STEM CELLS®
Rapid Communication

Enhancing and Diminishing Gene Function in Human Embryonic Stem Cells

LUDOVIC VALLIER,[a] PETER J. RUGG-GUNN,[a] ISABELLE A. BOUHON,[a] FRIDA K. ANDERSSON,[a] AMANDA J. SADLER,[b] ROGER A. PEDERSEN[a]

[a]Department of Surgery, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom; [b]School of Animal and Microbial Sciences, University of Reading, Reading, United Kingdom

Key Words. *Human embryonic stem cells · Fluorescent reporter genes · Green fluorescent protein · Red fluorescent protein · Transfection · Small interfering RNA · Gain-of-function · Loss-of-function · Stem cell therapy · Regenerative medicine*

ABSTRACT

It is widely recognized that gain- and loss-of-function approaches are essential for understanding the functions of specific genes, and such approaches would be particularly valuable in studies involving human embryonic stem (hES) cells. We describe a simple and efficient approach using lipofection to transfect hES cells, which enabled us to generate hES cell lines expressing naturally fluorescent green or red proteins without affecting cell pluripotency. We used these cell lines to establish a means of diminishing gene function using small interfering (si)RNAs, which were effective at knocking down gene expression in hES cells. We then demonstrated that stable expression of siRNA could knock down the expression of endogenous genes. Application of these gain- and loss-of-function approaches should have widespread use, not only in revealing the developmental roles of specific human genes, but also for their utility in modulating differentiation. ***Stem Cells*** *2004;22:2-11*

INTRODUCTION

The unique value of human embryonic stem (hES) cells for regenerative medicine lies in their capacity to differentiate into all body tissues [1, 2]. Mouse ES cells expressing lineage-specific transcription factors are capable of providing clinical benefits for immunodeficiency or Parkinson's disease when their in vitro differentiated derivatives are transplanted into recipient animal models [3, 4]. Thus, robust technologies for enhancing and diminishing gene function in hES cells can expedite their controlled in vitro differentiation into clinically useful cell types. This may involve, for example, achieving the expression of inductive genes, inhibiting mechanisms leading to competing pathways, and supplying reporter genes to indicate the status of cell differentiation. We used lipofection to generate hES cell lines expressing naturally fluorescent green or red proteins without changing their characteristics, specifically their capacity to differentiate into mesoderm, endoderm, and ectoderm cell types. We used these cell lines to examine the utility of small interfering (si)RNAs [5] as a means of diminishing hES cell gene function. We found that siRNAs were an effective and specific means of knocking down gene expression in hES cells whether administered transiently by lipofection or permanently by expression of hairpin-loop siRNA.

Correspondence: Ludovic Vallier, Ph.D., Box 202, Level E9, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, United Kingdom. Telephone: 44-1223-762025; Fax: 44-1223-410772; e-mail: lv225@cam.ac.uk *Received June 11, 2003; accepted for publication August 12, 2003.*

Materials and Methods

Human ES Cell Culture and Transfection

H9 hES cells (WiCell; Madison, WI; http://www.wicell.org) were cultured as described [1] in knockout Dulbecco's-modified Eagle's medium (KO-DMEM) supplemented with serum replacement (Invitrogen; Carlsbad, CA; http://www.invitrogen.com). Every 4 days, cells were harvested using 1 mg/ml collagenase IV (GIBCO; Carlsbad, CA; http://www.lifetech.com) and then plated into 60-mm plates. Culture dishes (Costar) were precoated with 0.1% porcine gelatin (Sigma; St. Louis, MO; http://www.sigmaaldrich.com) and 1×10^5 irradiated mouse embryonic fibroblasts. For stable transfection, three confluent 60-mm plates containing approximately 2,000 hES colonies each were plated onto one six-well gelatin-coated plate containing 5×10^4 feeders. After 48 hours, the cells were transfected using Lipofectamine 2000 (Invitrogen) or Exgen 500 (Fermentas; Hanover, MD; http://www.fermentas.com). Transfection using Exgen 500 was performed as described [6]. For Lipofectamine 2000, 10 μl transfection reagent was mixed with 250 μl KO-DMEM and incubated for 5 minutes. Four micrograms DNA were mixed with 250 μl KO-DMEM and combined with the Lipofectamine 2000 mix. After 20 minutes, the DNA-Lipofectamine 2000 was added to each well containing 2 ml of normal medium with 10% fetal bovine serum (HyClone; SH30070.03; Logan, UT; http://www.hyclone.com) instead of serum replacement.

The cells were incubated for 24 hours and then rinsed twice with phosphate-buffered saline (PBS). For transitory transfection, the cells were harvested 48 hours after transfection. For stable expression, the cells were passed 3 days after transfection onto 60-mm gelatin-coated tissue culture plates containing puromycin-resistant mouse fetal fibroblasts as feeders. After 3 days, puromycin (1 μg/ml final concentration) was added. Puromycin-resistant colonies that appeared by 12 days of selection were picked, dissociated, and plated onto 24-well gelatin-coated feeder-containing plates and expanded as described above for further analysis. The same procedure was used for stable cotransfection of the pSuper-HPRT vector with the puro-TK selection gene [7]. The DNA ratio was 3.5 μg of siRNA expression vector for 0.5 μg of selection gene. For pSuper-HPRT single transfection, hES cells were transfected on six-well matrigel-coated plates (Becton Dickinson; Franklin Lakes, NJ; http://www.bd.com) and then grown in feeder-free conditions as described [8] in order to avoid the use of 6-thioguanine (6-TG)-resistant feeder cells. After 4 days, 6-TG (3 μg/ml final concentration) was added to conditioned media. Selection was stopped 10 days later, and 6-TG-resistant colonies were picked after 2 additional days. Colonies were then dissociated, plated onto 24-well gelatin-coated feeder-containing plates, and expanded for further analyses as described above.

siRNA Design, Synthesis, and Transfection

siRNAs corresponding to hr-green fluorescent protein (hrGFP) or DsRed2 fluorescent reporter genes were designed as recommended [5] and synthesized using the Silencer siRNA Construction Kit (Ambion; Austin, TX; http://www.ambion.com). The following sequences were used: si-hrGFP6 sense 5′-CAACCACGUGUUCACCAUGUU-3′ and antisense 5′-CAUGGUGAACACGUGGUUGUU-3′; si-hrGFP14 sense 5′-CCUGAUCGAGGAGAUGUUCUU-3′ and antisense 5′-GAACAUCUCCUCGAUCAGGUU-3′; si-hrGFP22 sense 5′-GUUCUACAGCUGCCACAUGUU-3′ and antisense 5′-CAUGUGGCAGCUGUAGAACUU-3′; and si-DsRed2 sense 5′-CACCGUGAAGCUGAAGGUGUU-3′ and antisense 5′-CACCUUCAGCUUCACGGUGUU-3′. Transfection of siRNA was performed with Oligofectamine (Invitrogen) in 12-well plates, following manufacturer instructions. After 24 hours, the cells were retransfected as before. The fluorescence-activated cell sorting (FACS), real-time reverse transcription-polymerase chain reaction (RT-PCR), and the fluorescence microscopy analysis were done 48 hours after the second transfection.

For stable transfection, siRNA primers corresponding to hypoxanthine guanine phosphoribosyl transferase (HPRT) were designed using OligoEngine workstation software (Seattle, WA; http://www.oligoengine.com). The primers were subcloned downstream from the H1 promoter in the pSuper vector [9]. The resulting vector, pSuper-HPRT, was cotransfected into hES cells with the puro-TK vector for selection using puromycin [7], or alternatively with the hrGFP-pTP6 vector for selection using 6-TG. Preliminary observations revealed that 6-TG-treated hES cells tended to undergo differentiation, so only the hardiest colonies persisted under these conditions.

Real-Time and Semiquantitative RT-PCR

For real-time RT-PCR, total RNA was extracted from fluorescent green hES cells transfected with siRNA Anti-green 22 using the RNeasy Mini Kit (Qiagen; Valencia, CA; http://www.qiagen.com). Each sample was treated with RNase-Free DNase (Qiagen) in order to avoid DNA contamination. One-half microgram of total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). Real-time Taqman PCR was performed using an ABI 7700, with 1 × Mastermix (Eurogentec; Liège, Belgium; http://www.eurogentec.be), 500 nM of each primer, 200 nM Taqman probe, and 100 ng cDNA. Cycle conditions were as recommended by Eurogentec. Sequences were: porphobilinogen deaminase (PBGD)-FP: 5′-GGAGCCATGTCTGGTAACGG-3′; PBGD-BP: 5′-CCACGCGAATCACTCTCATCT-3′; PBGD Probe:

5′-TTTCTTCCGCCGTTGCAGCCG-3′; hrGFP-FP: 5′-CTTC GACATCCTGAGCCCC-3′; hrGFP-BP: 5′-GAAGTCGCTG ATGTCCTCGG-3′; and hrGFP Probe: 5′-TTCCAGTACGG CAACCGCACCTTC-3′.

For semiquantitative RT-PCR, total RNA was extracted from hES cells stably transfected with pSuper-HPRT vector and wild-type hES cells as described above. Total RNA was reverse transcribed using Superscript II Reverse Transcriptase. PCR reaction mixtures were prepared as described (Promega protocol for Taq polymerase) then were denatured at 94°C for 2 minutes and cycled at 94°C for 30 seconds, 60°C for 30 seconds (unless otherwise stated), and 72°C for 30 seconds. A final extension at 72°C for 10 minutes was performed after cycling. PCR primers were optimized for annealing temperature and a time course of cycle number was done, allowing semiquantitative comparisons within the log phase of amplification. Primers sequences were: HPRT-FP 5′-ATGCTGAGG ATTTGGAAAGG-3′; HPRT-RP 5′-TACTGGCGATGTCA ATAGG-3′; FGF-4-FP 5′-ACCTTGGTGCACTTTCTTCG-3′; FGF-4-RP 5′-CTCCACTGTTGCACCAGAAA-3′ (55°C); Cyclin D1-FP 5′-ATGAACTACCTGGACCGCTTCC-3′; Cyclin D1-RP 5′-ACAAGAGGCAACGAAGGTCTGC-3′; β-2 microglobulin (β_2M)-FP 5′-ACTGAAAAAGATGAGTA TGCCTGCCGTGTGAACC-3′; β_2M-RP 5′-CCTGCTCAGA TACATCAAACATGGAGACAGCACT-3′ (55°C).

Mouse ES Cell Culture and Transfection

The mouse ES cell lines R1 and CGR8 were cultured as described [10]. For transitory transfection, 1×10^7 cells were trypsinized and washed once in medium containing fetal calf serum (FCS; PAA Laboratories; Pasching, Austria; http://www.paa.at) and twice in DMEM without serum. The cells were then electroporated with a mix of 40 μg of circular DNA at 300 volts and 960 μF using the Gene Pulser II System (Bio-Rad Laboratories; Hercules, CA; http://www.bio-rad.com). The mix of DNA was made of 35 μg pTP6 vector plus 5 μg of a control vector containing a β-galactosidase reporter gene regulated by the CAGG promoter. The expression of the fluorescent reporter genes was analyzed 24 hours after electroporation. To assess experimental variations due to electroporation efficiency, a β-galactosidase essay was done on the same amount of protein for each sample. The fraction of cells was then normalized in function to the β-galactosidase activity. Stable transfection was performed as described [11]. Colonies selected for brightness using a Zeiss Axiovert 200 fluorescent microscope (Oberkochen, Germany; http://www.zeiss.com) were picked and expanded for further analysis.

Immunostaining

Human ES cells or their differentiated derivatives were fixed for 20 minutes in 4% paraformaldehyde and washed three times in PBS. Cells were incubated for 20 minutes at room temperature in PBS containing 10% goat serum (Serotec; Oxford, UK; http://www.serotec.co.uk) and subsequently incubated for 2 hours at room temperature with primary antibody diluted in 1% goat serum in PBS as follows: stage-specific embryonic antigen (SSEA)-1 (clone MC480, 1:50; Developmental Studies Hybridoma Bank [DSHB]; University of Iowa; http://www.uiowa.edu), SSEA-4 (clone MC813, 1:50; DSHB), Tra-1-60 (a gift from *Dr. P.W. Andrews*, 1:20), and β-tubulin (a gift from *Dr. S. Chandran*). Cells were then washed three times in PBS and incubated with fluorescein-isothiocyanate-conjugated anti-mouse IgG or IgM (Sigma; 1:200 in 1% goat serum in PBS) for 2 hours at room temperature. Unbound secondary antibodies were removed by three washes in PBS. Hoechst staining was added to the first wash (Sigma, 1:10,000).

Flow Cytometry

Mouse and human ES cells were dissociated with trypsin (0.25%) plus EDTA (1 mM; GIBCO), washed once in medium containing FCS, and washed twice in PBS containing 0.1% serum (hES). The cells were then immediately analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest acquisition and analysis software (Becton Dickinson).

Statistical Analysis

All results are presented as mean ± standard error. Comparisons between experimental and control data were made using Student's *t*-test (Excel, two-tailed). Responses were considered as different if $p < 0.05$.

Results and Discussion

Fluorescent reporter genes provide for a quantitative and qualitative estimation of specific gene expression directly in living cells, using simple assays such as fluorescence microscopy or FACS, which are capable of single-cell resolution [12]. However, the expression of fluorescent reporter proteins at a sufficient level to be readily detected can be toxic for some cell types [13].

We tested five different fluorescent proteins (destabilized d2GFP [Clontech; Franklin Lakes, NJ; http://www.cambrex.com], eGFP [Clontech], TauGFP [14], hrGFP [Stratagene; La Jolla, CA; http://www.stratagene.com], and DsRed2 [Clontech]) in mouse ES cells to identify those with maximal brightness and minimal toxicity. Each fluorescent reporter gene was subcloned downstream of the CAGG promoter into the pTp6 expression vector [14] (Fig. 1A) in order to obtain equivalent levels of expression (data not shown). The CAGG promoter confers strong and ubiquitous expression in mouse (m)ES cells and in mice derived

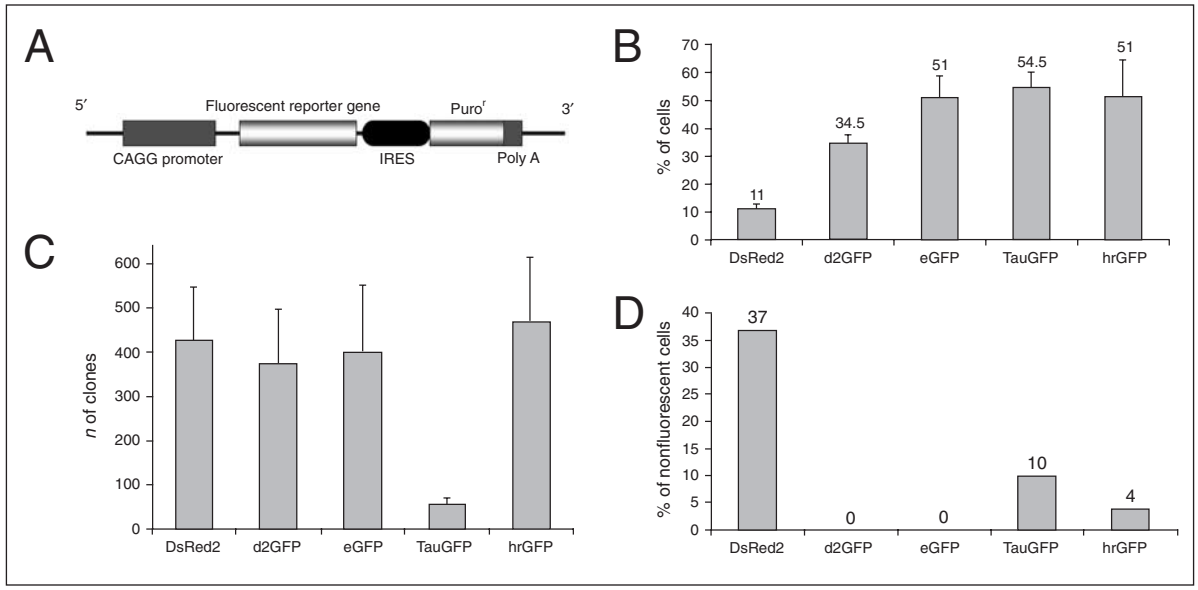

Figure 1. Toxicity and stability of different fluorescent reporter genes in mouse (m) ES cells. *A) Map of the pTP6 vector. pTP6 vector was developed by* Pratt et al. *[14]. It contains the CAGG promoter followed by the TauGFP gene and an IRES puromycin-resistance gene. All the GFP-pTP6 vectors were constructed replacing the TauGFP by the corresponding fluorescent reporter gene. Thus, the expression of each fluorescent gene was under the control of the CAGG promoter. B) Fraction of fluorescent cells after transitory electroporation. The R1 and CGR8 mES cell lines were transitorily electroporated with pTP6 vectors expressing DsRed2, d2GFP, eGFP, TauGFP, or hrGFP.*

*The electroporation conditions used allowed transfection of a large fraction of cells with a high quantity of DNA, resulting in strong expression. Consequently, if the fluorescent protein were toxic, the electroporated cells would be expected to die. A control vector was co-electroporated with each pTP6 vector to assess variations due to electroporation efficiency. hrGFP gave better results than DsRed2 and d2GFP (*p = *0.02, 10 degrees of freedom [d.f.]). However, the fraction of fluorescent cells obtained with TauGFP, eGFP, and hrGFP was not significantly different from each other (*p = *0.6, 10 d.f.). C) Stable expression. The R1 and CGR8 mES cell lines were stably electroporated with pTp6 vectors encoding each fluorescent protein. After 10 days of puromycin selection, the number of clones obtained with each reporter gene was counted. Because use of an IRES vector construction linked the level of fluorescence reporter gene expression to that of puromycin resistance gene expression, all the drug-resistant clones were fluorescent. Consequently, if the fluorescent protein were toxic, the clone would be expected to die during selection, so that fluorescent reporter genes with high clone yields can be regarded as low in toxicity and, conversely, those with low clone yields as toxic or detrimental. TauGFP gave the lowest number of clones (*p = *0.03, 4 d.f.). The number of clones generated with the other fluorescent genes was not significantly different from each (*p = *0.4, 4 d.f.). To assay the brightness of each fluorescent protein, we selected clearly positive clones using fluorescence microscopy and then evaluated their brightness using FACS (data not shown). D) Sensitivity to silencing. The brightest fluorescent clones for each reporter gene were selected by fluorescence microscopy and then grown for 7 weeks without puromycin selection. The fraction of fluorescent cells was analyzed by FACS to determine whether there was an increase in the number of the negative cells over time (defined as silencing). DsRed2 underwent silencing (>30% of cells), whereas other fluorescent genes did not to a substantial degree (<10%).*

from them [12, 14, 15]. Moreover, by linking the fluorescent reporter gene to the puromycin resistance gene through an internal ribosome entry site (IRES) [16], strong selective pressure could be applied on transgene expression during isolation and subsequent passaging of transfected colonies. Of the fluorescent reporter genes tested, hrGFP was the brightest and one of the least toxic for mES cells (Fig. 1B, 1C). Furthermore, silencing of this gene was observed only at low levels in mES cells (Fig. 1D), and the green protein was resistant to paraformaldehyde fixation, allowing its use for immunochemistry (data not shown). As a complementary fluorescent reporter gene, DsRed2 was chosen for its brightness and protein stability, although it showed a tendency towards silencing when mES cells were passaged repeatedly without drug selection (Fig. 1D). While recent results suggest lentiviral vectors have a high transfection efficiency when used in hES cells [17], they have the disadvantage of being complex to generate and accommodate a limited size of transgene. However, there is no clear consensus on relative efficiencies of the more conventional approaches to transfection of hES cells [6, 18]. We thus examined the efficiency of several transfection reagents in preliminary experiments (data not shown). On this basis, we then compared Lipofectamine 2000 and Exgen 500 [6] for stable transgene expression in hES cells using hrGFP and DsRed2 (Fig 2A, 2C). Colonies generated using the hrGFP vector with Lipofectamine transfection (n = 40), as well as those generated using Exgen 500 (n = 10) retained green fluorescence after five passages without puromycin selection. However, only 6 of 48 DsRed2-expressing clones

retained red fluorescence after five passages without selection. This reduction in fluorescence was lessened and sometimes reversed by resuming puromycin selection. Evidently, DsRed2 is sensitive to epigenetic silencing in hES cells, confirming the results obtained with mouse ES cells (Fig. 1D), thus requiring their continuous maintenance under drug selection. After 12 passages (2 months of culture), FACS analysis revealed that 99% of cells were fluorescent in several green- and red-fluorescing hES cell lines selected for optimal expression by fluorescent microscopy (Fig. 2B).

After hES cell differentiation in vitro, fluorescent protein expression was strongly detected in beating structures presumably containing cardiomyocyte (mesoderm)-like cells. Green or red fluorescent neuron (ectoderm)-like cells were also observed, and these cells expressed the specific neuronal marker β tubulin (Fig. 2D, 2E). Also, RT-PCR was used to detect the expression of

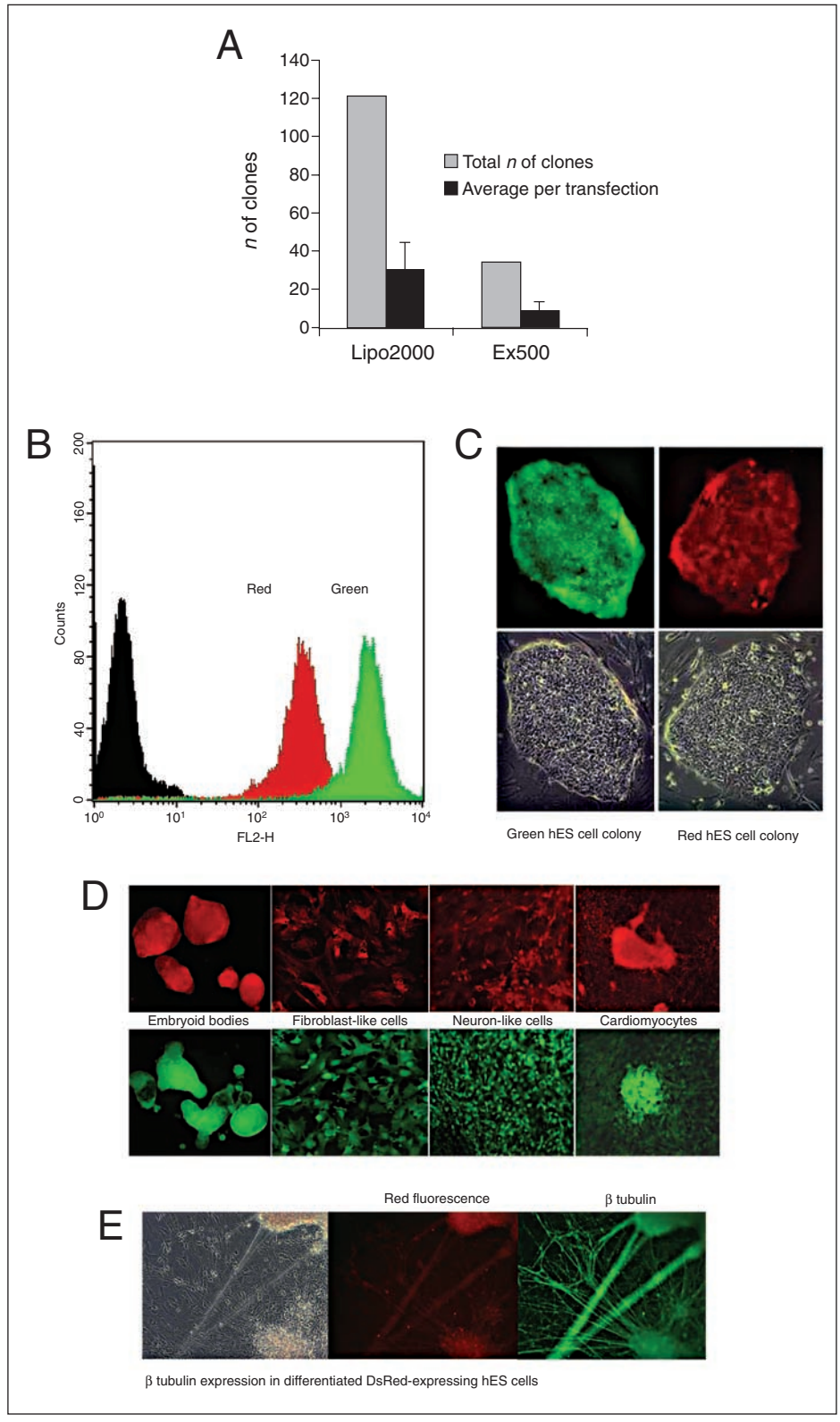

Figure 2. Generation of hES cell lines stably expressing red and green fluorescent reporter genes. *A) Number of transgenic colonies generated using Lipofectamine 2000 and Exgen 500. H9 hES cells were transfected with DsRed2-pTp6 or hrGFP-pTp6 as described in experimental protocols. The same number and condition of hES cells were used in each case. No significant differences were observed when transfecting linearized or circular DNA. The highest number of stably expressing cell lines was generated using Lipofectamine 2000* ($p = 0.05$, 6 *d.f.*). *B) FACS analysis of red and green hES cell lines that were selected by fluorescent microscopy as clearly expressing fluorescent protein. C) hES cell colonies expressing red and green fluorescent proteins (upper left and upper right, GFP fluorescence; corresponding phase contrast images are shown in lower left and right panel, respectively). D) Differentiated hES cells expressing red and green fluorescent proteins. Embryoid bodies were generated by growing hES cells in nonadherent conditions for 5 days, after which they were plated for 2 weeks on 12-well gelatin-coated plates. E) β tubulin expression in differentiated red fluorescent hES cells. Embryoid bodies were generated by growing hES cells in nonadherent conditions for 5 days, after which they were plated for 2 weeks on 12-well gelatin coated plates. Neurosphere-like structures appeared spontaneously 1 week after plating. These structures expressed the β tubulin protein (green fluorescence), which is a specific marker for neurons.*

α fetoprotein, albumin, and somatostatin, which are specific markers of endoderm [19] (data not shown). These results show that green and red fluorescent hES cells can be differentiated into all three germ layers. However, further studies will be needed to assess the functionality of differentiated cells derived from fluorescent hES cells. Finally, the pluripotent stem cell markers, Oct-4 and FGF-4, as well as the embryonic stem cell surface markers, SSEA-4, Tra-160, and Tra-181, were all expressed in fluorescent reporter transgene-expressing hES cells, whereas the differentiation marker SSEA-1 remained absent (data not shown). Furthermore, the karyotype and morphology of these cells were normal (data not shown), so despite the transfection and the selection process, the red- and green-fluorescing hES cells retained molecular markers of their undifferentiated state as well as the ability to differentiate into derivatives of each of the three germ layers. These findings provide additional compelling evidence that such genetic alteration of hES cells is compatible with maintenance of their pluripotency [6, 17, 18]. This expands the use of fluorescent reporter-expressing hES cell lines as powerful tools for cell tracing during tissue differentiation in in vitro and in animal models.

The principal approach that has been used to study gene function in mammals is gene targeting by homologous recombination in mES cells [20]. Such an approach recently has been used with hES cells to generate a mutation in a single allele of the *HPRT* and *Oct-4* genes [21]. However, generating a homozygous null mutation would require targeting of the second allele in the case of autosomal genes, or might be accomplished less reproducibly by gene conversion [22]. To circumvent this obstacle to perturbing gene function in hES cells, we evaluated the use of short-interfering double-stranded RNA, which has been shown to be efficient in mouse ES cells [23-26].

We first sought to define the efficacy of siRNA in hES cells by targeting the *hrGFP* and *DsRed2* genes in the fluorescent cell lines generated above using transitory transfection of siRNAs. Fluorescent reporter genes provide distinct advantages as siRNA targets, in that they are capable of single cell assessment, can sustain multiple observations in living cells without perturbing viability and development, and they encode readily detectable proteins, whose absence does not alter cellular phenotype or provide a proliferative disadvantage. We designed three siRNA oligonucleotides targeted against the mRNA of each gene and transfected them into stably expressing green or red hES cell lines using Oligofectamine. After 48 hours, we observed the disappearance of fluorescence in a fraction (5%-10%) of the cells. To increase this effect, cells were retransfected 24 hours after the initial transfection. The siRNA effect was analyzed 48 hours later by counting the number of colonies containing negative cells using fluorescence microscopy (data not shown), by counting the negative cells using FACS (Fig. 3A), or by direct measurement of fluorescent gene expression using real time RT-PCR (Fig. 3C). Two of three anti-green siRNAs (14 and 22) reduced the expression of the green protein in 10% and 20% of the cells, respectively (Fig. 3A) (*t*-test, $p = 0.007$), and negative cells were detected in all the colonies treated with them (Fig. 3B).

We observed similar effects with one of three siRNA against DsRed2 mRNA in red hES cells, where a majority of colonies contained negative cells (Fig. 3B and data not shown). The effect of the anti-red siRNA was more difficult to assay by FACS because of the stability of the red protein (data not shown). At the RNA level, anti-green siRNA 22 induced a 40% decrease in the green fluorescent gene transcript (Fig. 3C), suggesting that siRNA had a greater impact at the RNA level than at the protein level (Fig. 3A and 3C). Thus, transient transfection of siRNA led to a decrease of gene products in a substantial fraction of cells; however, the knock down apparently did not reach a null level.

The siRNA-induced knock down of gene function was specific to the targeted gene. The transfection of anti-red siRNA into green hES cells or anti-green siRNA into red hES cells had no effect, respectively, on hrGFP or DsRed2 expression as compared with the controls. Nonspecific effects were similarly absent when we examined the expression of endogenous genes using real-time PCR (Fig 3D). Likewise, siRNA transfection into hES cells did not alter expression of pluripotent markers such as Oct-4 (data not shown), SSEA-4, or Tra-1-60 (Fig. 3E). Therefore, siRNA does not appear to activate the interferon response or the PKR kinase pathway, which would lead to a generalized suppression of transcription, as with long double-stranded RNAs, nor does it affect the expression of markers indicative of the pluripotent state. Therefore, these findings provided proof of principle that siRNA knocks down gene expression in hES cells, although the fluorescent reporter genes were challenging targets for detecting such effects. First, the sensitivity of detecting the fluorescent proteins made it difficult to assess the degree of knock down at the protein level. This, together with the short-term, heterogeneous nature of transitory transfection, meant that we could not isolate a homogenous population of affected cells on which to accurately assess the extent of knock down at the mRNA level. Thus, the transient transfection data did not allow us to distinguish between an intermediate knock down efficiency at the mRNA level in most of the cells versus a high-efficiency knock down in a fraction of the cells.

Accordingly, we sought to establish hES cell sublines stably expressing hairpin-loop siRNA as a means of obtaining homogenous populations of cells. The characteristics of a desirable protein target for such an analysis would include

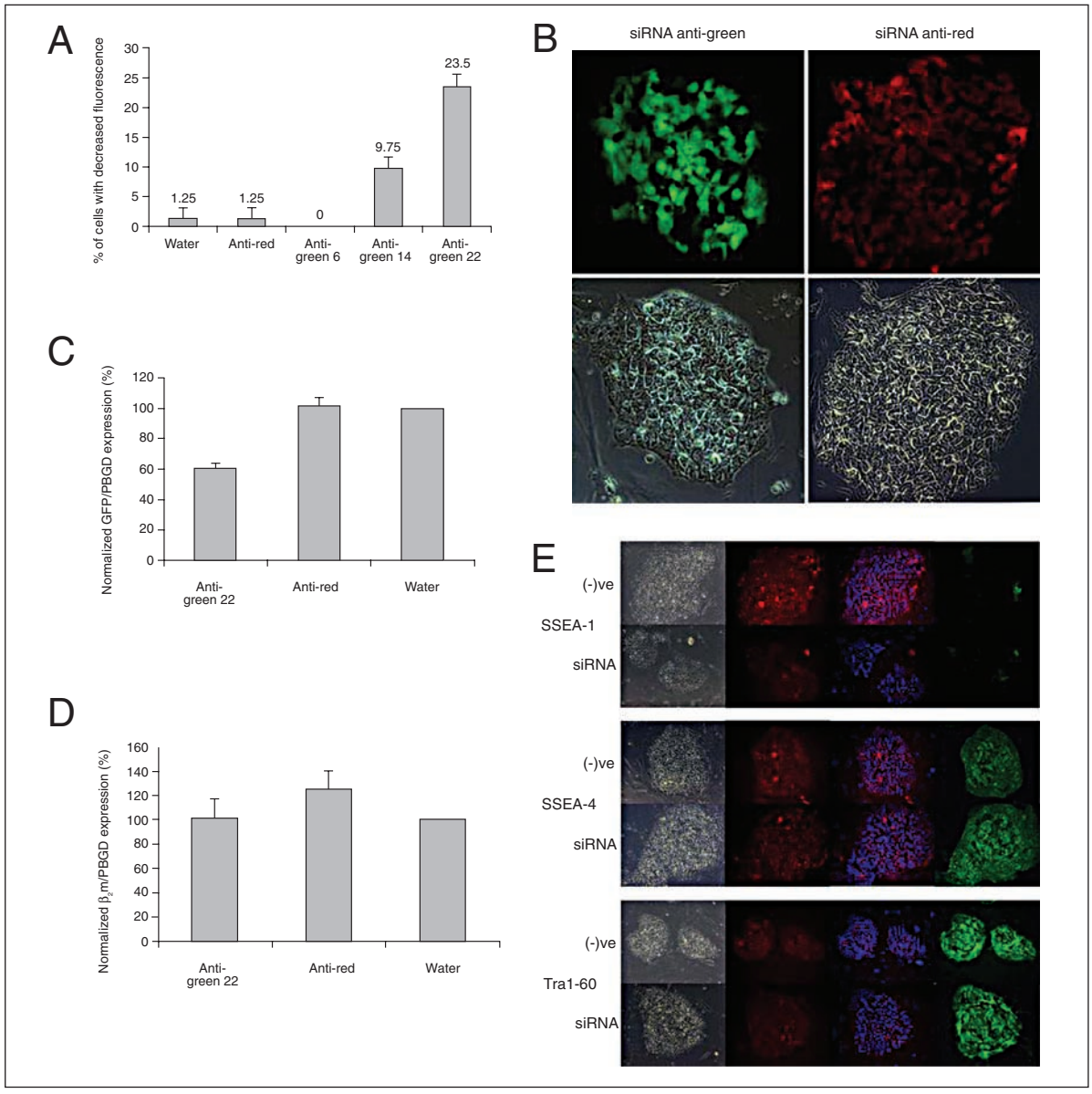

Figure 3. Effect of siRNAs on red and green fluorescent protein expression in hES cells. *A) FACS analysis of the siRNA effect on hrGFP expression. Three different siRNAs (anti-green 6, anti-green 14, and anti-green 22) designed against hrGFP mRNA were transfected twice into hES cells expressing the hrGFP fluorescent reporter gene. Two days after the second transfection, fluorescent cells were counted using FACS. No siRNA effect was detected 24 hours before or 24 hours after this time. The transfection procedure itself did not affect hrGFP expression, since no decrease in fluorescence was observed when only water was transfected (*p = *0.7, 2 d.f.). The transfection of anti-red siRNA did not diminish hrGFP expression, whereas it did diminish DsRed2 expression (*p = *0.007, 2 d.f.). B) Diminished gene expression induced by siRNA in fluorescent hES cells. Red and green fluorescent hES colonies containing negative cells induced by transfecting their respective siRNAs. C) Real-time RT-PCR analysis of the siRNA anti-green 22 effect on hrGFP expression of green fluorescent hES cells. Three days after the first transfection, normalized GFP/PBGD mRNA levels were measured using RT-PCR. Results are shown in comparison to untransfected control cells (100%). A significant decrease (40%) of the hrGFP RNA was observed with anti-green siRNA (*p = *0.006, 4 d.f.) but not in response to siRNA directed against DsRed2 or to water (*p = *0.1, 4 d.f.). D) Real-time RT-PCR analysis of the siRNA effect on endogenous gene expression. Green fluorescent hES cells were transfected as described in 4C. Two days after the second transfection, normalized β_2M/PBGD mRNA levels were measured by real-time PCR. Results are shown in comparison to untransfected control cells (100%). siRNA transfection did not affect the expression of the β_2M gene (*p = *0.8, 4 d.f.). E) Expression of pluripotent markers in siRNA transfected hES cells. Two days after the second transfection, hES cells were immunostained for the SSEA-1, SSEA-4, and TRA-1-60 surface antigens. The SSEA-1 antigen is a marker of differentiated cells, while SSEA-4 and TRA-1-60 are specific markers of undifferentiated hES cells. For each antigen, the first row [(-) ve] shows untransfected hES cell colonies and the siRNA row shows red fluorescent hES cell colonies transfected with anti-red siRNA. The green fluorescence corresponds to the surface-antigen staining. No differences were observed between untransfected and transfected cells, suggesting that siRNA transfection did not change the pluripotent state of hES cells. Moreover, the normal expression of these markers shows that the siRNA effect is specific for the targeted gene and did not interfere with gene expression in general.*

Table 1. Summary of siRNA-expressing hES cell sublines generated by stable transfection of the pSuper-HPRT vector

	Transfection	Clones	Screened	$HAT^r/6TG^r$	$HAT^s/6TG^r$
Co-Transfection					
pSUPER-HPRT/puroDTK	3	10	10	4	0
pSUPER-HPRT/hrGFP-pTP6	1	2	2	1	0
pSUPER/hrGFP-pTP6	1	2	2	0	0
Transfection					
pSUPER-HPRT	2	9	5	5	0

The first column shows the type of vector used. For cotransfection, the pSuper-HPRT vector was transfected either with the puro-TK selection gene or with the hrGFP-pTP6 vector. In both cases, cell lines resistant to 6-TG were generated (column $HAT^r/6TG^r$). The original pSuper vector was also cotransfected with the hrGFP-pTP6 vector as control. As expected, the two hrGFP-expressing cell lines generated were not resistant to 6-TG selection. For single transfection, the pSuper-HPRT vector was transfected alone and then stable transfectants were selected by adding 6-TG. None of the cell lines generated were both sensitive to HAT selection and resistant to 6-TG selection (column $HAT^s/6TG^r$).

being selectable and being nonessential for viability, pluripotency, or differentiation. On this basis, we targeted the endogenous HPRT gene, which encodes an enzyme involved in purine metabolism [27]. Because 6-TG is metabolized to a toxic compound by HPRT, it can be used to select for and identify drug-resistant cells that are deficient in HPRT. Using this approach, we generated five hES sublines that were 6-TG resistant among 10 sublines generated by cotransfection and selection for puromycin resistance, and another five sublines that were obtained by direct 6-TG selection (Table 1; Fig. 4A). Interestingly, none of the 10 hES sublines were sensitive to medium containing hypoxanthine, aminopterin, and thymidine (HAT), which blocks DNA synthesis in cells that are HPRT deficient, suggesting that while HPRT levels are diminished sufficiently to provide 6-TG resistance, they are not reduced to a null level. Consistent with this hypothesis, the levels of HPRT mRNA (analyzed by semiquantitative RT-PCR) were diminished to the borderline of detectability in siRNA-expressing hES cells (Fig. 4B).

Under the same assay conditions, HPRT expression was readily detectable in wild-type hES cells and in an hES cell subline that was puromycin resistant but 6-TG sensitive (HPRT2). We also assayed the effect of siRNA expression after hES-derived cells had differentiated into embryoid bodies for 6 days, were plated in normal medium for 5 more days, and, finally, were subjected to 6-TG selection for an additional 12 days. At the end of this selection regimen, few wild-type cells survived the 6-TG selection, whereas a majority of siRNA-expressing cells were still alive (Fig. 5A). Therefore, stable expression of siRNA is also effective in differentiated cells derived from hES cells. Our analysis of HPRT gene expression in differentiated cells using semiquantitative RT-PCR revealed almost undetectable mRNA levels, whereas wild-type hES cells expressed HPRT at high levels (Fig. 5B).

Housekeeping genes such as β_2M and *Cyclin D1* were expressed at wild-type levels in both control cells and in

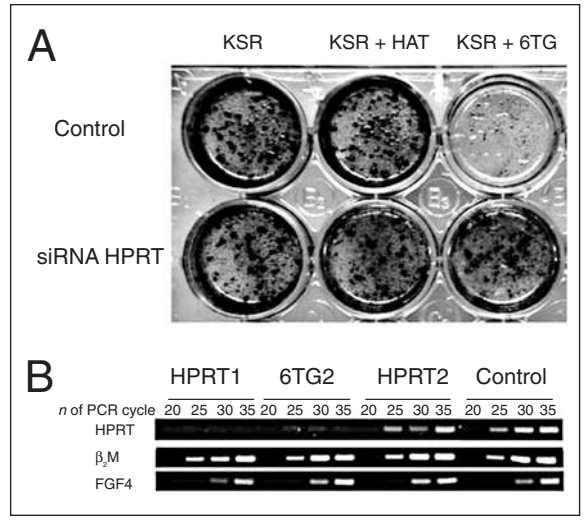

Figure 4. Knock down of HPRT gene in undifferentiated hES cells stably transfected with pSuper-HPRT hairpin loop vector. *A) 6-TG resistance of hES cells stably transfected with pSuper-HPRT vector. Nontransfected hES cells (control) and stably transfected hES cells (siRNA HPRT) were grown during 7 days in normal medium (knock out serum replacement [KSR]) or media containing either HAT (1×) or 6-TG (2 µg/ml). The surviving colonies were stained using crystal violet. No hES cell colony was detected in controls after 7 days of 6-TG selection (upper right well), while a majority of siRNA-expressing hES cell colonies survived (bottom right well). Both kinds of cells survived HAT selection, showing that HPRT is not knocked down to a null level in transfected hES cells. B) Analysis of HPRT expression in hES cells stably transfected with pSuper-HPRT vector. HPRT expression was analyzed using semiquantitative RT-PCR based on a time course of PCR cycle allowing comparison within the log phase. HPRT expression was almost undetectable in transfected hES cells resistant to 6-TG (HPRT1 and 6TG2), while it was readily detectable in nontransfected cells (control) or in transfected hES cells that were sensitive to 6-TG (HPRT2). FGF-4, a marker of pluripotency, and β_2M were both expressed at the same level in transfected hES cells (HPRT1, HPRT2, 6TG2) and in nontransfected cells (control), showing specificity of the siRNA effect.*

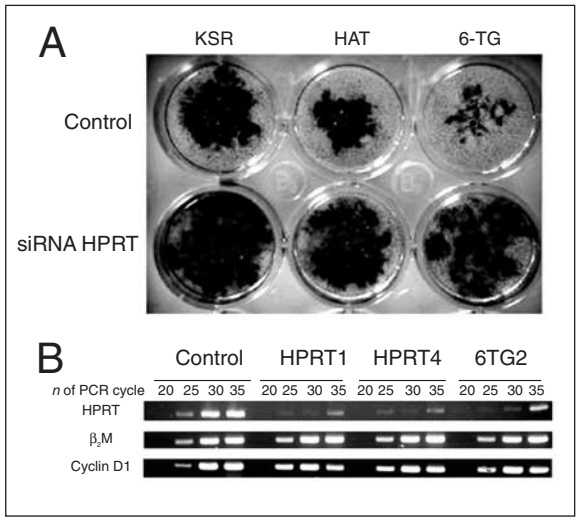

Figure 5. HPRT knock down in differentiated derivatives of hES cells stably transfected with the pSuper-HPRT vector. *A) 6-TG resistance of differentiated derivatives of transfected hES cells. Embryoid bodies (EBs) were generated from stably transfected hES cells by growing them in nonadherent conditions for 5 days. Then EBs were plated on gelatin-coated plates containing normal medium for 5 more days and, finally, were subjected to 6-TG (2 μg/ml) or HAT (1x) selection for an additional 12 days. The same number of EBs was plated for each well. After 2 weeks, surviving cells were stained using crystal violet. Nontransfected cells (control) did not survive in 6-TG-containing medium (top right well), while a majority of siRNA-expressing cells (siRNA HPRT) survived in 6-TG medium (bottom right well). Both kinds of cells survived in HAT medium (middle wells). (B) Analysis of HPRT expression in differentiated derivatives of hES cells stably transfected with the pSuper-HPRT vector. HPRT expression was analyzed using semiquantitative RT-PCR based on a time course of PCR cycle allowing comparison within the log phase. HPRT expression was barely detectable in differentiated derivatives of transfected hES cells resistant to 6-TG (HPRT1, HPRT4, and 6TG2), while it was readily detectable in nontransfected cells (Control). Cyclin D1 and β_2M were expressed at the same level in differentiated derivatives of transfected hES cells (HPRT1 and 6TG2) and in nontransfected cells (control) showing specificity of the siRNA effect.*

siRNA-expressing cells, showing the specificity of the siRNA effect on the HPRT target gene. Taken together, these results show that stable expression of siRNA knocks down both transgenic and endogenous genes expressed in hES cells and in their differentiated derivatives. While the knock down does not reach a null level, the resulting hypomorphic phenotypes can nevertheless provide both developmental and clinical models for understanding human functional genomics. For example, mouse mutants with various expression levels of vascular endothelial growth factor family genes have provided insights that could not be gained from the null mutation, which is lethal just after gastrulation stages [28, 29]. Similarly, a hypomorphic condition for HPRT may provide models not only for Lesch-Nyhan syndrome, which is associated with null or very low HPRT levels, but also for Kelley-Seegmiller syndrome and gout, which are associated with intermediate levels [30, 31]. In these cases, there are no animal models because HPRT-deficient mice are healthy. Thus, siRNA approaches involving hES cells and their differentiated derivatives can provide a key resource for human functional genomics, complementing gene targeting by homologous recombination that results in complete ablation of the targeted gene [21].

The potential for clinical application of hES cells is encouraging; however, this potential is still undergoing biological and technical evaluation. Beyond the obviously important task of controlling stem cell differentiation, realizing the clinical goal of cell-based transplantation therapies will require a means of matching donor tissues to the host immune system [32] and will also necessitate the derivation of new hES cell lines without using animal cells as feeders [33]. Each of these major tasks will benefit from robust approaches for regulating the expression of specific exogenous and endogenous genes. For example, use of siRNA could reveal the function of genes involved in the maintenance of pluripotency and thus lead to improvements in the efficiency of hES cell derivation, which would be necessary for generation of an hES cell bank. The results described here provide evidence that hES cells can undergo alteration in the expression of specific genes without effects on their pluripotency, a fundamental developmental characteristic of hES cells. Finally, because hES cells are capable of undergoing some of the differentiative events characteristic of early human development, these approaches for monitoring and modifying hES cell gene expression provide a useful model for human functional genomics. This in turn can reveal the molecular pathways of differentiation into the diversity of cells and tissues that ES cells are capable of forming.

ACKNOWLEDGMENTS

We thank *Dr. A. Nagy* for the R1 cells, *Dr. A.G. Smith* for the CGR8 cells, *Dr. T. Pratt* for the pTp6 vector, *Dr. Allan Bradley* for the puro-TK selection gene, *Dr. E. Delebesse* for PBGD primers and probe, *Dr. P.W. Andrews* for the TRA-1-60 antibody, *Dr. S. Chandran* for the β tubulin immunostaining, Dr. *J.A. Thomson* and Wicell for H9 cells, *Dr. E. Millan* for motivating discussions, and *Dr. M. Firpo* for providing access to ES cells and protocols. The SSEA-1 and SSEA-4 antibodies were obtained from the Developmental Studies Hybridoma Bank. This work was supported by the Medical Research Council.

REFERENCES

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-1147.

2 Reubinoff BE, Pera MF, Fong CY et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000;18:399-404.

3 Rideout WM 3rd, Hochedlinger K, Kyba M et al. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell 2002;109:17-27.

4 Kim JH, Auerbach JM, Rodriguez-Gomez JA et al. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 2002;418:50-56.

5 Elbashir SM, Harborth J, Lendeckel W et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494-498.

6 Eiges R, Schuldiner M, Drukker M et al. Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. Curr Biol 2001;11:514-518.

7 Chen YT, Bradley A. A new positive/negative selectable marker, puDeltatk, for use in embryonic stem cells. Genesis 2000;28:31-35.

8 Xu C, Inokuma MS, Denham J et al. Feederfree growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971-974.

9 Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002;296:550-553.

10 Robertson EJ. Derivation and maintenance of embryonic stem cell cultures. Methods Mol Biol 1997;75:173-184.

11 Chassande O, Fraichard A, Gauthier K et al. Identification of transcripts initiated from an internal promoter in the c-erbA alpha locus that encode inhibitors of retinoic acid receptor-alpha and triiodothyronine receptor activities. Mol Endocrinol 1997;11:1278-1290.

12 Hadjantonakis AK, Gertsenstein M, Ikawa M et al. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech Dev 1998;76:79-90.

13 Liu HS, Jan MS, Chou CK et al. Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 1999;260:712-717.

14 Pratt T, Sharp L, Nichols J et al. Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. Dev Biol 2000;228:19-28.

15 Chambers I, Colby D, Robertson M et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003;113:643-655.

16 Mountford P, Zevnik B, Duwel A et al. Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proc Natl Acad Sci USA 1994;91:4303-4307.

17 Ma Y, Ramezani A, Lewis R et al. High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. STEM CELLS 2003;21:111-117.

18 Lebkowski JS, Gold J, Xu C et al. Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications. Cancer J 2001;7(suppl 2):S83-S93.

19 Wells JM, Melton DA. Early mouse endoderm is patterned by soluble factors from adjacent germ layers. Development 2000;127:1563-1572.

20 Joyner AL. Gene Targeting: A Practical Approach. Oxford, UK: Oxford University Press, 2000.

21 Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. Nat Biotechnol 2003;21:319-321.

22 Lefebvre L, Dionne N, Karaskova J et al. Selection for transgene homozygosity in embryonic stem cells results in extensive loss of heterozygosity. Nat Genet 2001;27:257-258.

23 Rubinson DA, Dillon CP, Kwiatkowski AV et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 2003;33:401-406.

24 Carmell MA, Zhang L, Conklin DS et al. Germline transmission of RNAi in mice. Nat Struct Biol 2003;10:91-92.

25 Tiscornia G, Oded S, Ikawa M et al. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci USA 2003;100:1844-1848.

26 Kunath T, Gish G, Lickert H et al. Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. Nat Biotechnol 2003;21:559-561.

27 Albertini RJ. HPRT mutations in humans: biomarkers for mechanistic studies. Mutat Res 2001;489:1-16.

28 Pinter E, Haigh J, Nagy A et al. Hyperglycemia-induced vasculopathy in the murine conceptus is mediated via reductions of VEGF-A expression and VEGF receptor activation. Am J Pathol 2001;158:1199-1206.

29 Damert A, Miquerol L, Gertsenstein M et al. Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation. Development 2002;129:1881-1892.

30 McDonald JA, Kelley WN. Lesch-Nyhan syndrome: altered kinetic properties of mutant enzyme. Science 1971;171:689-691.

31 Kelley WN, Rosenbloom FM, Henderson JF et al. A specific enzyme defect in gout associated with overproduction of uric acid. Proc Natl Acad Sci USA 1967;57:1735-1739.

32 Bradley JA, Bolton EM, Pedersen RA. Stem cell medicine encounters the immune system. Nat Rev Immunol 2002;2:859-871.

33 Richards M, Fong CY, Chan WK et al. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002;20:933-936.