Transcriptional profiling of reporter genes used for molecular imaging of embryonic stem cell transplantation

Joseph C. Wu,1,2,* Joshua M. Spin,1:* Feng Cao,2 Shuan Lin,2 Xiaoyan Xie,2 Olivier Gheysems,2 Ian Y. Chen,2 Ahmad Y. Sheikh,3 Robert C. Robbins,3 Anya Tsalenko,4 Sanjiv S. Gambhir,2 and Tom Quertermous1

1Department of Medicine, Division of Cardiology, 2Department of Radiology and Bio-X Program, and 3Department of Surgery, Stanford University School of Medicine, Stanford; and 4Agilent Technologies, Palo Alto, California

Submitted 14 October 2005; accepted in final form 26 December 2005

Wu, Joseph C., Joshua M. Spin, Feng Cao, Shuan Lin, Xiaoyan Xie, Olivier Gheysems, Ian Y. Chen, Ahmad Y. Sheikh, Robert C. Robbins, Anya Tsalenko, Sanjiv S. Gambhir, and Tom Quertermous. Transcriptional profiling of reporter genes used for molecular imaging of embryonic stem (ES) cell transplanted profiling of murine ES cells. Murine ES cells were stably transduced with a self-inactivating lentiviral vector carrying a triple-fusion (TF) construct consisting of fluorescence, bioluminescence, and positron emission tomography (PET) reporter genes. Fluorescence-activated cell sorting (FACS) analysis allowed isolation of stably transduced populations. Microarray studies comparing gene expression in nontransduced control ES cells vs. stably transduced ES cells expressing triple fusion (ES-TF) revealed some increases in transcriptional variability. Annotation analysis showed that ES-TF cells downregulated cell cycling, cell death, and protein and nucleic acid metabolism genes while upregulating homeostatic and anti-apoptosis genes. Despite these transcriptional changes, expression of the TF reporter gene had no significant effects on ES cell viability, proliferation, and differentiation capability. Importantly, transplantation studies in murine myocardium demonstrated the feasibility of tracking ES-TF cells in living subjects using bioluminescence and PET imaging. Taken together, this is the first study to analyze in detail the effects of reporter genes on molecular imaging of ES cells.

Heart diseases; embryonic stem cells; microarray

ISCHEMIC HEART DISEASE is the number one cause of morbidity and mortality in the United States. Several studies have shown that transplantation of bone marrow stem cells (22), endothelial progenitor cells (22), and embryonic stem (ES) cells (18) can improve myocardial function after an ischemic insult. The mechanisms may be related to stem cells secreting paracrine factors, differentiating into cardiomyocytes, or recruiting peripheral stem cells to the ischemic territory (24). However, current methods of studying stem cell survival rely on conventional histology, which precludes longitudinal monitoring. Thus the ongoing development of reporter gene imaging technologies that could help determine the nature of engrafted cells is expected to have significant impacts on understanding stem cell biology and physiology in vivo (50).

In recent years, microarray studies have been widely employed to evaluate tens of thousands of gene transcripts simultaneously while studying numerous aspects of cardiovascular disease (17). Although previous studies have examined the transcriptional profiles of mouse ES cells and early embryos, the effects of reporter genes on ES cell biology and physiology have not yet been described (34, 43). Here we report the isolation and characterization of stable ES cells expressing triple fusion (ES-TF) cells that express monomeric red fluorescent protein (mRFP) for fluorescence-activated cell sorting (FACS) and single-cell microscopy imaging, firefly luciferase (Fluc) for high-throughput bioluminescence imaging, and herpes simplex virus type 1-truncated thymidine kinase reporter gene (tk) for positron emission tomography (PET) imaging. We first examine the effects of these reporter genes on the global gene expression profile of ES cells and identify differentially regulated pathways. We then evaluate the impact of these reporter genes on ES cell viability, differentiation, and function. Finally, we assess the feasibility of tracking ES-TF cells serially in the myocardium of living mice.

METHODOLOGY

Culture of undifferentiated ES cells. The murine ES-D3 cell line (CRL-1934) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were originally isolated from day 4 blastocysts of 129/Sv mice (12). ES cells were kept in an undifferentiated, pluripotent state by using 1,000 IU/ml leukemia inhibitory factor (LIF; Chemicon, ESGRO, ESG1107) and were grown on top of leukemia inhibitory factor (LIF; Chemicon, ESGRO, ESG1107) and were grown on top of a murine embryonic fibroblast feeder layer inactivated by 10 μg/ml mitomycin C (Sigma, St. Louis, MO). ES cells were cultured on 0.1% gelatin-coated plastic dishes in ES medium containing DMEM supplemented with 15% fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM glutamine, and 0.1 mM nonessential amino acids, as previously described (46).

Construction of the pUb-fluc-mrfp-ttk triple-fusion reporter gene. PCR amplification and standard cloning techniques were used to insert fluc and mRFP genes from plasmids pCDNA 3.1-CMV-fluc (Promega, Madison, WI) and pCDNA3.1-CMV-mrfp in frame with the tk gene into the pCDNA3.1-truncated sr39tk as described (36). This triple-fusion (TF) reporter gene fragment (3.3 kb) was released from the plasmid with NotI and BamHI restriction enzymes before blunt-end ligation into the multiple cloning site of lentiviral transfer vector, FUG, driven by the human ubiquitin-C promoter. Henceforth, fluc-mRFP-ttk refers to reporter genes, while FL-mRFP-TK refers to the corresponding reporter proteins.

Lentiviral production and transfection of murine ES cells. Self-inactivating (SIN) lentivirus was prepared by transient transfection of...
293T cells as previously described (29). Briefly, pFUG-TF containing the TF reporter gene was cotransfected into 293T cells with HIV-1 packaging vector (88.9) and vesicular stomatitis virus G glycoprotein-pseudotyped envelop vector (pVSVG). Lentivirus supernatant was concentrated by sediment centrifugation using a SW29 rotor at 50,000 g for 2 h. Concentrated virus was titered on 293T cells. Murine ES cells were transfected with LV-pUb-fluc-mrfp-ttk at a multiplicity of infection (MOI) of 10. The infectivity was determined by FACSscan (Becton Dickinson) using a Texas Red filter setting (585 ± 25 nm).

**Microarray hybridization and data acquisition.** RNA hybridizations were performed using the Agilent Mouse (Development) Oligo Microarray G4120A platform, consisting of 20,371 60-mer oligonucleotides representing over 20,000 known mouse genes and derived largely from sequences from the National Institute on Aging cDNA 7.4K and 15K mouse clone sets (11). A common reference consisting of RNA derived from whole 17.5-day mouse embryos was utilized as previously described (44). Briefly, 10 μg of total RNA were primed with 2 μl of 100 μM T16N2 DNA primer at 70°C for 10 min and then reversed transcribed at 42°C for 1 h in the presence of 400 U SuperScript II RTase (Invitrogen) and 100 μM each dATP, dTTP, and dGTP, with 25 μM dCTP, 25 μM Cy3- or Cy5-labeled dCTP (NEN Life Science, Boston, MA), and RNase inhibitor (Invitrogen). RNA was then degraded with RNase A, and labeled cDNAs were purified using QIAquick PCR columns (Qiagen). Oligoarray control targets and hybridization buffer (Agilent In Situ Hybridization Kit Plus) were added, and samples were applied to microarrays enclosed in Agilent SureHyb-enabled hybridization chambers. After hybridization, slides were washed sequentially with 6× SSC-0.005% Triton X-102 and 0.1× SSC-0.005% Triton X-102 before scanning. Slides were hybridized for 17 h at 60°C in a rotating oven, washed, and then scanned on an Agilent G2565AA scanner. Images were quantified using Agilent Feature Extraction Software (version A.7.5). All microarray data were submitted to the National Center for Biotechnology Information (NCBI)’s Gene Expression Omnibus (GEO Series ID GSE3432; http://www.ncbi.nlm.nih.gov/geo/).

**Microarray quality control analysis.** Box plots, log-log plots, and correlation scores examining expression value distribution and biological replicate consistency were generated and evaluated as previously described (41). One sample that showed a high degree of variability from other similarly treated biological replicates was removed from the data set and excluded from further analysis. The final analysis groups consisted of four control ES and three ES-TF replicates.

**Microarray data analysis.** Differential expression between ES and ES-TF cells was assessed using several parametric and nonparametric methods including t-test, threshold number of misclassifications (TNoM), and fold-change analysis (5, 37). Using GenBank accession numbers, UniGene cluster numbers, and Entrez gene symbols (NCBI, National Institutes of Health), the microarray was annotated as fully as possible using Gene Ontology (GO) annotation terms (2). The enrichment of these terms for ES and ES-TF groups was studied using GOMiner (51). Z-score cutoff for significance was P < 0.05, with a requirement of four or more genes per annotation group. Selected gene subsets were further analyzed using PathwayAssist 3.0.7 (Stratagene, La Jolla, CA), a literature-based gene networking program. Heat maps were created using HeatMap Builder 1.0 (created by E. A. Ashley, J. M. Spin, and C. Watt, Stanford University; http://quertermous.stanford.edu/heatmap.htm).

**Fig. 1.** Stable lentiviral transfection of embryonic stem (ES) cells with the triple-fusion (TF) reporter gene. A: schema of the TF reporter gene containing firefly luciferase (fluc), monomeric red fluorescence protein (mrfp), and truncated thymidine kinase (ttk). The reporter genes were joined by a 14- (LEN-SHASAGYQAST) and 8-amino acid (TAGPGSAT) long linker, respectively. The TF reporter gene was cloned into a self-inactivating (SIN) lentiviral vector downstream from the ubiquitin promoter. B: after lentiviral transduction, 29 ± 5% of murine ES cells were positive for mrfp [average of 3 fluorescence-activated cell sorting (FACS) analyses]. Fluorescence microscopy showed robust mrfp expression compared with control ES cells. C: stably transduced ES-TF cells showed significantly higher levels of FL enzyme activities on cell lysates compared with control ES cells. D: likewise, tTK enzyme activities were significantly higher in ES-TF cells. LVLTR, lentivirus long terminal repeat; cppt, central polyurine tract; WRE, woodchuck hepatitis B virus RNA regulatory element; SIN LTR, self-inactivating long terminal repeat.
Quantitative RT-PCR. We performed quantitative RT-PCR (qRT-PCR) to verify expression using the same microarray hybridization samples. Briefly, total RNA for each sample was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. Reactions were incubated at 25°C for 5 min, 42°C for 30 min, and then 85°C for 5 min. The resulting cDNA served as a template for real-time quantitative PCR, in which a fluorescent reporter dye (6-carboxy-fluorescein; FAM) was released and quantitated during each specific replication of the template. The cDNA (6 ng/μl) was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) for various specific genes. Six genes identified from pathway analysis were chosen (see RESULTS and DISCUSSION). Assays used included Mm00494175_m1 (Wee1), Mm00518586_m1 (Cul2), Mm00521735_m1 (Cdc51), Mm00484678_m1 (Gata1), Mm00514706_m1 (Ephx2), and Mm00519290_g1 (Ier3). Reactions were incubated in an Applied Biosystems Prism 7700 sequence detection system with the following parameters: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Quantities were expressed relative to 18S endogenous control. Fold changes were calculated by dividing the combined ES-TF cell results by the combined control ES cell results.

Effect of reporter genes on ES cell viability, proliferation, and differentiation. After lentiviral transfection, control ES and ES-TF cells were plated uniformly in 96-well plates at a density of 5,000 cells per well. The Trypan blue exclusion assay was used to assay for viability/cytotoxicity at 48- and 72-h time points. The CyQuant cell proliferation assay (Molecular Probes, Eugene, OR) was measured using a microplate spectrofluorometer (Gemini EM, Sunnyvale, CA) under the same conditions. Eight samples were assayed and averaged. Both ES-TF and control ES cells were differentiated into beating cardiomyocytes in vitro using the “hanging drop” method as described (8). At day 14, embryoid bodies derived from control ES and ES-TF cells were analyzed by RT-PCR.

RT-PCR analysis of embryonic- and ventricular-specific transcripts. RT-PCR was used to compare the expression of cardiac transcription factor (Nkx2.5), ventricular-specific proteins [myosin light chain (MLC)2v and β/-myosin heavy chain (β-MHC)], and
reporter genes (fluc) in control ES and ES-TF cells. Total RNA was prepared from ES-TF cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The primer sets used in the amplification reaction were as follows: Nkx2.5 forward primer was AGCAACTCTGGAACCTTTG, reverse primer was CGGGCTCATAGTTGGA; B-MHC forward primer was ACCCTCAGATATGGC, reverse primer was GTGACGTACTGTTGCC; MLCK forward primer was CAGCAGGCTCCTCGAACTCT, reverse primer was GTTTATTTGGCAGCAGCCT; Fluc forward primer was ATCTTACGAGCTGCATTAAAG, reverse primer was CAGCTTCTCTCAATAATCTACA. PCR products were separated by 1% agarose gel electrophoresis and quantified by using Labworks 4.6 Image Acquisition and analysis software (UVP Bio-imaging systems, Upland, CA).

Transplantation of murine ES-TF cells into the murine myocardium. Adult female 129/Sv mice (weighing 20–25 g) underwent aseptic thoracotomy. Animals received isoflurane (2%) for general anesthesia, banamine (2.5 mg/kg) for pain relief, and normal saline (200 μL) for volume replacement. Animals (n = 12) were injected intramyocardially with 1 × 10⁶ ES-TF cells in 50 μL of PBS. Control animals (n = 6) received the same number of nontransfected 1 × 10⁶ ES cells. All animals recovered uneventfully and underwent bioluminescence and small-animal PET imaging on week 1 and week 2 after transplant. Study protocols were approved by the Stanford Animal Research Committee.

Bioluminescence imaging of ES-TF cell transplantation. The same mice were scanned using the Xenogen In Vivo Imaging System (IVIS) after intraperitoneal injection of the reporter probe p-luciferin (375 mg/kg body wt; Promega) as described (49). Animals were imaged for 30 min using 1-min acquisition intervals. The gray scale photographic images and bioluminescence color images were superimposed and analyzed with Living Image software. A region of interest (ROI) was drawn over the heart, and the signal intensity was quantified as maximum photons per second per centimeter squared per steradian (photons·s⁻¹·cm⁻²·sr⁻¹).

Small-animal PET imaging of ES-TF cell transplantation. Animals were injected via tail vein with the PET reporter probe 9-(4-[¹⁸F]fluoro-3-hydroxymethylbutyl)-guanine ([¹⁸F]FHBG; 102 ± 12 μCi). After 1 h of uptake and clearance of radioactivity, imaging was acquired using the P4 microPET scanner (Concorde) for 15 min. Images were reconstructed by filtered back projection algorithm as described (48). ROIs were drawn over the heart, and signals were expressed as percentage of injected dose per gram of tissue (% ID/g). With the animals kept in the exact same position, [¹⁸F]fluorodeoxyglucose ([¹⁸F]FDG; 121 ± 22 μCi) was injected intravenously using the same acquisition sequence. Afterward, both the [¹⁸F]FDG (roadmap of the myocardium) and [¹⁸F]FHBG (location of transplanted cells) images were overlaid as a fusion image using the ASIPro VM software. Both [¹⁸F]FHBG and [¹⁸F]FDG were synthesized at the Stanford Cyclotron Facility.

Postmortem in vitro enzymatic analysis and histology. After completion of imaging analysis at week 2, mice were euthanized and their hearts homogenized in 4 ml/gm tissue of Passive Lysis Buffer (Promega). After freeze-thawing three times at −80°C for 15 min each, the homogenate was centrifuged at 14,000 rpm for 15 min. FL enzyme activity was assessed using 20 μl of supernatant with 100 μl of Luciferase Assay Reagent (Promega). The results were normalized to relative light units per microgram of protein (RLU/μg) as described (49). The same samples were assayed for thymidine kinase enzyme activity using [³H] penciclovir as the substrate, as described (48). For comparison, firefly luciferase and thymidine kinase enzyme activities were correlated with each other and with in vivo imaging results. Finally, hematoxylin and eosin stains (H&E) were performed in three heart samples for assessment of cell morphology and phenotype.

Data analysis for imaging and functional cell culture studies. Image data are given as means ± SD. For statistical analysis, the two-tailed Student’s t-test was used. Differences between control ES and ES-TF cells were considered significant at P < 0.05.

### Table 1. GO annotation analysis

<table>
<thead>
<tr>
<th>Changed</th>
<th>p value</th>
<th>GO term</th>
<th>Changed</th>
<th>p value</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.0036</td>
<td>aromatic compound metabolism</td>
<td>6</td>
<td>0</td>
<td>regulation of DNA metabolism</td>
</tr>
<tr>
<td>4</td>
<td>0.0107</td>
<td>positive regulation of development</td>
<td>10</td>
<td>0.0003</td>
<td>nucleocytoplasmic transport</td>
</tr>
<tr>
<td>9</td>
<td>0.0112</td>
<td>regulation of development</td>
<td>27</td>
<td>0.0007</td>
<td>cell cycle</td>
</tr>
<tr>
<td>6</td>
<td>0.0141</td>
<td>homeostasis</td>
<td>60</td>
<td>0.0015</td>
<td>nucleobase, nucleoside, nucleotide and nucleic acid metabolism</td>
</tr>
<tr>
<td>4</td>
<td>0.0169</td>
<td>negative regulation of development</td>
<td>17</td>
<td>0.0022</td>
<td>ubiquitin cycle</td>
</tr>
<tr>
<td>11</td>
<td>0.0181</td>
<td>cytoskeleton organization and biogenesis</td>
<td>58</td>
<td>0.0046</td>
<td>cellular protein metabolism</td>
</tr>
<tr>
<td>11</td>
<td>0.029</td>
<td>lipid metabolism</td>
<td>4</td>
<td>0.006</td>
<td>nucleosome assembly</td>
</tr>
<tr>
<td>11</td>
<td>0.029</td>
<td>lipid metabolism</td>
<td>4</td>
<td>0.0107</td>
<td>mitotic sister chromatid segregation</td>
</tr>
<tr>
<td>10</td>
<td>0.0356</td>
<td>negative regulation of apoptosis</td>
<td>6</td>
<td>0.0108</td>
<td>protein import</td>
</tr>
<tr>
<td>5</td>
<td>0.0367</td>
<td>negative regulation of programmed cell death</td>
<td>21</td>
<td>0.0155</td>
<td>intracellular transport</td>
</tr>
<tr>
<td>5</td>
<td>0.039</td>
<td>cell growth</td>
<td>26</td>
<td>0.0186</td>
<td>organelle organization and biogenesis</td>
</tr>
<tr>
<td>24</td>
<td>0.041</td>
<td>nucleotide binding</td>
<td>9</td>
<td>0.0187</td>
<td>translation</td>
</tr>
<tr>
<td>5</td>
<td>0.0426</td>
<td>regulation of cell size</td>
<td>16</td>
<td>0.0217</td>
<td>protein biosynthesis</td>
</tr>
<tr>
<td>4</td>
<td>0.0453</td>
<td>nucleotidyltransferase activity</td>
<td>8</td>
<td>0.0263</td>
<td>protein complex assembly</td>
</tr>
<tr>
<td>4</td>
<td>0.0469</td>
<td>regulation of cell differentiation</td>
<td>10</td>
<td>0.0456</td>
<td>chromosome organization and biogenesis</td>
</tr>
</tbody>
</table>

Gene Ontology (GO) annotation analysis. Term enrichment for genes identified during comparisons between control embryonic stem cells (ES) and ES cells expressing triple fusion (ES-TF) was performed as described in METHODS. The most highly significant terms are shown. Terms in boldface are discussed more extensively in the text. Some collapsing of similar terms has been performed for visual clarity. The number of significantly changed genes (Changed) and the enrichment p value for each term are shown.
RESULTS

Isolation and characterization of murine ES cells expressing the TF reporter gene. To develop a multimodality imaging approach, we used a TF reporter gene bearing a bioluminescence (fluc), fluorescence (mrfp), and PET reporter gene (tk) driven by a constitutive ubiquitin promoter (Fig. 1A). After lentiviral transduction, 29 ± 5% of the ES cells were positive for red fluorescence by FACS, which could also be detected by single-cell fluorescence microscopy (Fig. 1B). Compared with the control ES cells, the ES-TF cells showed significantly higher levels of FL enzyme activity (Fig. 1C) and tTK enzyme activity (Fig. 1D). To track cell transplant reliably, it is critical that the reporter gene activity correspond to cell numbers. We next assayed ES-TF cells (1 × 10^5 to 1 × 10^7) and showed there was a strong correlation between cell number vs. FL (r^2 = 0.95), cell number vs. tTK (r^2 = 0.91), and FL vs. tTK activities (r^2 = 0.85). These strict relationships suggest that reporter genes could be used to follow cell survival and proliferation with high fidelity.

Transcriptional profiling of control ES vs. ES-TF cells. Detailed transcriptional analysis of the differences between ES and ES-TF cells after several passages in culture was performed to identify effects of TF expression. Overall correlation of gene transcription between biological replicate arrays of control ES cells was high, with a coefficient of 0.8 for all arrays as shown in the similarity matrix (Fig. 2A). In comparison, the correlation coefficient for the transsected ES cells was lower at 0.6. This was not due to variation in array quality, as determined by various metrics (data not shown), but instead appears to reflect increased heterogeneity in the ES-TF cell population due to random chromosomal integration of the TF reporter gene. This idea is supported by the fact that the ES-TF arrays correlated similarly with each other and with the control arrays (0.6–0.7). Differential expression analysis was able to distinguish clearly the ES-TF arrays from control ES arrays. Using the very stringent criteria of expression fold change >1.5, t-test P value < 0.01, and TNOM score of 0, we identified 207 unique genes that were upregulated and 333 unique genes that were downregulated in ES-TF vs. control ES cells (Fig. 2B). A complete listing of these genes is found in Supplemental Table S1 (available at the Physiological Genomics web site).

Annotation analysis of control ES vs. ES-TF cells. Annotation analysis of the differentially regulated gene sets was performed, and significant terms were identified (P < 0.05). A total of 123 upregulated and 173 downregulated unique genes possessed GO annotation. Table 1 shows a summary of these significant term categories. Clear trends emerged from this analysis. Many of the significant genes found to be downregulated with TF reporter gene expression were involved in various aspects of mitosis, cell cycling, and nucleotide metabolism and included Wee1, Cdc5l, Tlk2, and Cct4. Analysis using the PathwayAssist software revealed the relationships of some of these genes and made it clear that many of the downregulated “cell cycling” genes also have a role in inducing cell death (Fig. 3A). In contrast, ES-TF cells upregulated several genes related to homeostasis, phosphorylation, and anti-apoptosis pathways (Fig. 3B). These genes included Mcl1, Ephp2, Pim2, Gata1, and Ier3. PathwayAssist confirmed that several of these genes are known to promote cell survival. It is perhaps notable, given that the TF reporter gene was driven by a constitutive ubiquitin promoter, that 17 significantly downregulated genes (control ES > ES-TF) were found to be “ubiquitin cycle” genes. Some of these (Cul2, Cul3, Gsp1, Rnf2) are also considered to be regulators of apoptosis and cell cycling, and suggest that some counterregulation might have taken place as a result of reporter gene expression. As ex-

---

Note: The Supplemental Material for this article (Supplemental Table S1 and Supplemental Video Clip) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00254.2005/DC1.
pected, relative expression changes correlated well between microarray and qRT-PCR data for all evaluated upregulated (Ephx2, Ier3, Gata1) and downregulated (Cul2, Wee1, Cdc51) genes (Fig. 3C).

Effects of reporter gene expression on ES cell viability, proliferation, and differentiation. Despite the up- and down-regulation of several unique genes, it was not clear what their overall effect would be on ES cellular characteristics. Therefore, we compared cell viability between control ES and ES-TF cells. We found no significant differences between the two cell populations (Fig. 4A). Likewise, the rate of cell proliferation between control ES and ES-TF cells was similar at 48 and 72 h (Fig. 4B). The ES-TF cells also stably expressed both mrfp and fluc for 30 passages as examined by FACS and enzyme assays, respectively (Fig. 4C). To assess whether the reporter genes could adversely affect ES-TF cell differentiation, we withdrew the LIF from cell culture. After 14 days of embryoid body differentiation, beating cardiomyocytes were clearly visible in both control ES and ES-TF cells (Supplemental Video Clip). There was no significant difference in chronotropicity between control ES and ES-TF cells (data not shown). Expression patterns of the cardiac transcriptional factor (Nkx2.5) and ventricular-specific proteins (MLC2v, β-MHC) were also similar between the two groups on RT-PCR analysis (Fig. 4D).

Tracking ES-TF cell survival using multimodality bioluminescence and PET imaging. To demonstrate the feasibility of imaging stem cells noninvasively after transplant, ES-TF cells (1 x 10^6) were injected into the myocardium of living mice, and scans were obtained on days 7 and 14 (Fig. 5A). The bioluminescence and PET signals were 1.51 x 10^8 ± 3.10 x 10^7 photons·s^{-1}·cm^{-2}·sr^{-1} and 0.63 ± 0.09% ID/g at day 7, respectively. Both signals increased significantly to 6.16 x 10^8 ± 1.04 x 10^8 photons·s^{-1}·cm^{-2}·sr^{-1} and 0.94 ± 0.13% ID/g by day 14 (P < 0.05 vs. day 7). Because the reporter genes are passed on from mother to daughter cells, these changes reflect donor cell survival and proliferation in the host. In contrast, control animals injected with nontransfected ES cells showed background signals of 3.91 x 10^4 ± 2.05 x 10^3 photons·s^{-1}·cm^{-2}·sr^{-1} and 0.03 ± 0.02% ID/g (P < 0.05 vs. study group). Quantification analysis showed a strong correlation (r^2 = 0.91) between the biolumines-

![Fig. 5. Multimodality imaging of ES cell transplant in living subjects. A: animals injected with control ES cells showed background activities in both bioluminescence (BLI; top) and positron emission tomography (PET; bottom) imaging. In contrast, a representative study animal injected with ES-TF cells showed significant bioluminescence and PET signals at days 7 and 14 (d7 and d14, respectively). B: in vivo bioluminescence signal activities for all animals quantified as units of photons per second per centimeter squared per steridian (P/s·cm^2·sr)(P = 0.05 vs. control). C: in vivo PET signal activities for all animals, quantified as percentage of injected dose of 9-(4-[18F]fluoro-3-hydroxyethylbutyl)-guanine ([18F]FHBG) per gram of heart tissue (% ID/g) (P = 0.05 vs. control). D: because the fluc and ttk reporter genes are linked together, a strong correlation existed between the bioluminescence and PET imaging signals (r^2 = 0.92). *P < 0.05 vs. control. **P < 0.05 vs. day 7.

Physiol Genomics • VOL 25 • www.physiolgenomics.org
cence and PET signals, which was expected because the \textit{fluc} and \textit{tk} reporter genes were fused together (Fig. 5, B–D). Likewise, there was a strong correlation between in vivo bioluminescence signal and ex vivo FL enzyme activity ($r^2 = 0.92$) as well as between in vivo PET signal and ex vivo TK enzyme activity ($r^2 = 0.88$). Finally, conventional histology using H&E stains confirmed the presence of ES-TF cells in the anterolateral wall (Fig. 6). Overall, these results validate the use of in vivo imaging in lieu of or in parallel with traditional in vitro assays for assessment of cell transfer.

**DISCUSSION**

The clinical application of human adult stem cells is arguably the most promising approach for cellular regeneration in the treatment of ischemic heart disease, yet we currently know very little about how these cells behave in vivo. For example, skeletal myoblasts have been shown to augment systolic and diastolic performance in animal models (31), but recent clinical studies suggest they may also trigger arrhythmias because of the lack of electromechanical coupling (24, 25). Mononuclear bone marrow cells or enriched hematopoietic stem cells have not caused any serious complications in clinical trials (19, 42), but their differentiation capacity into cardiomyocytes remains controversial in animal studies (3, 30). In contrast, ES cells are pluripotent and can differentiate into multiple lineages (13, 45), but their differentiation capacity into cardiomyocytes remains controversial in animal studies (3, 30). In contrast, ES cells may serve as a constant source of supply for cardiac cells. Several groups have recently reported that murine ES cells can improve cardiac function in mice and rats after myocardial infarction without any evidence of graft rejection, ventricular ectopy, or teratoma formation (4, 18, 26, 27). However, these studies relied on either green or cyan fluorescence markers, which required the animals to be killed at different time points for postmortem analysis.

To better understand stem cell fate in vivo, noninvasive imaging studies are needed. One approach is imaging of stem cells radiolabeled with [18F]FDG using PET (19). Because of the short physical half-life of [18F] radioisotope (~110 min), the imaging period is less than a day, which limits the biological and mechanistic data that can be obtained. Likewise, cells labeled with copper-64-pyruvaldehyde-bis(N4-methylthiosemicarbazone) ($^{64}$Cu-PTS) with longer half-life (~12.7 h) can only be tracked up to 24–36 h (1). On the other hand, MR imaging of iron-labeled cells can be tracked for a longer period of time but still cannot reliably distinguish viable from nonviable cells (10, 23). Taking advantage of the strengths and weaknesses of different modalities, we used a TF reporter gene with the following logic. The fluorescence reporter gene (\textit{mrfp}) allows imaging at the single-cell level by fluorescence microscopy and isolation of stable clone populations by FACS. The bioluminescence reporter gene (\textit{fluc}) can be used for high-throughput analysis of cell survival and proliferation in small animals at a relatively low cost per scan (49). The PET reporter gene (\textit{tk}) can be used for preclinical validation in living subjects (35).

A very important question with regard to reporter genes is whether they would affect cellular traits which may potentially hamper future efforts for clinical applications. To address this issue, we examined control ES vs. ES-TF cells using transcriptional profiling. While a small minority of the total number of examined genes showed differential regulation, enough differences were identified to clearly distinguish control ES from ES-TF cells. A large number of these genes belonged to apoptosis and cell cycling pathways as well as homeostasis and nucleotide metabolism pathways. Internal confirmation of the results is seen in the generally observed inverse regulation of gene families. That is, upregulation of anti-apoptosis/pro-homeostasis genes (e.g., Pim2, Eef1a2, Mcl1, Ier3; Refs. 14, 38, 47, 53) was accompanied primarily by downregulation of pro-apoptosis genes (e.g., Gsp1, Pcn1, Cct4, Pawr; Refs. 6, 9, 16, 54). As Fig. 3A shows, genes downregulated in ES-TF cells are involved in many aspects of cell cycling. For example, \textit{Wee1} (a tyrosine kinase, −1.54-fold) helps to coordinate the

---

**Fig. 6.** Postmortem histological analysis of transplanted ES cells. A: gross sample of the heart showing the transplanted ES-TF cell mass (black arrows). B: representative hematoxylin and eosin stain (H&E) image of ES-TF cells in the anterior left ventricular wall 2 wk after injection (×40). C: hemorrhagic formation (black arrow) in the center of the undifferentiated ES-TF cells (×100). D: high-power view showing viable undifferentiated ES-TF cells (black arrows) as well as degenerated cells with necrosis and apoptosis (green arrows) (×400).
transition between DNA replication and mitosis, maintaining mitotic timing (15). Bzw1 (-1.94-fold) regulates transcriptional control at the G1/S phase transition, enhancing histone H4 transcription through site II (28). Tlk2 (tosed-life kinase 2, -2.4-fold) is a nuclear serine/threonine kinase involved in the regulation of chromatin assembly. It displays maximal activity during S phase and is closely linked to DNA replication (39). Another downregulated gene, Cdc5 (1.56-fold), is a transcriptional regulator whose presence is required for mitotic entry from G2 (7). Genes involved in DNA/nucleotide metabolism (e.g., Set, Smarcad1, Tlk2) were generally downregulated as well.

Ubiquitination is closely tied to many of the processes already mentioned, and ubiquitin cycle genes were significantly suppressed in ES-TF cells when compared with control ES cells. These included Cul2, Cul3, and Cul4b. While Cul1 is considered a negative cell cycle regulator, Cul3 (a ubiquitin ligase) is thought to act in an opposite fashion, targeting cyclin E for ubiquitination and promoting cell cycling (40). Cul2 and Cul4b are also ubiquitin ligases. Cul2 is thought to act as a tumor suppressor, while Cul4b may stop aberrant reinitiation of DNA replication (33, 52). A trio of deubiquitinating enzyme genes was also downregulated in ES-TF cells, the ubiquitin-specific proteases Usp1, Usp9x, and Usp14. The TF reporter gene in our experiments was driven by the human ubiquitin-C promoter, and it seems possible that the subtle changes in gene regulation that we observed might relate to the nonspecific site integration of this promoter. Overall, it is important to recognize that, despite the observed transcriptional changes, we found no significant functional differences between ES and ES-TF cells in terms of viability, proliferation, chromotropism, or differentiation. One possibility is that the ES-TF cells activated anti-apoptotic/pro-homeostatic genes to successfully blunt any pro-apoptotic effects due to reporter gene expression. Alternatively, protein levels may have not been altered significantly by the observed transcriptional changes. This latter seems less probable, as a large proportion of the differentially regulated genes showed large fold changes. In ES-TF > ES comparisons, 91 genes increased >2.0-fold, and 13 changed 5.5-fold or more (Rab24 increased 46.6-fold), while for ES > ES-TF, 219 genes decreased >2.0-fold. Furthermore, we have repeatedly validated array results from this and similar array platforms with qRT-PCR/TaqMan (Applied Biosystems) and repeatedly validated array results from this and similar array platforms with qRT-PCR/TaqMan (Applied Biosystems) and have always achieved excellent correlation (21, 41, 44). Undoubtedly, future additional studies will be needed to further characterize these differences.

In conclusion, we report the first transcriptional profiling analysis on the effects of fluorescence, bioluminescence, and PET reporter genes in ES cells. Importantly, we demonstrated that ES cell survival and proliferation can be monitored noninvasively, repetitively, and quantitatively in the myocardium of living subjects. With further characterization and validation, we believe noninvasive imaging studies such as the techniques described by our study can help investigators better understand the biology and physiology of stem cells in vivo.

**GRANTS**

This work was supported in part by an Atorvastatin Research Award and grants from the American Heart Association and National Heart, Lung, and Blood Institute (J. C. Wu), as well as National Institutes of Health Grants NCI-ICMIC-P50CA114747 and NHLBI-R01-HL-078632 and grants from the National Cancer Institute Small Animal Imaging Resource Programs and Department of Energy (S. S. Gambhir).

**REFERENCES**


