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

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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).

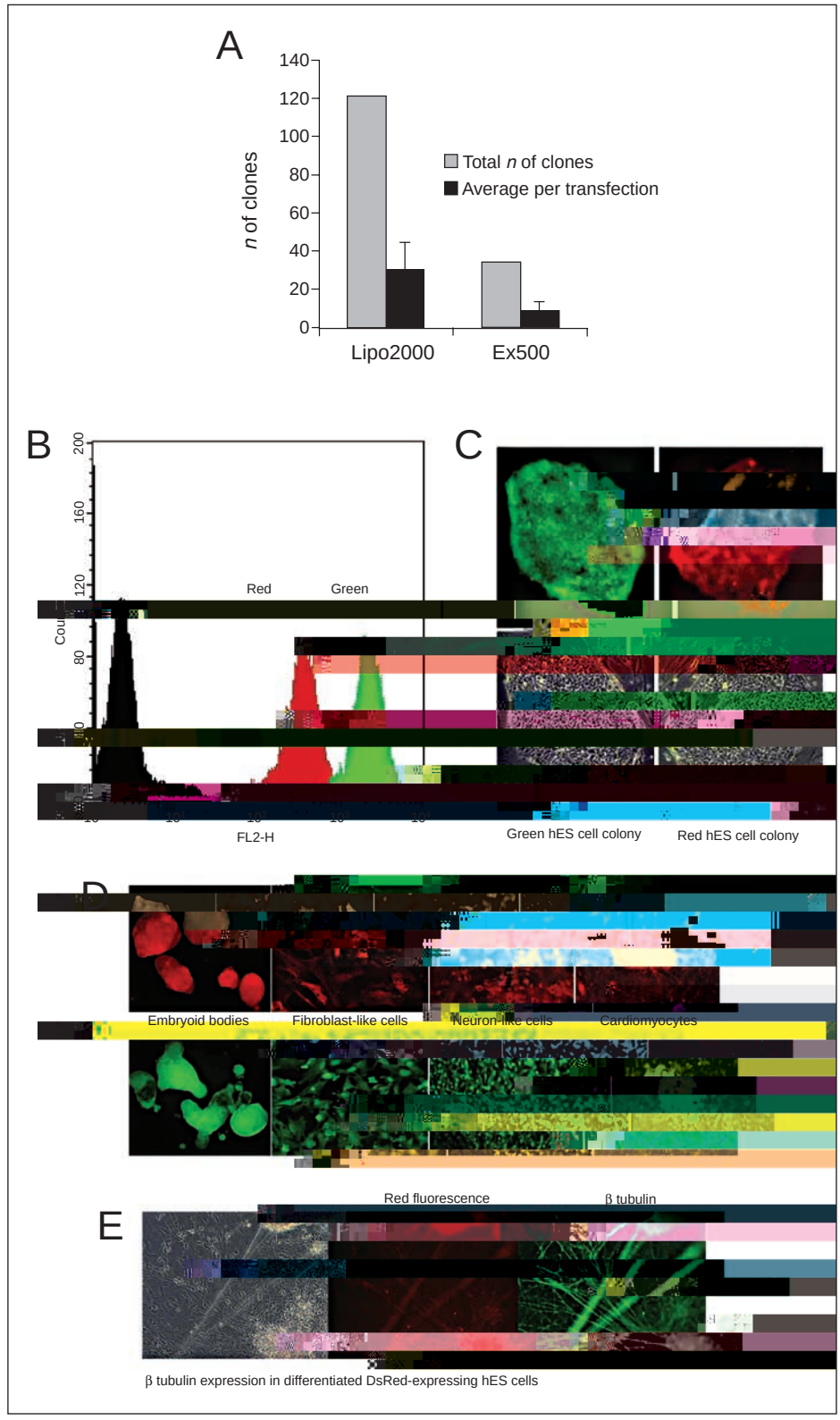
Statistical Analysis

All results are presented as mean ± standard error. Comparisons between experimental and control data were made using Student's *t*

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 isola-

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



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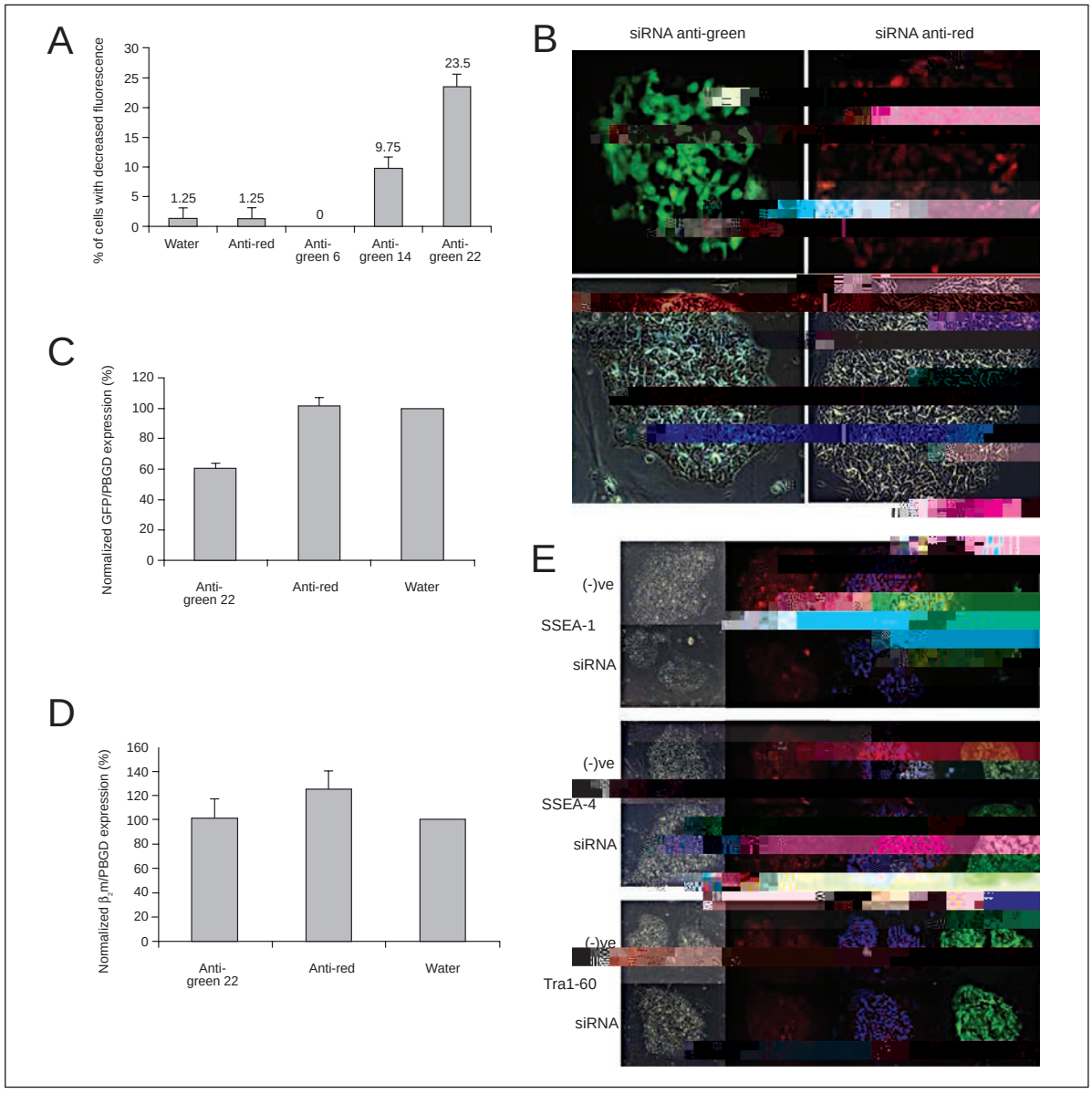
A) Number of transgenic colonies generated using Lipofectamine 2000 and Exgen 500. H9 hES cells were transfected with DsRed2-pTp6 or hrGFP-pTp6 as described in experimental protocols. The same number and condition of hES cells were used in each case. No significant differences were observed when transfecting 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



" #$% *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 com-*

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 u30W 6-TG slnll ce3lls (T[4:[]TN4tdslnll :.P]J.wTonormal lkh4normalls :.)(]Trmal.

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

REFERENCES

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic