-60, after treatment with siRNA to *Oct4* and the appearance of SSEA1, especially in 2102Ep and H7 cells, indicating differentiation. The percentage of cells in each population is shown in Table 1. Also note that  $\beta$ 2-microglobulin was down-regulated after treatment with  $\beta$ 2M siRNA but not *Oct4* siRNA in 2102Ep and H7 cells. The low and effectively undetectable level of  $\beta$ 2-microglobulin in the NTERA2 cells used in this experiment, in contrast to the experiment shown in Figure 1, reflects a commonly observed variability in MHC antigen expression by this particular hEC line in contrast to others [6]. **(C):** RT-PCR analysis of *hCG* and *Cdx2* expression after treatment with siRNA to *Oct4*.  $\beta$ -actin PCR was used as a template loading control.

**Table 1.** Changes in antigen expression following  $\beta 2M$  and *Oct4* siRNA–induced knockdown

Antigen	RNAi treatment	Measurement	NTERA2	2102Ep	H7
P3X	$\beta 2M$	% positive	2.9	2.6	0.8
	<i>Oct4</i>	% positive	1.5	3.0	1.6
SSEA1	$\beta 2M$	% positive	1.6	3.2	33.4
	<i>Oct4</i>	% positive	7.1	40.1	54.5
SSEA3	$\beta 2M$	% SSEA3 high <sup>a</sup>	91.7	81.3	66.8
	<i>Oct4</i>	% SSEA3 high <sup>a</sup>	17.5	50.6	58.1
TRA-1-60	$\beta 2M$	% TRA-1-60 high <sup>a</sup>	98.1	91.8	95.8
	<i>Oct4</i>	% TRA-1-60 high <sup>a</sup>	11.1	43.2	54.9
BBM1	$\beta 2M$	% positive	4.1	3.4	29.3
	<i>Oct4</i>	% positive	6.3	26.7	98.9

The table shows quantitative changes in the populations of antigen-expressing cells following RNA interference (RNAi) for  $\beta 2$ -microglobulin ( $\beta 2M$ ) and Oct4, shown in Figure 5. In the case of SSEA1 and  $\beta 2M$ , the percentage of cells fluorescing is compared with those fluorescing after reacting with the negative control antibody P3X63Ag8. In the case of SSEA3 and TRA-1-60, many of the cells would be scored positive by this criterion, following transfection with Oct4 short interfering RNA (siRNA), although a substantial reduction in expression was seen (5- to 10-fold) in terms of fluorescence intensity (Figure 5). Most likely, this is due to slow turnover of these antigens, which is seen in other studies of EC and ES cell differentiation [4, 6, 8, 12]. Therefore, in these cases we have shown the percentage of cells in the high-expressing populations identified in the  $\beta 2M$  siRNA control.

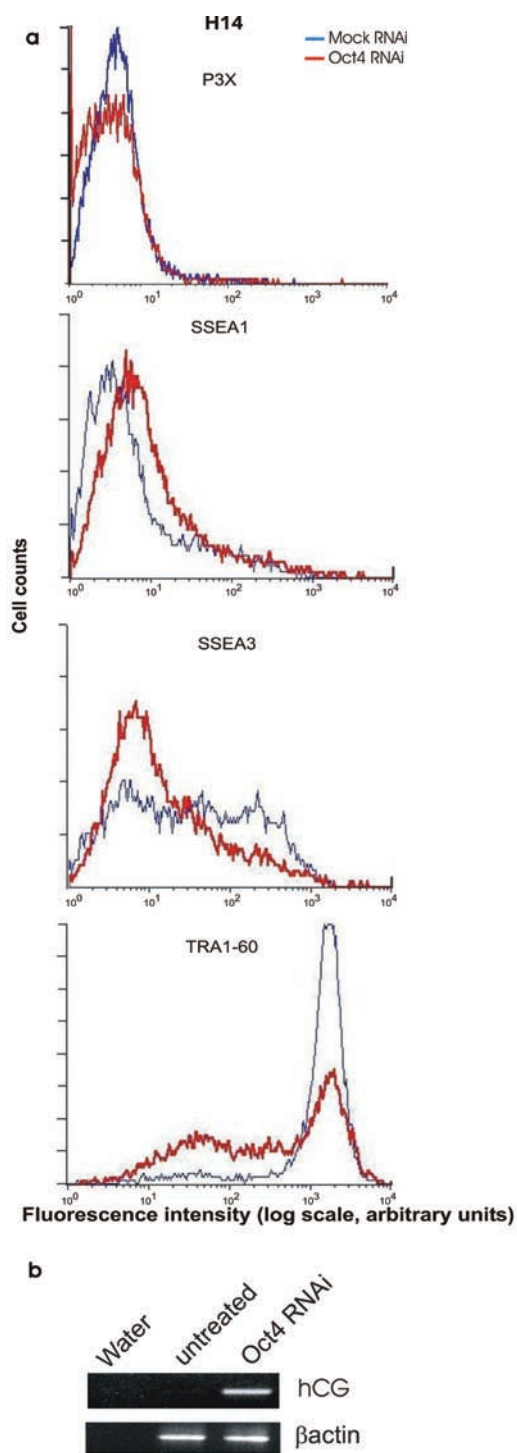
cells are currently difficult, precisely because of their pluripotency and propensity to differentiate and their requirements for complex culture systems that involve feeders or conditioned medium. By contrast, EC cells, while exhibiting many of the key features of the ES cells, are simpler to maintain and to analyze since their adaptation to tumor growth has reduced, but not eliminated, their capacity for differentiation and their demands on culture conditions. The two cell types thus provide complementary tools for investigation, allowing techniques and approaches to be developed in the simpler EC cell systems before application to ES cells. Further, germ cell tumors in which EC cells are the principle malignant component remain a significant medical problem, as they are the most common type of cancer occurring in young men. Understanding the relationships between EC and ES cells and the mechanisms that control their self-renewal will likely affect tumor biology, as well as embryology and regenerative medicine [19].

Human EC and ES cells differ in a number of ways from their murine counterparts, although they also have similarities [19]. For example, morphology and high expression of alkaline phosphatase and Oct4 are similar between mouse and human EC and ES cells. However, their surface antigen phenotypes are strikingly different, while the cytokine LIF is able to inhibit differentiation and promote self renewal of mouse ES cells but apparently has no effect on human ES cells [1].

Our results now show that RNA interference techniques using siRNA can be applied to human EC and ES cells to investigate the function of specific genes in control of their self-renewal and differentiation. The technique itself does not seem to have nonspecific effects on the cells, nor does it induce their differentiation; with the set of genes that we have studied—*GFP*,  $\beta 2$ -microglobulin, and *Oct4*—the effects on gene expression seem to be specific. Vallier et al. [20] have also recently demonstrated the efficacy of RNAi in human ES cells, where they used stable expression of siRNA to knock down expression of hypoxanthine phosphoribosyl transferase (HPRT). There might be concern that application of double-stranded RNA to EC and ES cells could induce expression of interferon, which itself might influence cell phenotype. Although we have not directly tested whether interferon is induced in these cells under the conditions of the experiments, our previous extensive studies of the effects of interferon on human EC [21] and ES [4] cells indicated that interferons do not induce the differentiation of these cells.

Taken together, the results also indicate that reduced expression of Oct4 in 2102Ep hEC cells and in H7 and H14 hES cells leads to their differentiation. Further, our data are consistent with the view that, as in murine ES cells, this differentiation is in the direction of trophectoderm. SSEA1 is expressed by trophectoderm in the early human embryo [16], as is hCG, and it has been shown that hCG expression is negatively regulated by Oct4 [22]. *Gcm1* has also been identified





**Figure 6.** The response of H14 hES cells to *Oct4* siRNA, in comparison with a mock-treated (dsRNA) control, 3 days after treatment. **(A):** Expression of surface antigens SSEA1, TRA-1-60, and SSEA3 3 days after treatment with *Oct4* siRNA (red histograms), or mock transfected cells, using transfection reagents and conditions but no RNA (blue histograms). **(B):** *hCG* expression in the same cells, analyzed by reverse transcription polymerase chain reaction (RT-PCR).

as a marker for trophoctodermal differentiation [23]. The results with expression of *Cdx2* are slightly less clear, though also consistent with trophoblast differentiation. In the mouse, *Cdx2* is not expressed by ES cells or their inner cell mass equivalents from the blastocyst but is induced in trophoctoderm derived from ES cells [3]. However, our results indicate that human EC and ES cells differ by initially expressing *Cdx2* at low levels. Nevertheless, in 2102Ep cells, there was indeed a marked upregulation of *Cdx2* following knockdown of Oct4 expression, and this is consistent with induction linked to trophoctoderm differentiation. In contrast, the initial levels of *Cdx2* in the human ES cells were somewhat higher than in 2102Ep, and no significant change was noted after Oct4 knockdown. The simplest explanation for this result is masking caused by the relative heterogeneity and spontaneous differentiation of the ES cell cultures compared with the 2102Ep EC cell cultures; *Cdx2* is not exclusively expressed in the trophoctoderm lineage [24].

By contrast to the 2102Ep EC cells and the ES cells, NTERA2 EC cells showed no induction of *hCG* or *Gcm1*, and downregulated *Cdx2*, in response to *Oct4* siRNA. This suggests that, although Oct4 is also required for the maintenance of an undifferentiated state in these EC cells, knockdown of Oct4 results in their differentiation along a distinct lineage or lineages. While closely resembling other human EC and ES cells, NTERA2 cells also exhibit some differences. For example, they do not express the placental-like form of alkaline phosphatase, commonly found in human EC cells [25]; they exhibit particularly low and variable levels of MHC antigen expression [6]; and their ability to differentiate in response to retinoic acid is rare among hEC cells. Taken together, these and the current observations might reflect an equivalence of NTERA2 cells to a later embryonic stage than 2102Ep or the hES cells.

In summary, we have shown that RNAi can be used specifically to downregulate gene expression in a transient way in human EC and ES cells, with resulting consequences for cell behavior. With an increased understanding of the genetic mechanisms that control cellular differentiation, regulation of gene activity by RNAi may prove a useful tool for manipulating the differentiation of hES cells. Our results further show that, in contrast to the role of LIF, another regulatory factor, Oct4, does seem to play the same role in human and murine EC and ES cells. Hay et al. [26] have also recently shown in two other human ES lines, H1 and H9, that knockdown of Oct4 results in their differentiation to trophoctoderm. Since EC cell proliferation was attenuated by knockdown of Oct4, our results also raise the possibility that Oct4 itself might be a useful target for therapy of testicular germ cell tumors in which EC cells are key contributors to malignancy.

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