LETTER

Oct4 RNA Interference Induces Trophectoderm Differentiation in Mouse Embryonic Stem Cells†

J. Matthew Velkey and K. Sue O'Shea*

Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, Michigan

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Summary: We examined whether suppression of Oct4 via RNA interference (RNAi) would affect embryonic stem (ES) cell lineage choice. Cells were transfected with plasmids containing an independently expressed reporter gene and an RNA polymerase type III promoter to constitutively express small stem-loop RNA transcripts corresponding to Oct4 mRNA. Cells transfected with Oct4 RNAi constructs demonstrated reduced levels of Oct4 mRNA and exhibited characteristics of trophectodermal differentiation. These findings support the critical role of Oct4 in regulating stem cell identity and suggest that future experiments using RNAi in ES cells can elucidate the roles of other genes affecting lineage specification during differentiation. genesis 37:18–24, 2003. © 2003 Wiley-Liss, Inc.

Key words: embryonic stem cells; Oct4; RNA interference; trophoblast; differentiation

The POU transcription factor Oct4 (encoded by *Pou5f1*) has been characterized as a regulator of ES cell pluripotency. Oct4 mRNA and protein are present in the inner cell mass (ICM) of mouse embryos (Palmieri *et al*., 1994), in cultured ES (Rosner *et al*., 1990), and embryonal carcinoma cells (Okamoto *et al*., 1990), but not in trophectoderm (Palmieri *et al*., 1994), or cultured trophoblast stem (TS) cells (Tanaka *et al*., 1998). Lineage tracing studies have established that ES cells inserted into morulae or blastocysts contribute to all tissues of the fetus but not the trophectoderm or primitive endoderm (Beddington and Robertson, 1989), whereas TS cells are conversely restricted to the trophoblast lineage (Tanaka *et al*., 1998). However, genetic studies have shown that altering expression levels of Oct4 can modify the early lineage restriction of ICM/ES cells. For example, Oct4-deficient embryos fail to form an ICM but instead consist primarily of cells committed to the trophoblast lineage (Nichols *et al*., 1998), and gene-targeted ES cells expressing reduced levels of Oct4 differentiate to trophectoderm in vitro while overexpression induces differentiation into primitive endoderm and mesoderm (Niwa *et al*., 2000). As a means of exploring the Oct4-mediated plasticity of ES cells (and with the understanding that the endogenous *Pou5f1* locus likely has regulatory roles for other transcription factors), we wished to determine both the efficacy and the developmental consequences of knocking down Oct4 expression in wildtype ES cells posttranscriptionally via RNA interference (RNAi).

RNAi is a widely used tool for suppressing gene expression in invertebrates and plants, but its application in mammals has been hindered until recently because the introduction of double-stranded RNA (dsRNA) longer than about 30 basepairs induced a presumably antiviral "interferon response" of nonspecific gene suppression (Zamore, 2001). Transfection of >30 bp dsRNA into undifferentiated mouse ES cells was reported to suppress function of a transgene in an RNAi-like manner, suggesting ES cells lack the interferon response (Yang *et al*., 2001), but the possibility remains that, with differentiation, gene suppression could become nonspecific. However, it has now been shown that the interferon response can be circumvented altogether in differentiated mammalian cells through the use of short (<30 bp) interfering RNA duplexes (siRNAs) (Elbashir *et al*., 2001). Moreover, siRNAs have been produced constitutively in mammalian cells using vectors containing RNA polymerase type III promoters such as the U6 small nuclear RNA promoter (U6) (Yu *et al*., 2002; Paul *et al*., 2002) or H1 promoter (Brummelkamp *et al*., 2002) driving expression of a single RNA transcript that folds into a hairpin siRNA. It has recently been shown that knockdown of p120-Ras GTPase-activating protein via hairpin siRNAs driven from the H1 promoter could recapitulate a *Rasa1* null phenotype in ES cell-derived embryos (Kunath *et al*., 2003). In each instance, significant reduction

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* Correspondence to: Dr. K. Sue O'Shea, Department of Cell and Developmental Biology, 4643 Medical Sciences II, University of Michigan Medical School, Ann Arbor, MI 48109-0616.

E-mail: oshea@umich.edu

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in the expression of targeted genes in a sequence-specific manner was observed and, because the siRNAs were constitutively expressed, long-term gene suppression could be achieved.

In designing our siRNA expression vectors, it was not known if the U6 or H1 promoters would exhibit any differences in activity in ES cells as it has been reported that different Pol III promoters can show variations in the level and localization of transcript expression (Ilves *et al.*, 1996). To test this, we transfected B5 mouse ES cells expressing an enhanced green fluorescent protein (eGFP) transgene (Hadjantonakis *et al.*, 1998) with plasmids (Fig. 1a) containing either U6 or H1 hairpin siRNA expression cassettes with coding sequences corresponding to eGFP mRNA (pU-eGFPi and pH-eGFPi, respectively). Cells transfected with either eGFPi plasmid showed significantly reduced levels of eGFP protein and mRNA when assayed at 48 and 72 h posttransfection as compared to controls transfected with the pBluescript II SK parent plasmid (pSK) alone (Fig. 2, Table 1), and we noted that cell cultures transfected with pH-eGFPi contained significantly more eGFP-negative cells after 72 h as compared to any other treatment group (Table 1). Overall, these results indicated that constitutively expressed hairpin siRNAs could effectively suppress gene expression in ES cells and that the H1 promoter appeared to be slightly more effective at maintaining hairpin siRNA-mediated RNAi. We then wished to determine whether RNAi targeting of an endogenous regulatory gene such as Oct4 would have consequences in lineage segregation or developmental potential.

To identify transfected cells, pRed expression constructs (Fig. 1b,c) were made in which a red fluorescent protein (DsRed) is driven from the simian cytomegalovirus immediate-early enhancer/promoter in the pCS2 vector (Turner and Weintraub, 1994). In the second multiple cloning site we placed either U6 or H1 hairpin siRNA expression cassettes containing coding sequences corresponding to Oct4 mRNA (pCU-Octi and pCH-Octi, respectively). D3 mouse ES cells were transfected with pCU-Octi, pCH-Octi, or pRed alone (day 0), cultured in ES cell medium supplemented with leukemia inhibitory factor (LIF) to inhibit differentiation (Williams *et al.*, 1988), then enriched by fluorescence activated cell sorting (FACS) based on expression of DsRed on day 1.5. At 1, 3, 5, or 7 days after transfection, cells were fixed for immunohistochemical analysis and RNA isolated from cell lysates for RT-PCR assays.

As shown in Figure 3, cells transfected with pRed (Fig. 3e–g) were similar to untransfected ES cells, having a high nucleus:cytoplasm ratio and multiple nucleoli typical of ES cells. In contrast, many cells transfected with either pCU-Octi (Fig. 3h–j) or pCH-Octi (Fig. 3k–m) were morphologically similar to trophoblast giant cells (epithelioid cells with large nuclei and dark perinuclear deposits) (Tanaka *et al.*, 1998). When viewed with the scanning electron microscope, wildtype D3 ES cells (Fig. 3a) were compact, with filopodia but few lamellipodia. In contrast, the trophoblast-like cells (Fig. 3b) were highly spread, exhibiting a large nuclear bulge and an expansive cytoplasm that frequently surrounded neighboring cells. Immunostaining showed that the trophoblast-like cells in the experimental groups expressed a trophectodermal marker, TROMA-I (Hashido *et al.*,

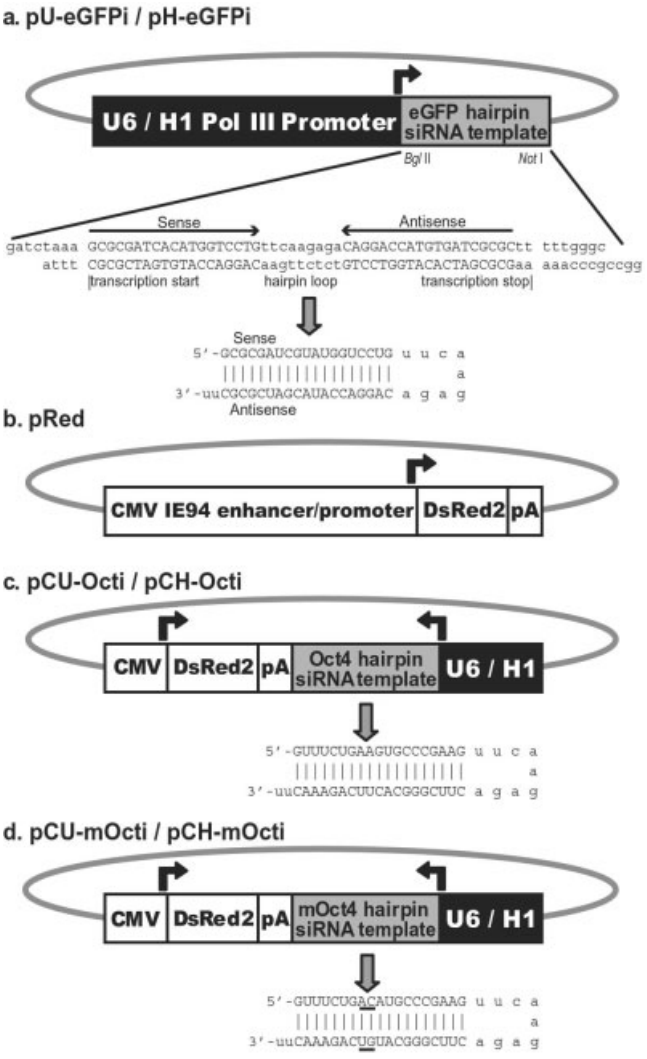

FIG. 1. Hairpin siRNA and DsRed expression constructs. **a**: Diagram of U6 and H1 RNA Pol III promoter-based hairpin siRNA expression vectors used to target eGFP in B5 ES cells. The U6 or H1 promoter has been cloned into pSK plasmids with an engineered *Bgl*II site at position –9 to –4. Templates encoding hairpin siRNAs with 19-nt of homology to the eGFP transgene were synthesized as 64-mer DNA oligonucleotides, annealed in vitro, and inserted as shown. Base pairing between sense and antisense strands of the transcript is predicted to produce a hairpin siRNA with a 9-nt loop. **b**: The pRed vector contains a red fluorescent reporter (DsRed2) with an SV40 polyA signal sequence driven from the constitutively active simian cytomegalovirus immediate-early RNA Pol II enhancer/promoter. **c**: Oct4 RNAi reporter constructs contain the DsRed reporter and either a U6 or H1-based RNAi cassette in the opposite orientation. Pol II transcription from the CMV promoter (right arrow) produces DsRed, whereas Pol III transcription from U6 or H1 (left arrow) produces hairpin siRNAs targeting Oct4. **d**: "Mutant" Oct4 RNAi (mOcti) constructs are identical to the Octi constructs tested except for a 2-nt substitution (underlined) in the middle of the hairpin stem.

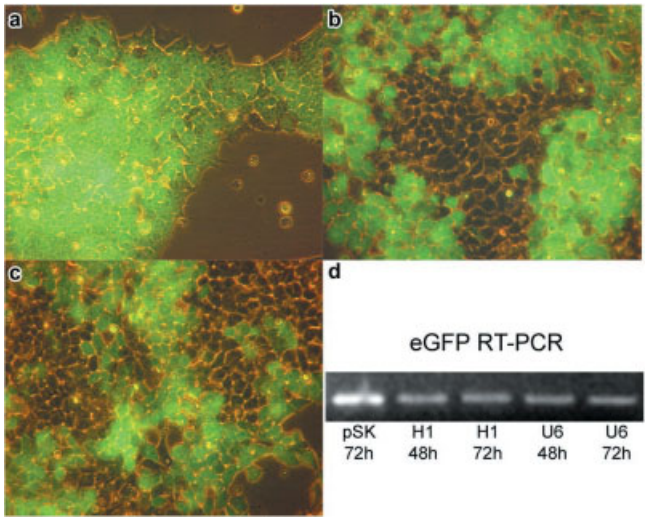

FIG. 2. Suppression of eGFP via RNAi. **a**: B5 ES cells 72 h after electroporation with control plasmid, pSK, show little to no loss of eGFP expression. **b,c**: B5 ES cells 72 h after electroporation with either pH-eGFPi (**b**) or pU-eGFPi (**c**) show a significant reduction in the number of eGFP-positive cells and the clonal distribution pattern observed suggests that the eGFP suppression is stable and heritable. A detailed statistical analysis indicated that pH-eGFPi was slightly more effective in suppressing eGFP fluorescence than pU-eGFPi at 72 h. **d**: RT-PCR analysis shows reduced levels of eGFP mRNA at 48 and 72 h in cells electroporated with either eGFP RNAi construct as compared to the control group at 72 h.

Table 1
eGFP Expression in B5 ES Following Treatment With eGFPi or Control Plasmids

Treatment group	$eGFP^+$ cells	$eGFP^-$ cells
48h, 72h pSK	2054	80
48h, 72h pU-eGFPi	2437*	854*
48h pH-eGFPi	1261*	582*
72h pH-eGFPi	1267*	874*

*$P < 0.001$, eGFPi vs. pSK.

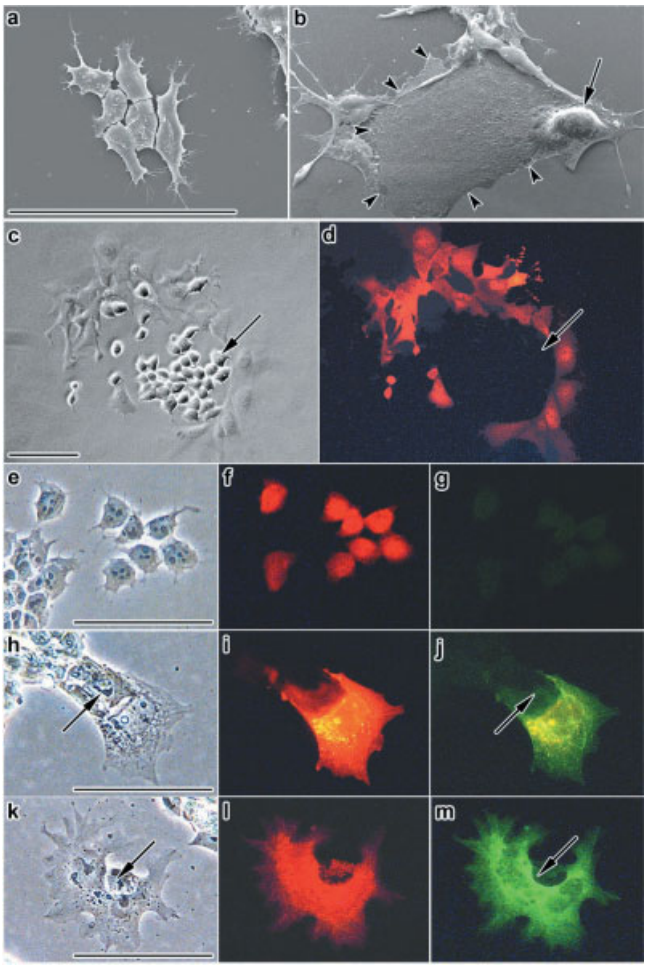

FIG. 3. Suppression of Oct4 via RNAi induces trophectodermal differentiation in wildtype ES cells. **a,b**: When viewed with an SEM, cultures of D3 ES cells transfected with control plasmid (**a**) exhibit a typical ES cell morphology and contain only compact clusters of cells with filopodia and few lamellipodia. In contrast, cultures of cells transfected with either pCU-Octi (**b**) or pCH-Octi (not shown) contain very large epithelioid cells with features characteristic of trophoblast giant cells such as an extensive, thinly spread cytoplasm (boundary indicated by arrowheads) and a pronounced nuclear bulge (arrow). **c,d**: Live-cell phase contrast (**c**), and epifluorescence microscopy (**d**) images of cells transfected with pCH-Octi and enriched by FACS show the presence of untransfected, DsRed-negative cells (arrow) that retain an ES cell morphology. The Octi-transfected cells, however, appear more epithelioid and surround the clusters of ES cells in a manner reminiscent of a "blastocyst." **e–m**: D3 ES cells transfected with pRed alone (**f**) retain an ES cell morphology (**e**) and do not react with the trophoblast giant cell-specific antibody, TROMA-I (**g**). Cells transfected with pCU-Octi (**h–j**) or pCH-Octi (**k–m**) change their ES cell morphology as they differentiate into large, flattened cells exhibiting characteristics of trophoblast giant cells such as large nuclei, extensive cytoplasmic spreading, dark perinuclear deposits (**h,k**) and TROMA-I immunostaining (**j,m**). Panels **h–m** show other examples in which the transfected trophectoderm-like cells can be seen enveloping untransfected ES cells (arrows). Scale bars in **a–m** = 100 μm.

1991) (Fig. 3j,m), whereas pRed-transfected cells were consistently TROMA-I negative (Fig. 3g). It should be noted that in both the pCU-Octi and pCH-Octi treated groups, the sorted cell populations initially were 90–95% pure (data not shown). However, the small fraction of untransfected cells not eliminated by FACS expanded significantly in culture by d5 and 7, likely due to the favorable conditions imparted by the culture medium. The presence of untransfected cells had an interesting effect in the experimental groups in that we observed several instances where the Octi-treated cells surrounded untransfected cells in a manner reminiscent of a blastocyst (Fig. 3c,d). That is, we frequently saw clusters of untransfected ES cells completely or partially enveloped (as in Fig. 3h–m) by transfected trophectoderm-like cells.

We used RT-PCR to assay for expression of Oct4 mRNA and one of its downstream target genes, *Fgf4* (Nichols *et al.*, 1998), as well as markers of proliferating trophoblast, *mEomes* (Russ *et al.*, 2000) and *Fgfr2* (Orr-Urtreger *et al.*, 1993), and trophoblast giant cells, *Pl-I* (Faria *et al.*, 1991). As shown in Figure 4, we detected

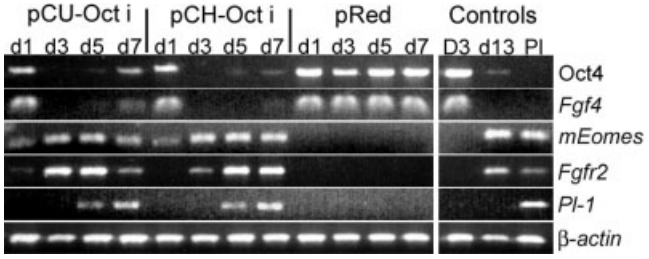

FIG. 4. Changes in gene expression patterns following suppression of Oct4 via RNAi. RT-PCR analyses of RNA isolated from cell lysates illustrates the abrogation of Oct4 mRNA transcripts and suppression of a downstream target gene, *Fgf4*, in cells transfected with either pCU-Octi or pCH-Octi. Concomitant with the suppression of Oct4 and *Fgf4*, the upregulation of early trophoblast markers, *mEomes* and *Fgfr2*, is observed by d3 in the Octi treatment groups, while expression of a late trophoblast marker, *Pl-I*, is not observed until d5. Cells transfected with control plasmid (pRed) show no reduction in Oct4 or *Fgf4* nor upregulation of any trophoblast markers at any time point assayed. Cells were sorted 1.5 days after transfection; thus, d1 lanes represent data from mixed cultures, whereas d3, d5, and d7 lanes represent enriched (sorted) cultures. The expression of Oct4 and *Fgf4* observed in the Octi treatment groups at d5 and d7 is likely due to the proliferation of untransfected ES cells that were not excluded by FACS (see text for details). Analyses of lysates from undifferentiated D3 ES cells (D3), day 13 mouse embryos (d13), and day 9 mouse placenta (Pl) all show appropriate expression levels of the markers tested. Expression of β-actin is included as a positive control.

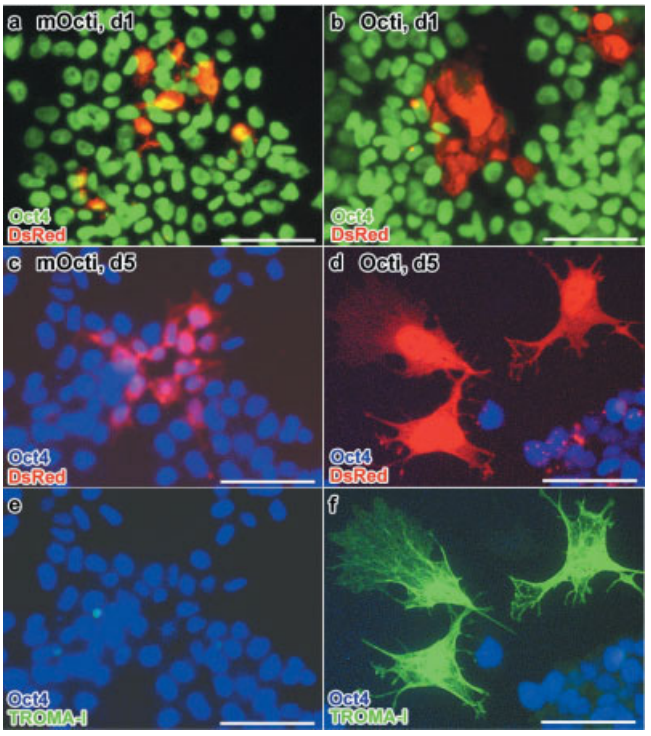

FIG. 5. Knockdown of Oct4 by hairpin siRNA constructs is sequence-specific. **a,b**: Merged images illustrating the expression of Oct4 (green) and DsRed (red) in unsorted D3 ES cell cultures 24 h after treatment with control (**a**) or Octi (**b**) constructs. Untransfected ($DsRed^-$) ES cells present in both cultures show nuclear expression of Oct4 confirmed by Hoechst 33258 co-staining (not shown). Cells transfected with pCU-mOcti (**a**) also show nuclear expression of Oct4 (yellow), whereas cells transfected with pCU-Octi (red cells in **b**) are $Oct4^-$. **c–f**: Merged images showing the expression of Oct4 (blue) and either DsRed (red in **c,d**) or TROMA-I (green in **e,f**) in unsorted D3 ES cell cultures 5 days after treatment with control (**c,e**) or Octi (**d,f**) constructs. Cells transfected with pCH-mOcti (red cells in **c**) coexpress Oct4 and do not exhibit any immunoreactivity to TROMA-I (**e**). In contrast, cells transfected with pCH-Octi (red cells in **d**) are $Oct4^-$ and exhibit overt signs of trophectodermal differentiation, including TROMA-I immunoreactivity (**f**). Scale bars in **a–f** = 100 μm.

upregulation of early trophoblast markers by d1 (*mEomes*) and d3 (*Fgfr2*) and a later marker for trophoblast giant cells (*Pl-I*) by d5 in the experimental groups, consistent with the trophectodermal phenotype observed in our morphological analyses. Other lineage-restricted genes such as *Sox2* (neuroectoderm) and *Brachyury* (mesoderm) were not expressed in the Octi treatment groups (data not shown), further indicating that the differentiation induced by Oct4 RNAi was trophoblast-specific. Reduced expression of Oct4 and *Fgf4* was only slightly apparent at d1 (prior to sorting), presumably due to the large number of untransfected cells present that were still expressing these markers in the unsorted cultures. Purification of the cultures by FACS and subsequent analysis indicated an abrogation of Oct4 and *Fgf4* mRNA in both experimental groups at d3. However, we saw a slight increase in Oct4 and *Fgf4* expression at d5 and d7, presumably due to the expansion of untransfected ES cells that remained in each culture, as discussed above.

While the increase in Oct4 and *Fgf4* in the Octi cultures could be attributed to expansion of the untransfected ES cells not eliminated by FACS, the possibility remains that the suppression of Oct4 was transient or incomplete. In order to assess Oct4 expression at the protein level in individual cells, we performed additional transfections followed by immunohistochemical analyses using TROMA-I and Oct4-specific antibodies. In addition, although RNAi has been reported to be highly sequence-specific (Brummelkamp *et al.*, 2002; Yu *et al.*, 2002), we reasoned that the effects observed in our previous experiments could have been a generic response of the cells to hairpin siRNAs, so we also transfected cells with pCU-mOcti and pCH-mOcti constructs (Fig. 1d) containing a 2-nucleotide "mutation" in the middle of the hairpin stem as an additional control. Cells were transfected with either control or Octi constructs, cultured for 1 day, plated at low density (150 cells mm^{-2}), and allowed to grow for an additional 2–4 days. The cultures were not sorted by FACS so that untransfected $Oct4^+$ ES cells would remain as internal positive controls for the Oct4 antibody.

We assayed the cells at 1, 3, or 5 days following transfection and found that cells transfected with either mOcti (Fig. 5a) or pRed constructs (not shown) exhibited normal nuclear expression of Oct4 protein as indicated by Hoechst 33258 co-staining (not shown) after 1 day as compared to untransfected cells. In contrast, cells

Table 2
Oct4 and TROMA-I Expression Profiles of ES Cells Transfected With Octi or Control Plasmids

Treatment group	Oct4$^+$	TROMA-I$^+$	Oct4$^-$/TROMA-I$^-$	Total red$^+$ cells
Day 1: Controls	631	0	0	631
U-Octi	10*	0	191*	201
H-Octi	9*	0	226*	235
Day 3: Controls	524	0	30	554
U-Octi	8*	57*	27*	92
H-Octi	4*	53*	35*	92
Day 5: Controls	546	0	34	580
U-Octi	4*	56*	6*	66
H-Octi	1*	76*	4*	81

*$P < 0.001$, Octi vs. controls.
All TROMA-I$^+$ cells were Oct4$^-$; conversely, all Oct4$^+$ cells were TROMA-I$^-$.

transfected with either pCH-Octi (Fig. 5b) or pCU-Octi (not shown) constructs were noticeably devoid of Oct4 protein within 1 day of treatment. We did not observe TROMA-I$^+$ cells in any of the d1 cultures (not shown), but this might be expected, given that it is a marker of differentiated trophoblast giant cells. By day 5, however, ~95% of the Octi-transfected cells we observed were Oct4$^-$ (Fig. 5d,f) and TROMA-I$^+$ (Fig. 5f), whereas cells in the control groups remained Oct4$^+$ (Fig. 5c, e) and TROMA-I$^-$ (Fig. 5e). To quantify these observations, we carried out cell counts (Table 2) of transfected (DsRed$^+$) cells based on Oct4 and TROMA-I immunoreactivity in 10 randomly selected fields from three replicate samples. We did not observe any statistically significant variation ($P > 0.05$) between the control groups (pRed, pCU-mOcti, and pCH-mOcti) at each time point, indicating that the effects of transfection with either mOcti construct were no different than treatment with the pRed construct alone. Similarly, no significant differences ($P > 0.05$) were observed between the U-Octi and H-Octi groups at each time point. However, significant ($P < 0.001$) differences were observed when comparing the controls versus the Octi groups at any of the time points. In addition, when we performed multiple comparisons between each control and Octi group, we also observed differences in the populations between time points. That is, the Octi treatment groups were significantly different from each other ($P < 0.001$) at d1, d3, and d5, probably a reflection of the temporal delay between suppression of Oct4 and development of TROMA-I immunoreactivity. The control groups also differed significantly when comparing d1 versus d3 and d1 versus d5 ($P < 0.001$) but not d3 versus d5 ($P = 0.99$), most likely due to random differentiation occurring as a result of low-density culture (Robertson, 1987).

Our results indicate that expression vectors containing either U6 or H1 RNA Pol III promoters can be used to generate hairpin siRNAs that effectively and rapidly suppress gene expression in ES cells in a sequence-specific manner. Whereas most vector-based RNAi studies to date have used U6 or H1 promoters exclusively, we have considered the possibility that these promoters exhibit different levels of activity in ES cells. The clearest differences are observed in our eGFPi experiments (Table 1); however, we also see subtle variations in the Octi data as well. For example, the RT-PCR data in Figure 4 reveal slight differences between the U6 and H1 vectors in both the suppression of Oct4 and *Fgf4* (d7 lanes) and induction of early (*Fgfr2*) and late (*Pl-I*) trophoblast genes (d3, d5, and d7 lanes). In the Octi experiments (Table 2), there were fewer Oct4$^+$ cells in the H-Octi treatment groups at d3 and d5 compared to the U-Octi group, although these differences were not statistically significant ($P = 0.16$ for d3; $P = 0.11$ for d5). Taken together, our data suggest that H1-mediated RNAi appears to be more effective at suppressing gene expression compared to the U6 promoter. Recent studies comparing U6 and H1-mediated RNAi knockdown of VEGF in cultured cells have observed similar (although not statistically significant) differences in overall efficacy (Zhang *et al.*, 2003). While it is possible that the differences observed merely reflect variations in transfection and/or integration efficiency as has been proposed (Zhang *et al.*, 2003), we believe our findings suggest the need for more rigorous quantitative analysis of the relative activities of these promoters in vector-based RNAi applications.

We have also shown that RNAi-mediated suppression of a developmentally relevant gene such as Oct4 has significant effects on lineage choice and plasticity in wildtype ES cells and induces differentiation even in the presence of serum and LIF, inhibitors of ES cell differentiation. The fact that we observe trophectodermal differentiation further supports an emerging model of developmental plasticity whereby stem cells may be capable of undergoing "de-differentiation" to a more primitive lineage, thus acquiring fates from which they were originally thought to be restricted (Rossant, 2001; Hübner et al., 2003). Recent work has shown that human ES cells are similarly capable of dedifferentiation to the trophoblast lineage when treated with bone morphogenetic protein 4 (Xu *et al.*, 2002). In this context, RNAi appears to be a valuable tool in stem cell biology for dissecting the pathways involved in lineage segregation. Hairpin siRNA expression vectors should also be useful for developmental studies in vivo, as it has been recently demonstrated that expression vectors (including those for hairpin siRNAs) can be injected into the tail vein of a

pregnant mouse and delivered to the developing embryo (Gratsch et al., 2003). Numerous developmental studies have taken advantage of inducible or tissue-specific promoter systems to elucidate the effects of extrinsic and intrinsic cell signals in development. We believe that conditionally expressed hairpin siRNAs could be used in a similar manner to address the effects of a specific gene product during particular stages of embryogenesis or differentiation of ES cells as embryoid bodies.

MATERIALS AND METHODS

Plasmid Construction

Mouse U6 and H1 promoters were PCR-amplified from sv129 mouse genomic DNA, digested with *EcoR*I and *BamH*I and cloned into pBluescript II SK (pSK) phagemids (Stratagene, La Jolla, CA) to make the subclones pU6 and pH1. Hairpin siRNA templates corresponding to nt 1325–1341 of pEGFP-N1 (ClonTech, Palo Alto, CA) were designed as previously described (Brummelkamp *et al*., 2002), synthesized as DNA oligonucleotides, annealed in vitro as described (Yu *et al*., 2002), and ligated within the *Bgl*II and *Not*I sites of pU6 or pH1 to make pU-eGFPi and pH-eGFPi. The red fluorescent protein, DsRed2, was PCR amplified from pDsRed2-1 (ClonTech), digested with *BamH*I and *Xba*I, and cloned into pCS2+ (provided by David Turner) to make pRed. The U6 and H1 promoters were released from pU6 and pH1 by digestion with *Kpn*I and *Not*I and moved into the corresponding sites of pRed to give the subclones, pCU and pCH. Hairpin siRNA templates corresponding to nt 670–688 of Oct4 (GenBank X52437) were inserted into pCU or pCH digested with *Bgl*II and *Not*I to construct pCU-Octi and pCH-Octi. DNA oligonucleotide templates with "mutations" corresponding to an AG→CA substitution at nt 678–679 in the Oct4 open reading frame were similarly inserted into pCU or pCH to produce pCU-mOcti and pCH-mOcti. Sequences for PCR primers and DNA oligonucleotide templates used in plasmid construction are provided in the on-line supplementary material.

Cell Culture, Transfection, and FACS

B5 and D3 mouse ES cells were cultured on 0.1% gelatin-coated substrates in ES medium consisting of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% ES-tested FBS (Atlanta Biologicals, Norcross, GA), 10^{-4}M β-mercaptoethanol (Sigma, St. Louis, MO), 0.224 μg/ml L-glutamine (Invitrogen), 1.33 μg ml^{-1} HEPES (Invitrogen), and 1,000 units ml^{-1} human recombinant LIF (Oncogene Research Products, San Diego, CA). 1.5×10^6 B5 ES cells suspended in 800 μl of ES medium were electroporated with 20 μg of *Not*I-linearized plasmid (pSK, pU-eGFPi, or pR-eGFPi) using a BioRad (Hercules, CA) Genepulser (0.3 kv current, 250 μF capacitance) and seeded onto six-well plates at 2.5×10^5 cells per well. For the Oct4 RNAi studies, wildtype D3 mouse ES cells were seeded onto six-well plates at 7.5×10^5 cells per well and transfected with 1 μg DNA/well (pRed, pCU-Octi, or pCR-Octi) 24 h later using Lipofectamine/Plus Reagent (Invitrogen) in serum-free DMEM. After 3 h, the transfection medium was replaced with ES medium; we designated this timepoint as d 0. At d 1.5, cells were dissociated with 0.25% trypsin/1mM EDTA, resuspended in PBS, and sorted based on expression of DsRed2 with a Coulter Elite ESP Cell Sorter using a 514 nm argon laser excitation source and 575 nm bandpass filter. Suspensions of the sorted cells were gently added to gelatin-coated 35 mm dishes or 13 mm diameter Thermanox® coverslips (Nalge Nunc Intl., Rochester, NY) at a density of ~150 cells mm^{-2} and then cultured up to d 7 in ES medium containing 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Microscopy and Immunohistochemistry

All light microscopy was performed on a Leitz Flouvert microscope equipped for phase contrast and epifluorescence microscopy and outfitted with a Nikon DXM1200 digital camera. Images were captured using Nikon ACT-1 digital imaging software and then imported into PhotoShop 6.0 (Adobe Systems, San Jose, CA) to compose the figures. For the eGFP RNAi studies, live B5 ES cells were imaged 48 and 72 h following transfection using a combination of low-light phase contrast and eGFP epifluorescence. For the Oct4 RNAi studies, live-cell phase contrast and DsRed epifluorescence microscopy was performed at 24-h intervals to track general changes in cell morphology, DsRed2 expression, and cell number. At the time-points assayed, cells were fixed with 2% paraformaldehyde/PBS, washed, and stored at 4°C in PBS. Nonspecific antibody binding was blocked by incubating in 10% donkey serum, then cells exposed to anti-cytokeratin endoA (TROMA-I ascites, Developmental Studies Hybridoma Bank, U. of Iowa, 1:50) and Oct4 antibody (sc-8628, Santa Cruz Biotechnology, Santa Cruz, CA, 1:100), followed by appropriate secondary antibodies conjugated to FITC (TROMA-I) or AMCA (Oct4). To confirm nuclear localization of Oct4 protein, selected samples were stained with Hoechst 33258 (Sigma). In these samples, the secondary antibodies were reversed (AMCA:TROMA-I; FITC:Oct4) since the cytoplasmic signal from TROMA-I could be clearly discerned from the Hoechst nuclear stain. For statistical analyses, cell counts were obtained from at least 10 randomly selected fields (25× objective) of treatment and control groups at each time point indicated and the data entered into Microsoft Excel spreadsheets for Chi-square statistical analysis (with Yates and Bonferroni inequality corrections where appropriate). For SEM analysis, cells grown on Thermanox coverslips were fixed in 2% glutaraldehyde / 0.1M Sorensen's phosphate buffer, then postfixed in 1% OsO_4/0.1M Sorensen's phosphate buffer. Samples were dehydrated through a graded ethanol series, rinsed twice with hexamethyldisilazane, and air-dried. Specimens were mounted on conductive stubs, sputter-coated with Au-Pd, and viewed with an Amray 1000B SEM (10 keV) equipped with a SEMICAPS SEMSYNC digital imaging system.

RT-PCR

Cytoplasmic RNAs were extracted from lysates of cells treated with TRIzol reagent (Invitrogen), DNAsed, and quantified by spectrophotometry. For the eGFP RNAi studies, cell lysates were collected 48 and 72 h after electroporation. For the Oct-4 RNAi studies, lysates were collected at 1, 3, 5, and 7 days after transfection. RNAs isolated from untreated D3 ES cultures, gestation day 13 mouse embryos, and gestation day 9 mouse placental tissue were included as additional controls. RNAs (1 μg each) served as templates in reverse transcription reactions with oligo-dT primers, and 1/20 of the single-strand cDNA products were used in each PCR amplification. General PCR conditions were 94°C / 3 min, 94° / 30 s, 53–63°C / 1 min, 72°C / 1 min for 25–40 cycles. Specific information regarding PCR primer sequences and reaction conditions is provided in the online supplementary material. The PCR products (10 μl each) were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, then photographed in a UV light box.

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