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In Vivo Visualization of Embryonic Stem Cell Survival, Proliferation, and Migration After Cardiac Delivery

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Abstract

Background—Recent studies have shown that stem cell therapy can promote tissue regeneration; however, monitoring stem cells in vivo remains problematic owing to limitations of conventional histological assays and imaging modalities.

Methods and Results—Murine embryonic stem (ES) cells were stably transduced with a lentiviral vector carrying a novel triple-fusion (TF) reporter gene that consists of firefly luciferase, monomeric red fluorescence protein, and truncated thymidine kinase (fluc-mrfp-ttk). ES cell viability, proliferation, and differentiation ability were not adversely affected by either reporter genes or reporter probes compared with nontransduced control cells (P=NS). Afterward, 1×10^7 of ES cells carrying the TF reporter gene (ES-TF) were injected into the myocardium of adult nude rats (n=20). Control animals received nontransduced ES cells (n=6). At day 4, the bioluminescence and positron emission tomography signals in study animals were $3.7\times10^7\pm5.8\times10^6$ photons $\cdot$ s^{-1} $\cdot$ cm^{-2} per steradian (sr) and 0.08±0.03% injected dose/g, respectively (P<0.05 versus control). Both signals increased progressively from week 1 to week 4, which indicated ES cell survival and proliferation in the host. Histological analysis demonstrated the formation of intracardiac and extracardiac teratomas. Finally, animals (n=4) that were treated with intraperitoneal injection of ganciclovir (50 mg/kg) did not develop teratomas when compared with control animals (n=4) treated with saline (1 mL/kg).

Conclusion—This is the first study to characterize ES cells that stably express fluorescence, bioluminescence, and positron emission tomography reporter genes and monitor the kinetics of ES cell survival, proliferation, and migration. This versatile imaging platform should have broad applications for basic research and clinical studies on stem cell therapy.

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Disclosures

None.

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Keywords

heart diseases; stem cells; positron emission tomography; nuclear medicine; molecular imaging

Ischemic heart disease is the number one cause of morbidity and mortality in the United States. The economic cost of cardiovascular diseases is estimated at a staggering $350 billion per year.[1] Transplantation of bone marrow stem cells,[2] endothelial progenitor cells,[3] and embryonic stem (ES) cells[4] has recently been shown to improve myocardial function. The mechanisms may be related to stem cells differentiating into cardiomyocytes, secreting paracrine factors, or recruiting peripheral stem cells to the ischemic territory.[5] However, current methods of studying stem cell engraftment rely on postmortem histological sampling, which precludes longitudinal monitoring. Thus, the development of "molecular markers" that could determine the nature of engrafted cells and their progeny would be extremely useful.[6]

To understand the fate of stem cells in vivo, imaging techniques have recently been proposed and evaluated in a limited number of cell-delivery models. One technique is radionuclide imaging of stem cells labeled with F-18 fluorodeoxyglucose ([^{18}F]-FDG); however, the short physical half-life of this radioisotope (T1/2 ≈ 110 minutes) limits the imaging protocol to within the first day of cell transplantation.[7] Another technique is MRI of stem cells labeled with iron oxide particles[8] or perfluoropolyether agents,[9] which has the advantage of providing detailed anatomic location but is unable to quantify cell signal activity reliably because of dilutional effects during cellular division.[8] In addition, the engulfment of labeled radioisotopes, iron oxide particles, or perfluoropolyether agents by neighboring macrophages after donor cell death limits the ability of both techniques to distinguish viable from nonviable cells.[7–9] The third modality is based on the reporter gene imaging approach that our group has validated previously.[10] Although this prior study was able to image transplanted rat embryonic cardiomyoblasts noninvasively, accurate monitoring of cell survival, proliferation, and migration was not feasible because of the transient nature of adenoviral transduction.

Here, we report several significant improvements toward reporter gene imaging of stem cells. We first designed a versatile triple-fusion (TF) reporter gene construct carried by a self-inactivating lentiviral vector capable of stably transducing ES cells. We next showed that expression of reporter genes and their interaction with reporter probes did not adversely affect ES cell viability, proliferation, and differentiation. We then imaged ES cell fate in living rats and importantly confirmed that cell imaging signals correlated strongly with traditional ex vivo assays. Finally, we demonstrated that the positron emission tomography (PET) reporter gene (ttk, or truncated thymidine kinase) could also serve as a suicide gene, thus providing an extra safety mechanism against cellular misbehavior.

Methods

Culture of Undifferentiated ES Cells

The murine ES-D3 cell line (CRL-1934) was obtained from the American Type Culture Collection (ATCC; Manassas, Va). ES cells were kept in an undifferentiated, pluripotent state with 1000 IU/mL leukemia inhibitory factor (LIF; Chemicon, ESGRO, ESG1107) and grown on top of the murine embryonic fibroblasts feeder layer inactivated by 10 μg/mL mitomycin C (Sigma). ES cells were cultured on 0.1% gelatin-coated plastic dishes in ES medium containing Dulbecco modified Eagle medium supplemented with 15% fetal calf serum, 0.1 mmol/L β-mercaptoethanol, 2 mmol/L glutamine, and 0.1 mmol/L nonessential amino acids as described previously.[11] The ES culture medium was changed every day, and ES cells were passaged every 1 or 2 days. To confirm their undifferentiated state, ES cells were labeled with 4′,6-diamidino-2-phenylindole (DAPI) and Oct4 antibodies (Sigma) as nuclear marker and stem cell marker, respectively.[4] Images were obtained with a Zeiss Axiovert microscopy (Sutter Instrument Co., USA).

Construction of pUb-fluc-mrfp-ttk and Lentiviral Transduction of ES Cells

In our previous work, the TF reporter gene was downstream from a cytomegalovirus promoter.[12] This gene fragment (3.3 kbp) was released from the plasmid with *Not*1 and *Bam*H1 restriction enzymes before blunt-end ligation into the multiple cloning site of the lentiviral transfer vector, FUG, driven by the human ubiquitin-C promoter.[13] A self-inactivating lentivirus was prepared by transient transfection of 293T cells as described previously.[14] Briefly, pFUG-TF containing the TF reporter gene was cotransfected into 293T cells with HIV-1 packaging vector (δ8.9) and vesicular stomatitis virus G glycoprotein-pseudotyped envelop vector (pVSVG). Lentivirus supernatant was concentrated by sediment centrifugation with SW29 rotor at 50 000 g for 2 hours. Concentrated virus was titrated on 293T cells. Murine ES cells were transduced with LV-pUb-fluc-mrfp-ttk at a multiplicity of infection (MOI) of 10. The infectivity was determined by monomeric red fluorescence protein (mrfp) expression as analyzed on FACScan (Becton Dickinson). For comparison, ES cells were also transduced with lipofectamine (Qiagen) and electroporation (Bio-Rad Gene Pulser) techniques according to manufacturer's protocol.

Effect of Reporter Genes and Reporter Probes on ES Cell Viability and Proliferation

Cells were plated uniformly in 96-well plates at a density of 5000 cells per well. The control nontransduced ES and ES-TF cells were incubated with bioluminescence reporter probe D-luciferin (100 μmol/L) or PET reporter probe 9-(4-[^{18}F]-fluoro-3hydroxymethylbutyl)guanine ([^{18}F]-FHBG; 100 μCi) for 6 hours before the media were changed on a weekly basis. The CyQuant cell proliferation assay (Molecular Probes) was measured with a microplate spectrofluorometer (Gemini EM) at week 1, 2, 3, and 4 time points. Eight samples were assayed and averaged. The trypan blue exclusion assay was used to assess the viability and cytotoxicity under the same conditions.

Embryoid Body Formation and Cardiomyocyte Differentiation

ES cells were differentiated into beating cardiomyocytes in vitro by the "hanging drop" method as described previously.[15] Briefly, the main steps included withdrawal of LIF and cultivation of 400 cells in 18-μL hanging drops to produce embryoid bodies for 3 days, followed by cultivation as a suspension in ultra-low-cluster 96-well flat-bottom plates for 2 days. Next, the embryoid bodies were seeded onto 48-well plates. Over the next 3 weeks, the beating rates (chronotropicity) of these embryoid bodies were compared between control ES and ES-TF cells.

Reverse-Transcription Polymerase Chain Reaction Analysis of Embryonic and Ventricular Specific Transcripts

Reverse-transcription polymerase chain reaction (RT-PCR) was used to compare the expression of embryonic marker (Oct4), cardiac transcription factor (Nkx2.5), ventricular-specific proteins (β-MHC [myosin heavy chain] and MLC_{2v} [myosin light chain 2]), and reporter genes (fluc, or firefly luciferase) between control nontransduced ES and ES-TF cells. Total RNA was prepared from ES-TF cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The primer sets used in the amplification reaction were as follow: Oct4 forward primer GGCGTTCTCTTTGCAAAGGTGTTC, reverse primer CTCGAACCACATCCTTCTCT; Nkx2.5 forward primer AGCAACTTCGTGAACTTTG, reverse primer CCGGTCCTAGTGTGGA; MLC_{2v} forward primer CAGCAGGCTCCTCGAACTCT, reverse primer GTTTATTTGCGCACAGCCCT; and Fluc forward primer ATCTACTGGTCTGCCTAAAG, reverse primer CAGCTCTTCTTCAAATCTATAC. PCR products were separated on 1% agarose gel electrophoresis and quantified with Labworks 4.6 Image Acquisition and analysis software (UVP Bio-Imaging Systems).

Transplantation of Murine ES Cells Into Rat Myocardium

Adult female nude athymic rats (weight 200 to 250 g) underwent aseptic lateral thoracotomy. Animals received isoflurane (2%) for general anesthesia, banamine (2.5 mg/kg) for pain relief, and normal saline (2 to 3 mL) for volume replacement. Harvested control ES and ES-TF cells were kept on ice for <30 minutes for optimal viability before injection into the rat myocardium. Animals (n=20) were injected intramyocardially with 1×10^7 of ES-TF cells in 50 μL of PBS. Control animals (n=6) received 1×10^7 nontransduced ES cells instead. All animals recovered uneventfully and underwent bioluminescence and small-animal PET imaging later. Study protocols were approved by the Stanford Animal Research Committee.

Optical Bioluminescence Imaging of ES Cell Transplantation

Cardiac bioluminescence imaging was performed with the Xenogen In Vivo Imaging System. After intraperitoneal injection of the reporter probe D-luciferin (375 mg/kg body weight), animals were imaged for 30 minutes with 1-minute acquisition intervals. The same rats were scanned repetitively for a 4-week period according to the specific study design. Bioluminescence was quantified in units of maximum photons per second per centimeter squared per steradian ($P \cdot s^{-1} \cdot cm^{-2} \cdot sr^{-1}$) as described previously.[10]

Small-Animal PET Imaging of ES Cell Transplantation

Cardiac PET imaging was acquired with the P4 Concorde MicroPET system. Animals were injected with the reporter probe [^{18}F]-FHBG (0.92±0.19 mCi), and images from 60 to 75 minutes after injection were reconstructed by filtered back-projection algorithm as described. Regions of interest (ROIs) were drawn over the anterolateral wall (horizontal view). The counts per pixel per minute were converted to counts per milliliter per minute with a calibration constant obtained from scanning a cylindrical phantom. The ROI counts per milliliter per minute were converted to counts per gram per minute (assuming a tissue density of 1 g/mL) and divided by the injected dose to obtain an image ROI-derived [^{18}F]-FHBG percentage injected dose per gram of heart (% ID/g) as described previously.[10] With the animals kept in the same position, [^{18}F]-FDG (1.06±0.32 mCi) was injected intravenously to acquire the myocardial image. Afterward, both the [^{18}F]-FHBG and [^{18}F]-FDG images were overlaid as a fusion image with ASIPro VM software.

Postmortem Immunohistochemical Stainings

After imaging, all animals were euthanized by protocols approved by the Stanford Animal Research Committee. Explanted hearts and extracardiac teratomas were routinely processed for hematoxylin- and-eosin staining. Slides were interpreted by an expert cardiac pathologist blinded to the study (A.J.C.).

Statistical Analysis

Data are given as mean±SD. For statistical analysis, the 2-tailed Student *t* test was used. Differences were considered significant at $P<0.05$.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Efficient and Stable Transduction of ES Cells With Self-Inactivating Lentivirus

To develop a multimodality imaging approach, we used a TF reporter gene–bearing fluc-mrfp-ttk driven by a constitutive human ubiquitin promoter (Figure 1a). Compared with plasmid lipofectamine and electroporation, lentiviral transduction of murine ES cells with the TF reporter gene was the most efficient (29±4%) based on the average of 3 fluorescence-activated cell sorter (FACS) scans with a Texas red (585±25 nm) filter setting (Figure 1b). Both nontransduced ES cells (control) and ES cells carrying TF (ES-TF) showed similar morphology on brightfield microscopy and expression of stem cell marker Oct4 on immunofluorescence stainings (Figure 1c). Recent data suggest that gene expression from retrovirus-transduced ES cells can be silenced by DNA methylation and histone deacetylation.[16] To examine whether similar events could occur with lentiviral transfection of ES cells, we performed Fluc enzyme assays (Figure 1d) and mrfp FACS analysis (Figure 1e) and found relatively stable activities for 40 passages over 6 weeks. Likewise, we also examined embryoid bodies and found stable Fluc and ttk enzyme activities from day 0 to day 21 of differentiation (data not shown). Thus, the lack of significant transgene silencing

is consistent with other reports using lentiviral transduction of murine and human ES cells.[17,18]

Reporter Genes and Reporter Probes Do Not Affect ES Cell Viability and Proliferation

Reporter genes have been used to track tumor metastasis in the oncology literature,[19] but few studies have addressed potential adverse cellular effects. To study this issue, we examined control ES and ES-TF cell viability and proliferation at several time points and observed no significant changes between the 2 populations (Figures 2a and 2b). We also incubated ES-TF cells with combined D-luciferin and [^{18}F]-FHBG, which are reporter probes for fluc and ttk reporter genes, respectively. The ES-TF cells were exposed for 6 hours on a weekly basis over a month to simulate the longitudinal imaging protocol. Likewise, there were no significant adverse cellular effects (Figure 2c). By our estimate, the dosage of D-luciferin (100 μmol/L) and [^{18}F]-FHBG (100 μCi) used for cell culture experiments would be equivalent to ≈5× peak serum levels of reporter probes used for in vivo imaging. Finally, we separately transduced 3 other cell lines (rat embryonic H9c2 cardiomyoblast, mouse C2C12 myoblast, and mouse H1 atrial myocyte) with the TF reporter gene and did not notice any significant cellular effects (data not shown).

TF Reporter Gene Expression Does Not Affect ES Cell Differentiation Into Cardiomyocytes

In the literature, both human and murine ES cells have well-documented differentiation and replication capacities.[20,21] We examined whether the TF reporter gene could adversely affect ES cell differentiation. After 7 to 10 days of differentiation, beating cardiomyocytes were clearly visible in ES-TF cells (Data Supplement Movie). At day 12 and day 20, the beating rates per minute were similar between control ES (71±19 and 153±33 bpm) and ES-TF cells (64±17 and 143±54 bpm; P=NS), which suggests that the cardiomyocyte chronotropicity was not affected (Figure 3a). Next, we used RT-PCR analysis and showed that the TF reporter gene did not affect the expression of stem cell marker (Oct4), early cardiac-specific transcriptional factor (Nkx2.5), or ventricular chamber–specific genes (β-MHC or MLC_{2v}; Figure 3b). To confirm that the levels of reporter gene activities correlated with cell numbers, ES cells (1×10^5 to 2×10^7) were assayed for fluc and ttk enzyme activities. Overall, there was a robust relationship between cell number versus fluc (r^2=0.93) and ttk (r^2=0.90), as well as fluc versus ttk (r^2=0.84; Figures 3c through 3f).

Imaging ES Cell Survival, Proliferation, and Migration in Living Mice

To assess ES cell engraftment, stably transduced ES-TF cells were transplanted into athymic nude rat hearts (n=12). Noninvasive imaging was performed for 4 weeks with the bioluminescence charge-coupled device (CCD) camera and small-animal PET scanner (Figure 4a). At day 4, the bioluminescence and PET signals in study animals were $3.7\times10^7\pm5.8\times10^6$ P · s^{-1} · cm^{-2} · sr and 0.08±0.03% ID/g, respectively (P<0.05 versus control). Both signals increased progressively from week 1 to week 4, which indicated ES cell survival and proliferation in the host (Figure 4b). Cell proliferation was most robust from week 2 to 3, with a 295±11% increase in cell imaging signals. Quantification of cell signal activity showed a robust correlation between bioluminescence and PET imaging, which is expected because the 2 reporter genes are linked and expressed as fusion proteins

with preserved fluc and ttk activities (r^2=0.94; Figure 4c). Likewise, there was a robust correlation between in vivo bioluminescence and in vitro fluc enzyme assay (r^2=0.95), as well as in vivo PET image and ex vivo ttk enzyme assay (r^2=0.91). In contrast, background signals of $3.2\times10^4\pm3.5\times10^3$ P · s^{-1} · cm^{-2} · sr and 0.03+0.01% ID/g were seen in control animals for all time points. To further define the location of ES cell survival within the myocardium, animals underwent [^{18}F]-FHBG reporter probe imaging followed by [^{18}F]-FDG myocardial viability imaging. Fusion of the 2 images allowed identification of transplanted ES cells in detailed anatomic and tomographic resolution (Figures 5a and 5b). Because the small-animal PET scanner is simply a miniaturized version of the clinical PET scanner, similar studies should be feasible in humans using PET or PET-CT studies in the future.

ES Cell–Derived Teratoma Formation Can Be Selectively Ablated by Ganciclovir Therapy

The possibility of teratoma formation after transplantation poses a daunting challenge for clinical application of ES cells. To date, no study has addressed this issue from an imaging standpoint. We hypothesized that a PET reporter gene (ttk) could also serve as a suicide gene using ganciclovir treatment. Longitudinal imaging was performed in another group of animals (n=8). Animals were randomized into an experimental group treated with intraperitoneal injection of ganciclovir (50 mg/kg twice daily; n=4) versus a control group treated with saline (1 mL/kg twice daily; n=4) starting at week 3 (Figure 6a). At week 5, none of the ganciclovir-treated animals developed teratomas, whereas all of the saline-treated animals showed intracardiac and extracardiac signals. These imaging signals were confirmed to be teratomas by postmortem histology (Figure 6b). The teratomas were predominately solid with intermixed areas of endodermal, mesodermal, and ectodermal differentiation.

Discussion

Stem cell therapy offers exciting promises for tissue regeneration, yet currently, we know very little how they behave in vivo. To better understand stem cell activity in vivo, noninvasive imaging studies are needed. The ideal imaging platform should have the following characteristics: (1) biocompatibility, safety, and nontoxicity; (2) single-cell detection capability; (3) quantification of cell number; (4) no dilution with cell proliferation; and (5) noninvasive imaging in living subjects. At present, no single imaging modality possesses all of these parameters.[22]

Taking advantage of the strengths and weaknesses of different modalities, we used a novel TF reporter gene with the following logic. The mrfp allows imaging at the single-cell level by fluorescence microscopy and isolation of stable clonal population by FACS. The fluc is used for high-throughput bioluminescence imaging of cell survival, proliferation, and migration in small animals at relatively low cost per scan. Both fluorescence and bioluminescence imaging are based on low-energy photons (2 to 3 electronvolts [ev]), which become attenuated within deep tissues. Therefore, PET imaging, with its high-energy photons (511 kev), is more suitable for future clinical application. The PET reporter gene (ttk) used in the present study is an improved version of the herpes simplex virus mutant

thymidine kinase (HSV1-sr39tk).[10] It has a deletion in the first 135 bp that contains the nuclear localization signal. The ttk enzyme accumulates more in the cytoplasm rather than the nucleus and results in less cellular toxicity and improved image-signal activity.[12] Compared with MRI, the detection threshold of PET is ≈7 log order more sensitive (10^{-4} versus 10^{-11} mol/L, respectively),[19] which is one of the reasons why PET imaging of reporter gene expression has now been successfully translated into clinical trials involving patients with recurrent glioblastoma[23] and hepatocellular carcinoma.[24]

Previously, we had used E1-deleted adenovirus carrying a single reporter gene to transiently transduce embryonic rat H9c2 cardiomyoblasts.[10] This was not the ideal design for several reasons. Despite deletion of the E1 region, leaky expression of immunogenic adenoviral proteins occurs, which could lead to host immune response against H9c2 cells expressing the reporter gene.[25] Second, the adenoviral transduction leads to episomal gene expression, because the reporter gene is not integrated into the chromatin of mother or daughter cells. This can be a severe limitation if the desired goal of noninvasive imaging is to track stem cell survival and proliferation longitudinally. Consequently, neither radiolabeling nor ferromagnetic imaging could achieve either purpose, because they are not genetic-based markers as discussed previously.[7–9] Because of these drawbacks, we have now switched our delivery system from adenovirus- to lentivirus-based vectors in most of our molecular imaging applications. Lentiviral vectors have several advantages: They can stably integrate into the cell chromatin with minimal cytotoxicity, infect dividing and nondividing cells, and are not subjected to gene silencing.[17]

Another important question is whether reporter genes would affect cellular traits such that future clinical applications may be hampered. We examined ES cell proliferation and viability and observed no significant changes between control ES and ES-TF populations. Likewise, we confirmed that the TF reporter gene did not inhibit ES cell differentiation into cardiomyocytes based on RT-PCR analysis of cardiac-specific transcriptional factors and ventricular chamber–specific genes. We also documented that the reporter probes (D-luciferin for fluc and [^{18}F]-FHBG for ttk) caused no negative effects on ES-TF cells. Whereas the D-luciferin is unlikely to be used clinically because the low-energy photons are subject to attenuation within deeper tissues, the radiotracer [^{18}F]-FHBG has been shown to be safe and pharmacokinetically stable in human volunteer subjects.[26] Importantly, the Food and Drug Administration has recently approved [^{18}F]-FHBG as an investigational new drug submitted by our group. Ongoing studies involving proteomic and genomic analysis will further characterize these observations.

The potential value of transplanting any type of stem cells also carries with it inherent risks because some of the transplanted cells may misbehave over a lifetime. This misbehavior could take the form of malignant stem cells that retain an unchecked growth rate or hyperfunctioning stem cells that oversupply regulated levels of missing proteins, such as insulin for diabetes treatment. In the present study, we noted that all of the animals transplanted with murine ES cells eventually developed intracardiac or extracardiac teratomas. In contrast, several groups have recently reported that transplantation of murine ES cells can improve cardiac function in mice and rats after myocardial infarction without any evidence of graft rejection or teratoma formation.[4,27–29] Although the exact reason for

the discrepancy is unclear, it is possible that conventional histological samplings may not have identified all the potential sites of cell survival, migration, and subsequent teratoma formation. Thus, the results of the present study caution against the use of undifferentiated ES cells for myocardial regeneration. In parallel, the concept of introducing a reporter-suicide gene into stem cells that serve as a safety mechanism against cellular misbehavior is an attractive option and warrant further investigation. In this case, the ttk acts as a PET reporter gene when the [^{18}F]-FHBG reporter probe is used in the picomolar to nanomolar concentrations, but it behaves like a suicide gene when ganciclovir (a guanosine analog that inhibits DNA replication of HSV-tk) is administered in milligram amounts to animals.[30,31]

In conclusion, we report the first proof-of-principle study for lentivirus-transduced ES cells that stably express fluorescence, bioluminescence, and PET reporter genes. We demonstrated how this novel molecular imaging platform can be used to monitor the kinetics of stem cell survival, proliferation, migration, and ablation of teratoma sites. The same approach could also permit experiments to evaluate the contribution of stem cells to organ function at different time points after stem cell delivery. Future imaging studies will need to specifically address the temporal sequence of ES cell differentiation into various tissue types, which perhaps may be monitored with tissue-specific promoters driving reporter genes. Likewise, the kinetics of cell survival will need to be assessed in different myocardial milieus (eg, normal versus acute infarction versus chronic ischemia). If the clinical potential of stem cell therapy is to be realized, we believe detailed evaluation of stem cell biology and physiology will most likely need to be conducted in vivo. For these reasons, noninvasive molecular imaging such as the techniques described by the present study will play an important role as stem cell biology moves from histology-based to living subject–based systems in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PERSPECTIVE

Molecular imaging is a rapidly advancing biomedical discipline. Compared with traditional histological sampling, molecular imaging has the advantages of allowing noninvasive, quantitative, and repetitive imaging of targeted biological processes at the molecular and cellular levels within living subjects. This is an extremely powerful technique with numerous applications, including analysis of gene transfer, the expediting of drug discovery, and the tracking of stem cell delivery, as described here. In this report, we studied the fate of murine embryonic stem (ES) cells carrying fluorescence, bioluminescence, and positron emission tomography reporter genes transplanted into the heart. We demonstrated that the ES cell survival, proliferation, and migration could be imaged by a bioluminescence charge-coupled device (CCD) camera and positron emission tomography scanner. We also raised caution against the use of transplanting undifferentiated ES cells for myocardial regeneration, which can lead to intracardiac and extracardiac teratoma formation. For these reasons, we believe further development of novel imaging techniques will contribute important insights into the biology and physiology of transplanted stem cells, leading to expanding, significant clinical applications for years to come.

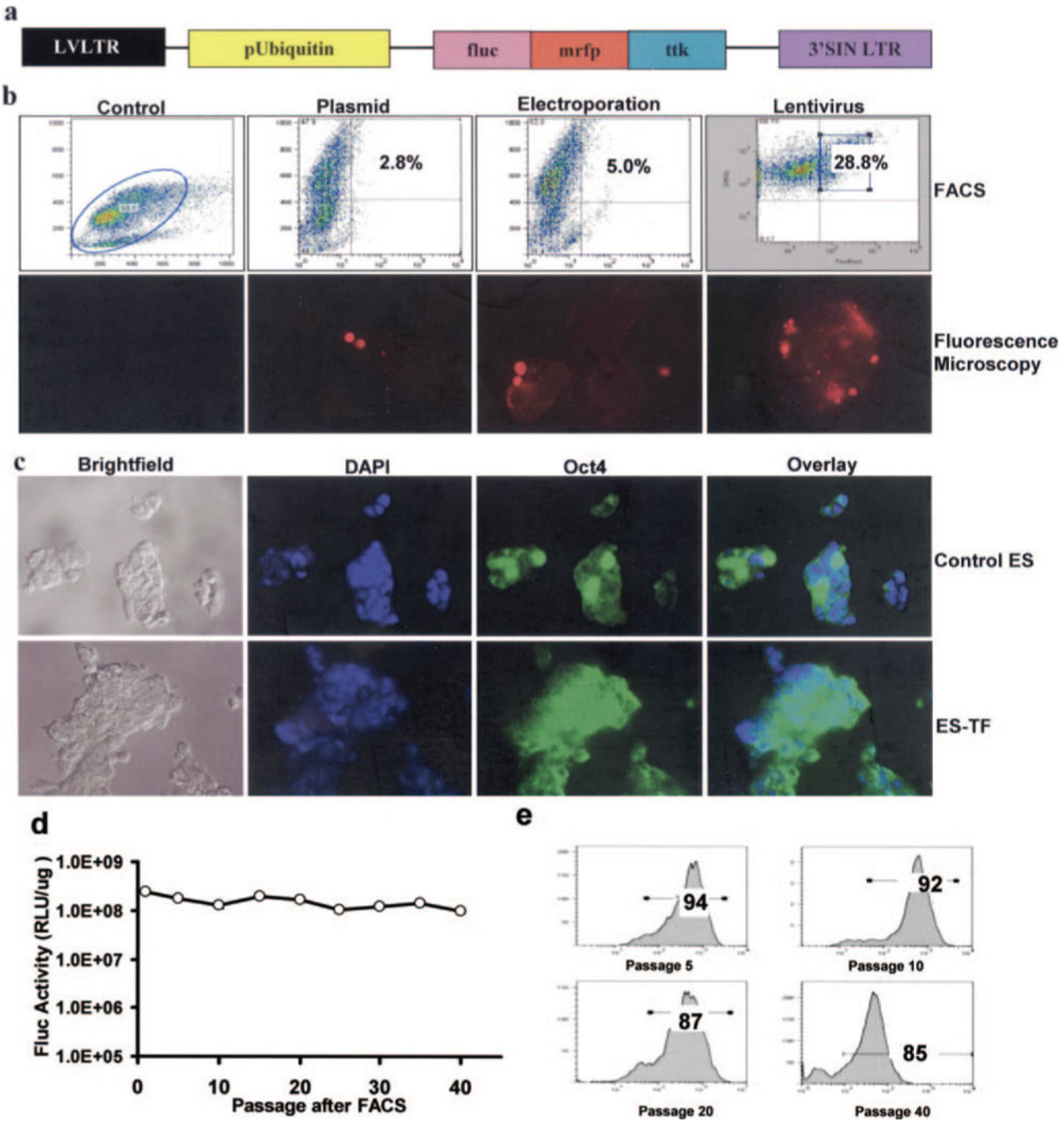

Figure 1.

Stable lentiviral transduction of ES cells with the TF reporter gene. a, Schema of the TF reporter gene containing fusion of fluc-mrfp-ttk. The TF reporter gene was cloned into a self-inactivating lentiviral vector downstream from the ubiquitin promoter. The 3 fusion proteins are joined by a 14-amino acid (LENSHASAGYQAST) and 8-amino acid (TAGPGSAT) linker, respectively. b, FACS histograms of ES cells 48 hours after transduction with plasmid lipofectamine, electroporation, and lentivirus carrying the TF reporter gene. c, Control ES and ES-TF cells showed similar morphology and stem cell

marker Oct4 expression on brightfield and fluorescence microscopy, respectively. DAPI staining is used as a nuclear marker. The stability of TF reporter gene expression was measured quantitatively by both (d) fluc enzyme assays and (e) mrfp FACS for 40 passages. LVLTR indicates lentivirus long-terminal repeat.

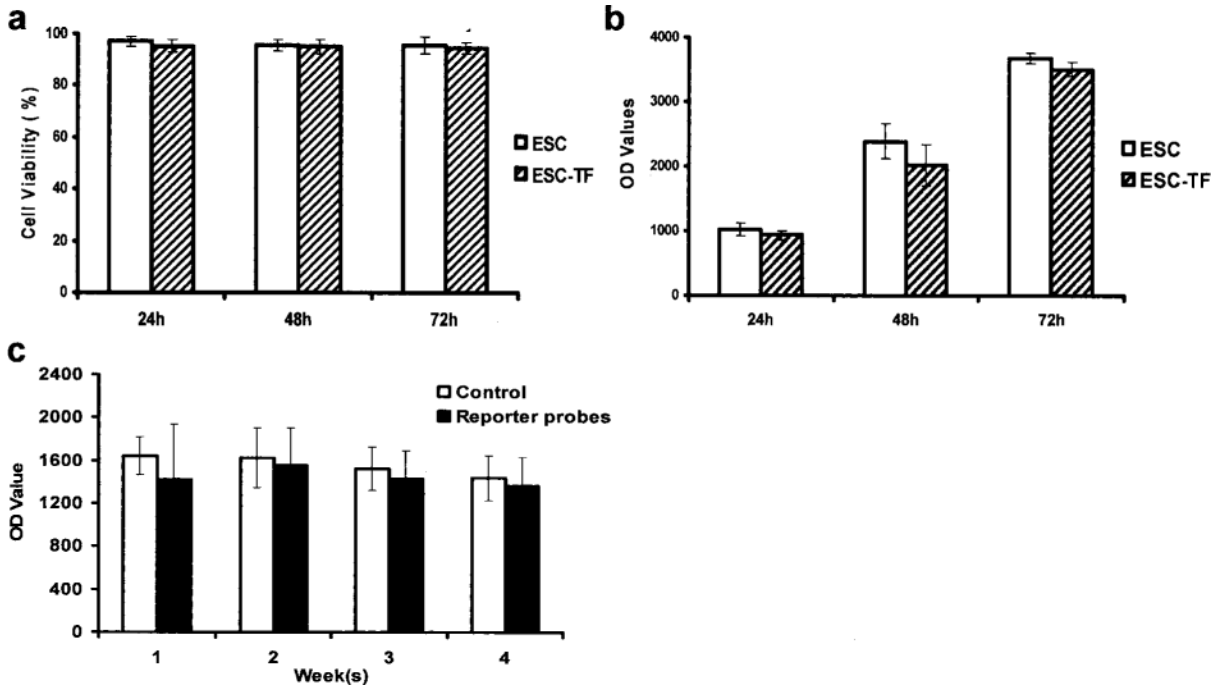

Figure 2.

Effects of reporter genes and reporter probes on cell viability, proliferation, and differentiation. Trypan blue cell viability (a) and CyQuant cell proliferation assay (b) both showed no significant difference between control ES and ES-TF cells at various time points. c, Weekly exposure of ES-TF cells to combined bioluminescence reporter probe D-luciferin (100 μmol/L) and PET reporter probe [^{18}F]-FHBG (100 μCi) had no significant adverse effects on ES-TF cell proliferation in vitro over 1 month.

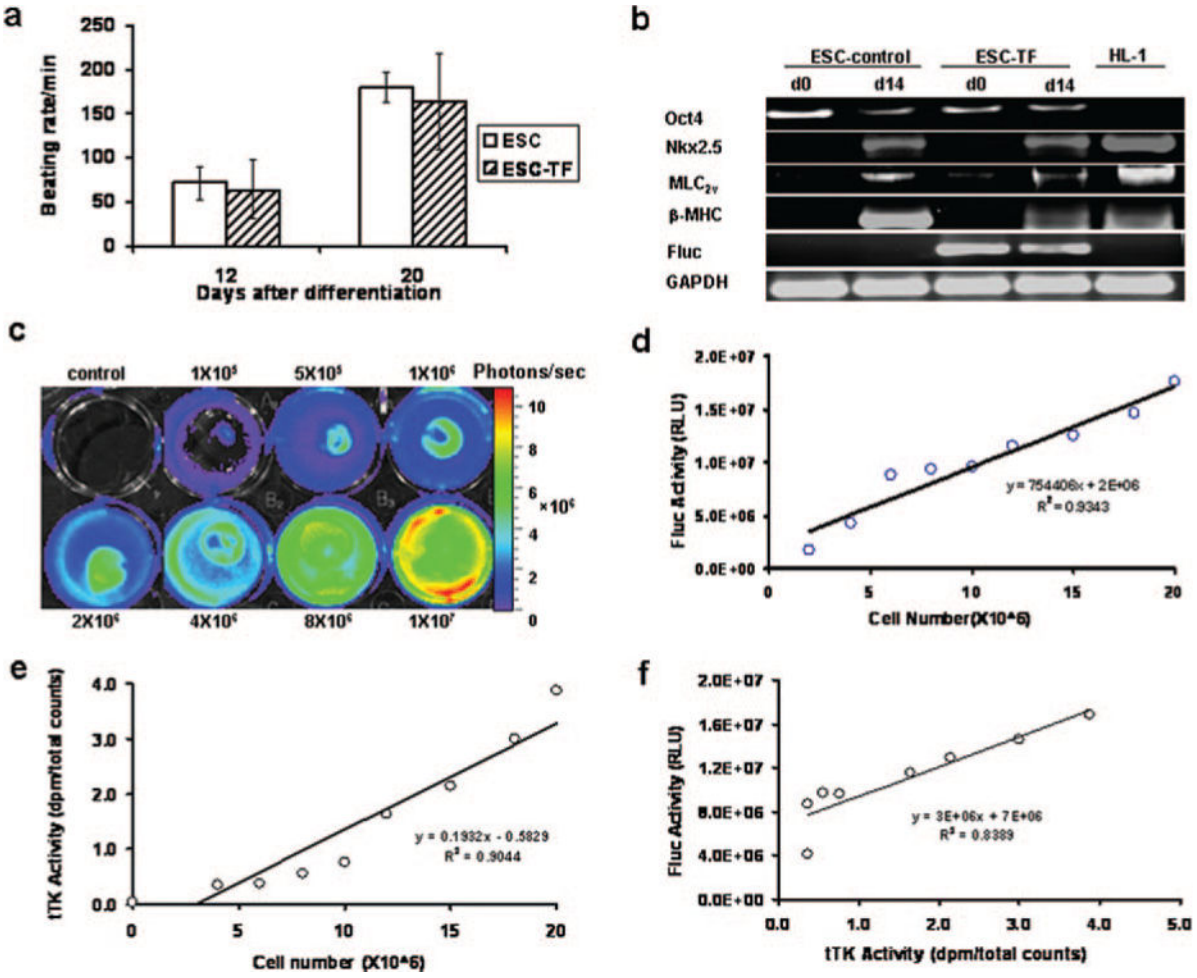

Figure 3.

Effect of TF reporter gene expression on ES cell differentiation. a, Both control ES and ES-TF cells showed similar beating rates per minute at day 12 and day 20 of embryoid body differentiation ($P<0.05$ vs day 12). b, RT-PCR analysis showed the levels of cardiac transcriptional factor (Nkx2.5) and ventricular-specific marker (β-MHC, MLC_{2v}) increased from day 0 to day 14 of embryoid body differentiation, whereas the stem cell markers (Oct4) decreased during the same period. Fluc is present only within ESC-TF cells, as expected. HL-1 is a control mouse cardiomyocyte cell line that is positive for Nkx2.5 and β-MHC but negative for Oct4. GAPDH is a loading control for all cells. c, Bioluminescence imaging of varying numbers of ES-TF cells plated on 24-well plates with increasing signals. P/sec indicates photons $\cdot$ sec^{-1} $\cdot$ cm^{-2} $\cdot$ sr^{-1}. Stably transduced ES-TF cells showed robust correlation with (d) fluc enzyme activity (r^2=0.93) and (e) ttk enzyme activity (r^2=0.90). f, Fluc and ttk activities also correlated well with each other (r^2=0.83). RLU indicates relative light units; dpm, disintegrations per minute.

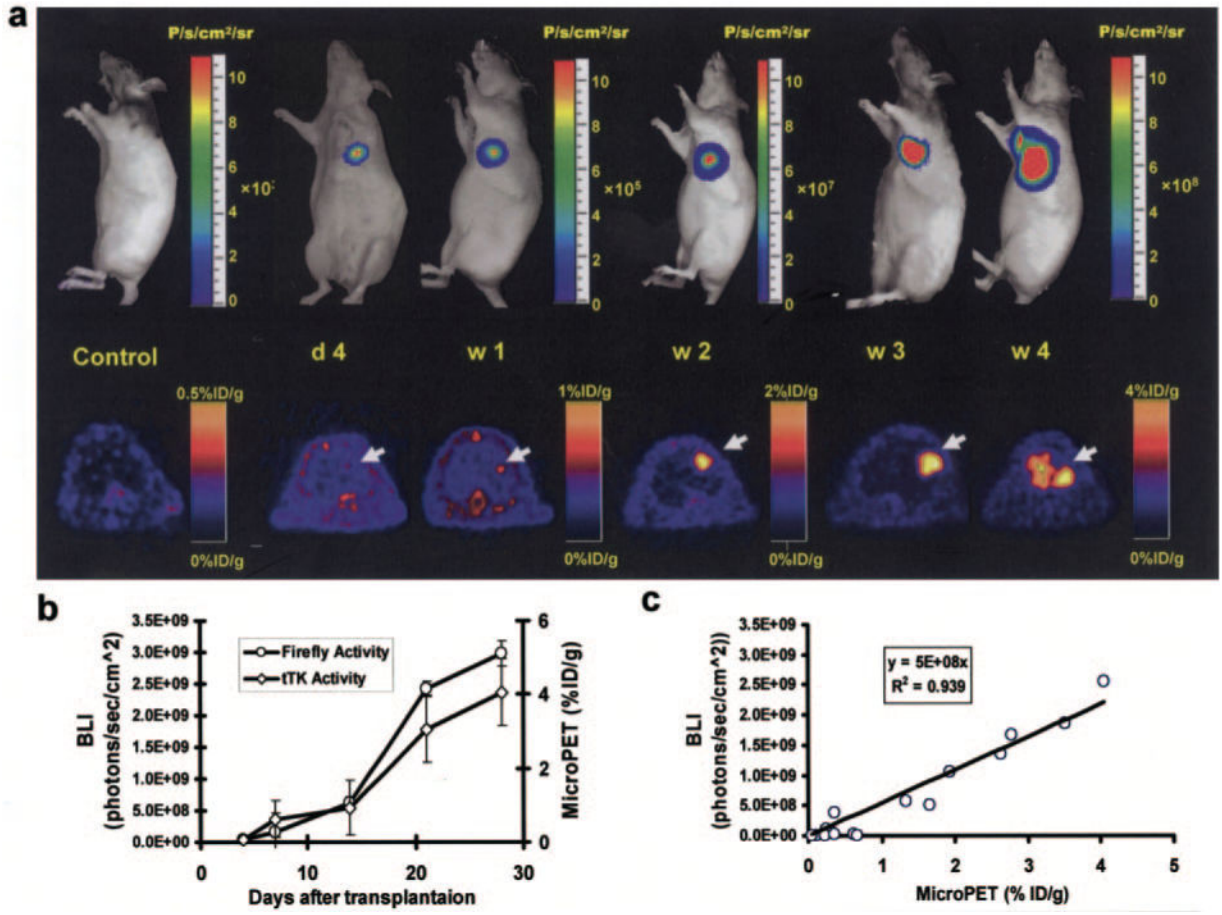

Figure 4.

Molecular imaging of transplanted ES cells with bioluminescence and PET imaging. a, To assess longitudinal cell survival, animals were imaged for 4 weeks. A representative study animal injected with ES-TF cells showed significant bioluminescence (top) and PET (bottom) signals at day 4, week 1, week 2, week 3, and week 4. In contrast, control animals had background activities only. b, Quantification of imaging signals showed a drastic increase of fluc and ttk activities from week 2 to week 4. Extracardiac signals were observed during subsequent weeks. c, Quantification of cell signals showed a robust in vivo correlation between bioluminescence and PET imaging (r^2=0.92). BLI indicates bioluminescence.

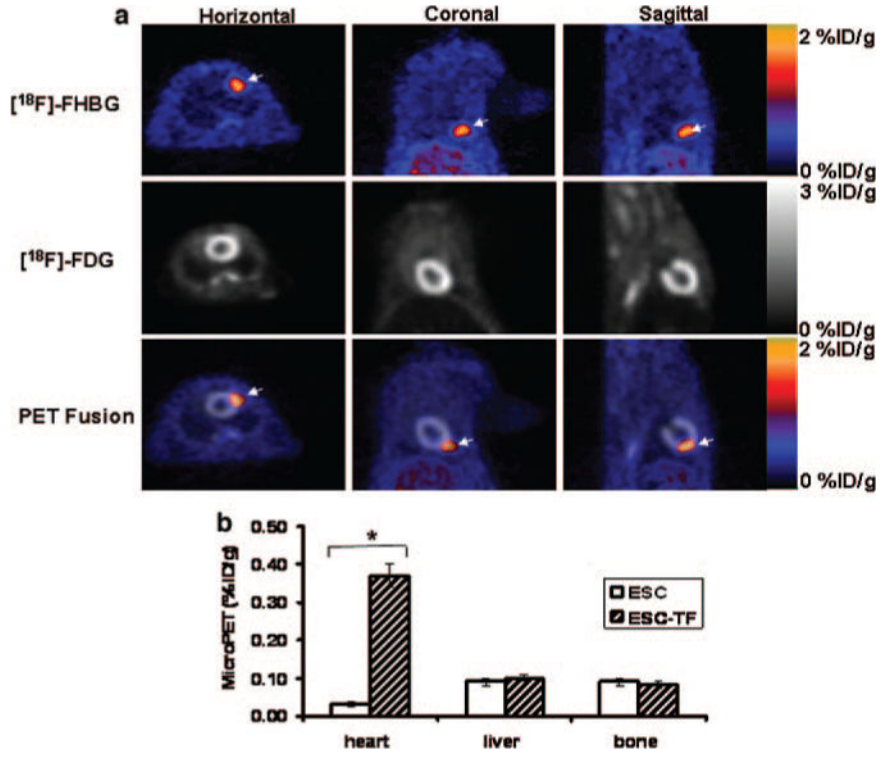

Figure 5.

Defining the tomographic location of transplanted ES cells in the myocardium. a, Two weeks after cell transplantation, animals underwent [^{18}F]-FHBG reporter probe imaging (top row) followed by [^{18}F]-FDG myocardial viability imaging (middle row). Fusion of [^{18}F]-FHBG and [^{18}F]-FDG images (bottom row) shows the exact anatomic location of transplanted ES-TF cells (arrow) at the anterolateral wall in horizontal, coronal, and sagittal views. b, The cardiac [^{18}F]-FHBG activities between control ES and ES-TF animals were 0.03±0.01% ID/g and 0.37±0.05% ID/g, respectively (*P<0.05). Notice that for the [^{18}F]-FHBG image, there is residual activity in the liver and bone due to hepatic clearance of tracer and free fluoride in systemic circulation.

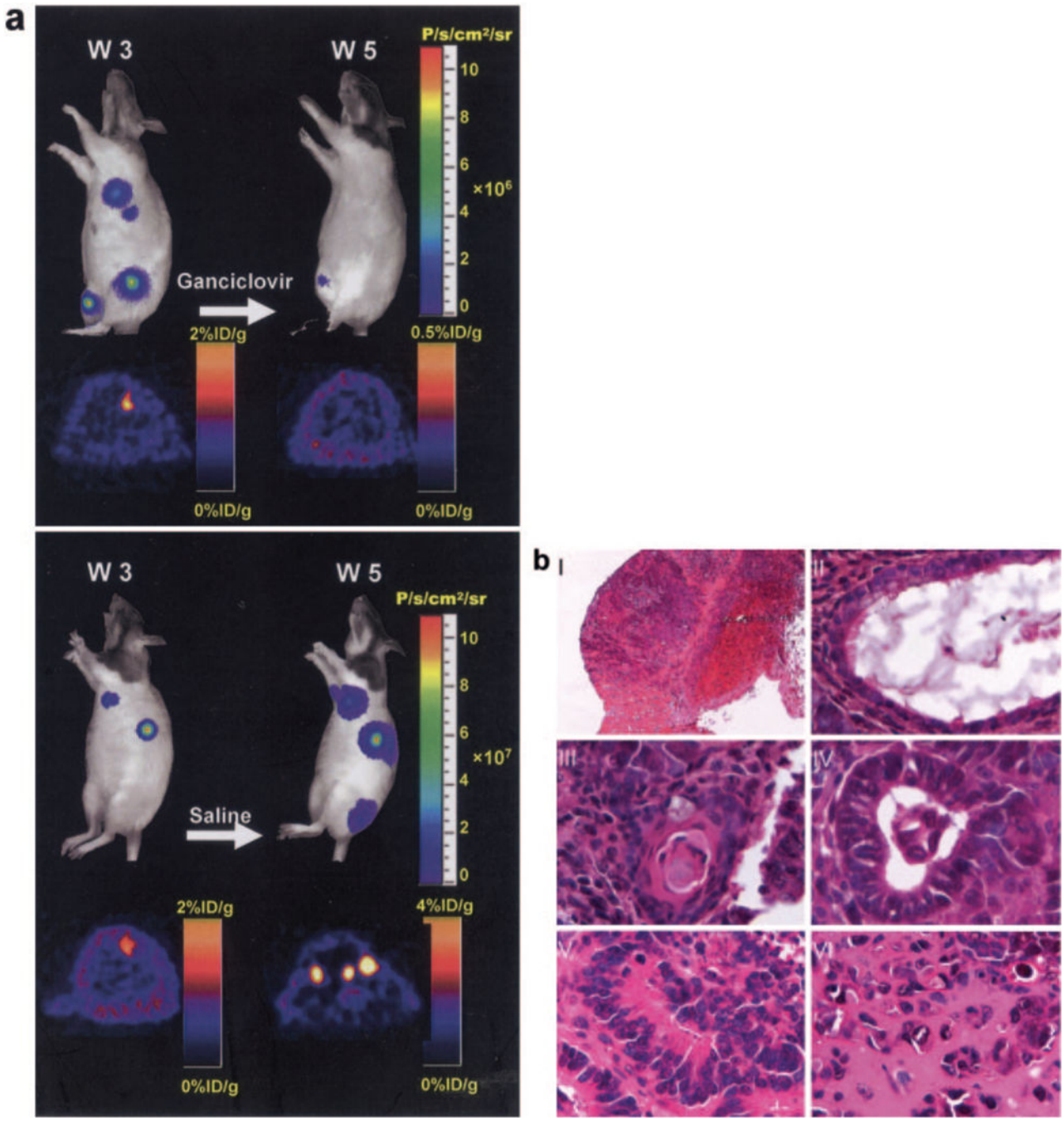

Figure 6.

Ablation of teratoma formation with ttk as both a reporter gene and a suicide gene. a, Treatment of control animals with saline resulted in multiple teratoma formation by week 5. In contrast, study animals treated with ganciclovir for 2 weeks showed abrogation of both bioluminescence and PET imaging signals. b, Postmortem histological analysis of a representative explanted heart showed (I) anterior wall of the myocardium injected with cells surrounding area of hemorrhage (40×); (II) respiratory epithelium with ciliated columnar and mucin-producing goblet cells (1000×); (III) squamous cell differentiation with

keratin pearl; (IV) nonciliated columnar gland (1000×); (V) rosette consistent with neuroectodermal differentiation (1000×); and (VI) osteoid (nonmineralized bone) formation (1000× magnification).