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Effects of Epigenetic Modulation on Reporter Gene Expression: Implications for Stem Cell Imaging

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Abstract

Tracking stem cell localization, survival, differentiation, and proliferation following transplantation in living subjects is essential for understanding stem cell biology and physiology. In this study, we investigated the long-term stability of reporter gene expression in an embryonic rat cardiomyoblast cell line and the role of epigenetic modulation on reversing reporter gene silencing. Cells were stably transfected with plasmids carrying cytomegalovirus promoter driving firefly luciferase reporter gene (CMV-*Fluc*) and passaged repeatedly for 3–8 months. Within the highest expressor clone, the firefly luciferase activity decreased progressively from passage-1 (843 ± 28) to passage-20 (250 ± 10) to passage-40 (44 ± 3) to passage-60 (3 ± 1 RLU/ μ g) ($P < 0.05$ vs. passage-1). Firefly luciferase activity was maximally rescued by treatment with 5-azacytidine (DNA methyltransferase inhibitor) compared to trichostatin A (histone deacetylase inhibitor) and retinoic acid (transcriptional activator) ($P < 0.05$). Increasing dosages of 5-azacytidine treatment led to higher levels of firefly luciferase mRNA (RT-PCR) and protein (Western blots) and inversely lower levels of methylation in the CMV promoter (DNA nucleotide sequence). These *in vitro* results were extended to *in vivo* bioluminescence imaging (BLI) of cell transplant in living animals. Cells treated with 5-azacytidine were monitored for 2 weeks compared to 1 week for untreated cells ($P < 0.05$). These findings should have important implications for reporter gene-based imaging of stem cell transplantation.

Keywords

gene silencing; reporter genes; molecular imaging; stem cell transplant

INTRODUCTION

Stem cell therapy offers exciting promises for the treatment of neurodegenerative disorders, cancer, ischemic heart disease, and metabolic defects (1). To fully understand the beneficial effects of stem cell therapy, investigators must be able to track the biology and physiology of transplanted cells in living subjects over time. Traditionally, markers such as green

fluorescent protein (GFP) and β -galactosidase (*LacZ*) have been the mainstays of cell labeling (2). However, identification of these cells by conventional microscopy requires histologic tissue sampling that can be labor intensive. The invasive nature of classical pathology also precludes serial assessment within the same subject. Thus, the recent development of novel molecular imaging techniques for visualizing cell survival and proliferation has attracted much deserved attention (3).

To date, three major imaging modalities have been used to noninvasively track stem cells in living subjects. These include radiolabel, ferromagnetic, and reporter gene labeling. In radiolabel technique, cells are incubated with radioactive probes such as ^{111}In -oxine (^{111}In -oxine) prior to transplantation (4). The main limitation of ^{111}In is its physical half-life of 62.7 hours, so cell distribution can be studied for only 5–7 days. In ferromagnetic labeling, cells can be loaded with superparamagnetic iron oxide (SPIO) particles prior to transplantation (5). However, due to the engulfment of these SPIO particles by surrounding macrophages following cell death, one cannot distinguish viable from non-viable cells. Additionally, the amount of iron particles within stem cells becomes diluted after cellular division, leading to difficulty in accurate quantification of cell signal intensity (6). The cellular dilution problem also applies to the radiolabel strategy based on ^{111}In (4) or other isotopes such as Copper-64 (7).

In reporter gene imaging, stem cells can be genetically engineered to express various reporter genes prior to transplantation. The reporter gene expression could be detected by ultra-sensitive imaging devices such as an optical charged coupled device (CCD), single photon emission computed tomography (SPECT), positron emission tomography (PET), or magnetic resonance imaging (MRI) (8, 9). The conceptual basis of reporter gene imaging is elegantly simple. After transplant, if cells are alive and functional within the host milieu, the reporter genes will be expressed. If the transplanted cells are dead or apoptotic, the reporter genes will be degraded. If the transplanted cells with stably integrated reporter genes divide and proliferate, these reporter genes will be passed on to progeny cells. Thus, reporter gene imaging currently represents a powerful approach to study the physiology and biology of transplanted cells *in vivo*.

Despite the many advantages of molecular imaging, the issue of reporter gene silencing has not been systemically evaluated. Specifically, within each cell type, an interplay of several proteins helps cells coordinate and maintain tissue specific patterns of gene expression, endogenous or exogenous. For example, DNA methylation and histone deacetylation have been shown to play important roles in mammalian development (10), tumor transformation (11), and stem cell differentiation (12). In a classic study by Makino *et al.* treatment of murine bone marrow stromal cells with 5-azacytidine led to changes in stromal cells with fibroblast-like morphology into spontaneously beating cardiomyocytes (13). Subsequent studies have shown that bone marrow stromal cells can be differentiated into hepatocytes (14) or neuronal cells (15) after exposure to 5-azacytidine.

DNA methylation is mediated by a class of enzymes called DNA methyltransferases (DNMTs) that covalently link a methyl group to the cytosine residue within 5'-CpG-3' islands at the promoter region (16). Following DNA methylation, a separate group of proteins containing a methylcytosine-binding domain (MBD) is recruited and bound to these methylated CpG sites, which then block the access of transcription factors that typically bind to the promoter. MBD proteins also recruit histone deacetylase enzymes (HDACs), which catalyze the removal of acetyl groups from the ϵ -amino groups of specific lysine residues, and cause a tighter packing of DNA. The end result is a condensed chromatin that further decreases the access of transcription factors to their promoter binding sites, eventually resulting in gene silencing (10).

In this study, we hypothesize that reporter gene silencing due to DNA methylation and histone deacetylation could affect *in vivo* cellular and molecular imaging. To test this model, we first created several stable clones of rat H9c2 embryonic cardiomyoblasts that express a firefly luciferase reporter gene. We next evaluated the extent of reporter gene silencing in the same clones for 3–8 months. We then assessed the ability of a methylation inhibitor (5-azacytidine), a histone deacetylation inhibitor (trichostatin A), or a transcriptional activator (retinoic acid) to rescue the silenced gene. Finally, we tracked the longitudinal survival of these treated cells within the skeletal muscles of living rodents using *in vivo* bioluminescence imaging.

METHODS

Cell culture

The rat H9c2 embryonic cardiomyoblast cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM medium (Invitrogen, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS), 10 units/ml penicillin, and 10 µg/ml streptomycin. Although the H9c2 cell line was derived originally from embryonic rat myocardium, its morphologic, biochemical, and electrophysiologic properties more closely resemble those of a skeletal muscle cell line (17).

Cloning and transfection

Firefly luciferase cDNA was inserted into plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) using *Nhe*I and *Xho*I restriction enzyme sites. The final construct contained the human cytomegalovirus promoter driving firefly luciferase and an SV40 promoter driving the aminoglycoside phosphotransferase (*Neo*) cDNA, followed by an SV40 poly-A fragment (pCMV-*Fluc*-SV40-*neo*). H9c2 cells were stably transfected using the Superfect protocol (Invitrogen, Carlsbad, CA). Cells were plated at a density of 1×10^3 cells/cm² with G418 sulfate (neomycin; 0.5 mg/ml). Colonies resistant to G418 were selected and subjected to further subcloning. Five clones that stably expressed *Fluc* were identified and designated as H9c2-*Fluc*.1, H9c2-*Fluc*.2, H9c2-*Fluc*.3, H9c2-*Fluc*.4, and H9c2-*Fluc*.5. Henceforth, the firefly luciferase gene and enzyme are referred to as *Fluc* and FL, respectively.

Treatment with 5-azacytidine (5-Aza), Trichostatin A (TSA), and Retinoic acid (RA)

All H9c2-*Fluc* clones were passaged for 3 months. The highest expressor clone (H9c2-*Fluc*.3) was passaged for an additional 5 months to evaluate the full extent of reporter gene silencing. The H9c2-*Fluc*.3 cells were grown in 100 mm plates. After a 24 hour incubation, cells were treated with 5-Aza (Sigma, St. Louis, MO) at different concentrations (1, 5, 10, 20, 50, 100, and 250 µM). After 48 hours, treated cells were trypsinized and lysed with the Passive Lysis Buffer (Promega, Madison, WI). FL activity was assayed using 20 µL of the supernatant with 100 µL of Luciferase Assay Reagent (Promega, Madison, WI). Protein content was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). A luminometer (Turner TD-20/20, Sunnyvale, CA) was used to measure total light emission. Samples were performed in quadruplicates. Results were expressed as relative light unit normalized to milligram of protein (RLU/µg). The same assay conditions were also performed for TSA (dosages 10, 20, 50, 100, 200, 400, and 800 nM) and RA (1, 5, 10, 20, 50, 100, and 250 µM).

Cell proliferation and cell viability after drug treatment

H9c2-*Fluc*.3 cells were distributed uniformly in 96-well plates at a density of 10,000 cells per well. Cells were treated with 5-Aza at 1, 5, 10, 20, 50, 100, and 250 µM. For each concentration, 8 samples were assayed and the highest and lowest values were discarded.

The CyQuant cell proliferation assay (Molecular Probes, Eugene, OR) was used according to the manufacturer's protocol. After 24, 48, and 72 hours of treatment, the density of surviving cells was measured using a microplate spectrofluorometer (Gemini EM, Sunnyvale, CA). The same assay conditions were performed for TSA (dosages 10, 20, 50, 100, 200, 400, and 800 nM) and RA (1, 5, 10, 20, 50, 100, and 250 μ M). The Live/Dead kit (Molecular Probes, Eugene, OR) was also used to assay for viability/cytotoxicity for all three drug dosages.

Western blot analysis

H9c2-*Fluc*.3 cells were washed twice in phosphate buffered saline (PBS) and lysed mechanically in a buffer containing 10 mM Tris•HCl (pH 8.0), 1 mM EDTA, 1 mM DTT with 20% glycerol, and 0.1 mM PMSF. The samples were centrifuged at 4°C and $9,300 \times g$ for 5 min. Protein was quantified and 10 μ g from each sample was mixed with two volumes of sample buffer and boiled for 5 min. Denatured samples were resolved in a 12% acrylamide gel and transferred to poly(vinylidene difluoride) membrane by using a Hoefer semi-dry blotting apparatus. The membrane was immediately transferred to PBS containing 3% milk powder and blocked for 3 h with proper mixing. The membrane was incubated with a primary polyclonal anti-*Fluc* antibody (Promega, Madison, WI) overnight at room temperature with proper shaking. The washed membrane was incubated for 1 h with donkey anti-mouse IgG-HRP conjugate for 1 h. Immunochemical detection was carried out by using the substrates from the Amersham ECL kit.

RT-PCR analysis

Total RNA was prepared from 5-Aza-treated H9c2-*Fluc*.3 cells using a Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To prepare first strand cDNA, 1 μ g of total RNA was incubated in 20 μ l of the reaction mix containing 2 μ l of first strand buffer (10 $\times$), 1 μ l dNTP mix (10 mM each), 2 μ l 100 mM DTT, 4 μ l of MgCl₂ (25mM), 1 μ l Superscript II Reverse transcriptase (50 U) (Invitrogen, Carlsbad, CA) and 5 μ l random primers (50 ng/ μ l) (Invitrogen, Carlsbad, CA) at 42°C for 1 hour. The reaction was terminated by incubating at 70°C for 15 minutes and chilled immediately on ice. RNase H was added and incubated for 20 minutes at 37°C before proceeding to amplification of target cDNA gene products. The cDNA was amplified with primers specific for firefly luciferase or α -tubulin. The amplification reactions were carried out in a solution containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM Magnesium acetate, 1 unit Triple Master Taq DNA polymerase (Eppendorf-Brinkmann Instruments, Inc, NY), 200 μ M dNTPs, and 100 pmol of forward and reverse primers in 50 μ l reaction volume. The cyclic conditions were as follows: 94°C for 30sec, 56°C for 30 sec, 72°C for 45 sec for 30 times and a final extension step at 72°C for 5 min in a DNA Engine Thermal Cycler (MJ Research, Waltham, MA). The primer sequences were as follows: firefly luciferase forward primer, 5'-GCAGCTAGCGCCACCATGGAAGAC-3'; firefly luciferase reverse primer, 5'-ACCGGCGTCATCGTCGGAAGACC-3'; α -tubulin forward primer, 5'-AGAGATCAACCAATGCCTGCT-3'; α -tubulin reverse primer, 5'-ACTGGATGGTACGCT TGGTC-3'. All amplification products were subjected to 1% agarose gel electrophoresis in TBE buffer. The resulting bands were quantified by using Labworks 4.6 Image Acquisition and analysis software (UVP Bio-imaging systems, Upland, CA).

Quantification of DNA methylation sites on CMV promoter

Genomic DNA was prepared by using DNazol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 10 μ g of high molecular weight genomic DNA was treated with an EZ DNA methylation kit as per the manufacturer's protocol (ZYMO Research Orange CA). Afterwards, 100 ng of sodium bisulfite modified DNA was used to amplify 152 bp PCR product of the CMV promoter sequence (588 bp total). The

PCR reaction was performed with 100 pmol of each forward and reverse primers (5'-GGGATTTTAAAGTTTTATTATTGA-3' and 5'-AACTCTACTTATATAAACCTCCCACC-3') (position on CMV promoter sequence 437–463 and 562–588), 2.5 mM dNTPs, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM magnesium acetate, and 1 unit Triple Master Taq DNA polymerase in 50 µl volume (Eppendorf-Brinkmann Instruments, Inc, NY). After initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 5 min program was performed. The PCR products were purified by a Qiagen PCR purification kit (Qiagen, Valencia, CA). Nucleotide sequencing was performed with an ABI 3100 automated sequencer. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines to uracils (which are replicated as thymines after 2 rounds of PCR amplification), while methylated cytosines are not affected by this chemical process and therefore are replicated as cytosines during PCR. The ratio of the cytosine peaks to the sum of the cytosine and thymine peaks (C/T+C) at the original location of CpG dinucleotides determined the degree of DNA methylation. Final data were expressed as percentage of DNA methylation at each of the 8 CpG sites (ratio of C/T+C).

Bioluminescence imaging (BLI) in cell cultures and living animals

Firefly luciferase enzyme activities within the five H9c2-*Fluc* clones were confirmed using the Xenogen IVIS 200 system (Alameda, CA). The five H9c2-*Fluc* clones (1×10^6 cells in each 35-mm well) were incubated each with 20 µl of D-Luciferin (15 mg/ml) and signal activities were quantified using the Xenogen Living Image software. For *in vivo* imaging, H9c2-*Fluc*.3 cells were implanted into skeletal muscles of male Sprague Dawley rats (n = 10). Within each animal, the left thigh was injected with 1×10^6 of treated H9c2-*Fluc*.3 cells (50 µM of 5-Aza for 48 hours) at passage 60 (study group), the right thigh was injected with 1×10^6 of untreated H9c2-*Fluc*.3 cells at passage 60 (positive control), and the right arm was injected with 1×10^6 of control H9c2 cells (negative control). Note this experimental design allows the same animal to serve as both the study and control groups and avoids inter-subject variability as a confounding factor. The same animals were imaged repetitively from 6 hour to 2 weeks after transplant. The animals were first placed supine in a light-tight chamber, and a gray scale reference image was obtained under low-level illumination. BLI was performed after intraperitoneal injection of the reporter substrate D-Luciferin (375 mg/kg body weight) as previously described (18). Signal intensities from region of interest (ROI) were drawn over skeletal muscles. Data from each image were analyzed using both maximum and mean photons/second/square centimeter/steradian (photons/s/cm²/sr). We found however that the mean ROI values can vary for any given region depending on the total number of pixels selected over the skeletal muscles. Therefore, all of our results are expressed as maximum photons/s/cm²/sr to avoid any confounding factor due to pixel variation from one image to another. The gray-scale photographic images were superimposed onto color images using the LivingImage software overlay (Xenogen Corp., Alameda, CA) and Igor image analysis software (Wavemetrics, Lake Oswego, OR). Final annotations were added using another graphics software package (Photoshop, Mountain View, CA).

Statistics

All results are expressed as mean $\pm$ standard deviation. The Student's *t* test was used and *P* values of <0.05 were considered to indicate significant differences between two groups.

RESULTS

Silencing of *Fluc* expression after repeated cell passages

After transfecting the rat H9c2 embryonic cardiomyoblast cell line with the pCMV-*Fluc*-SV40-*neo* plasmid, single clones resistant to G418 were selected. Brightfield microscopy showed no difference in gross morphology of transfected H9c2-*Fluc* cells compared to untransfected H9c2 cells (Figure 1). Clone number 3, denoted as H9c2-*Fluc*.3, had the strongest signal as determined by BLI on 6-well tissue culture plates. The cell proliferation assay showed no significant difference at 24, 48, and 72 hours between control H9c2 (847 ± 70) and H9c2-*Fluc*.3 (892 ± 32 fluorescence units) clones ($P=NS$). To assess the stability of *Fluc* transgene expression, all 5 clones were passaged serially. After 3 months, the average *Fluc* activity at passage-25 (123 ± 35) was only $28 \pm 7\%$ compared to passage-1 (647 ± 155 RLU/ μ g) for all 5 clones. Clone H9c2-*Fluc*.3 was followed for an additional five months. The FL enzyme activity for this clone decreased significantly from passage-1 (843 ± 28) to passage-20 (250 ± 10) to passage-40 (44 ± 3) to passage-60 (3 ± 1) ($P < 0.05$ vs. Passage-1). At 8 months, the H9c2-*Fluc*.3 activity at passage 60 (3 ± 1) was only 0.01% compared to passage-1 (843 ± 28 RLU/ μ g). Finally, H9c2-*Fluc*.3 cells that have been stored at -80°C at earlier passages resume the same rate of *Fluc* expression loss upon re-plating on cell culture dishes (data not shown).

Rescue of *Fluc* expression by 5-Aza, TSA, and RA

We hypothesize that multiple epigenetic pathways may be involved in silencing the *Fluc* reporter gene activity. Accordingly, we compared the effects of treating the H9c2-*Fluc*.3 clone at passage-60 with 5-Aza (DNA methyltransferase inhibitor), TSA (histone deacetylase inhibitor), and RA (transcriptional activator). Among these three agents, 5-Aza induced the highest level of FL activity (Figure 2A). At $50 \mu\text{M}$ of 5-Aza, the FL activity was 24-fold higher compared to untreated H9c2-*Fluc*.3 cells (73 ± 2 vs. 3 ± 1 RLU/ μ g protein). Treatment with TSA also showed remarkable enhancement of FL activity with increasing drug dosages ($P < 0.05$ for 200 – 800 nM vs. control). Interestingly, after a 48 hour exposure to 5-Aza or TSA, the FL activity in H9c2-*Fluc*.3 cells increased initially but decreased gradually to baseline levels over the next 5–10 passages (data not shown). Likewise, continuous exposure to 5-Aza or TSA rescued FL activity but was not able to maintain it at the highest levels for more than 5–10 passages. Finally, RA had no significant induction of FL activity ($P=NS$).

Effects of 5-Aza, TSA, and RA on cell proliferation and viability

To ensure that the above drug treatment regimen did not adversely affect normal cellular physiology, we carefully examined the clones for changes in both cell proliferation and viability. Using the CyQuant fluorescent dye which binds to nucleic acids as a marker of cell proliferation, we define a proliferation rate of less than 80% of control untreated H9c2-*Fluc* cells as having a negative effect on cellular physiology. This cutoff was purposely set to be more stringent than the standard IC₅₀ (inhibitory concentration 50%). Figure 2B shows the relative ranges of 5-Aza, TSA, and RA dosages that can induce *Fluc* gene expression without affecting H9c2 proliferation. These dosages were $50 \mu\text{M}$ for 5-Aza, 50 nM for TSA, and 10 nM for RA. Figure 3 shows that cells treated with the triple drug combination had higher induction of FL than any single agent alone (567 ± 42 RLU/ μ g with $50 \mu\text{M}$ of 5-Aza, 200 nM of TSA, and $20 \mu\text{M}$ of RA) ($P < 0.05$). This suggests a synergistic or additive effect among these agents but at the expense of depressed cell proliferation rate ($39 \pm 6\%$ of control). For the triple drug treatment, the 80% cell proliferation threshold is $5 \mu\text{M}$ of 5-Aza, 20 nM of TSA, and $3 \mu\text{M}$ of RA, which yielded 81 ± 2 RLU/ μ g instead. Finally, the Live/Dead assay, which uses a two-color fluorescence to measure both intracellular esterase

activity and plasma membrane integrity, was used to assess cell viability. The results showed similar patterns compared to the CyQuant cell proliferation assay (data not shown).

Dissecting the molecular mechanisms of CMV driven *Fluc* gene silencing

To assess if the loss of *Fluc* activity was due to excessive DNA methylation of the CMV promoter, which can prevent binding of transcriptional factors, we treated H9c2-*Fluc*.3 cells at passage 60 with increasing concentrations of the DNA methyltransferase inhibitor 5-Aza for 48 hours. Afterwards, cell lysates were subjected to the FL enzyme assay, Western blot of FL protein (Figure 4A), RT-PCR of *Fluc* mRNA (Figure 4B), and bisulfite genomic sequencing of the CMV promoter to assess methylation changes at CpG dinucleotides (Figure 4C). Increasing dosages of 5-Aza treatment led to increased levels of *Fluc* mRNA and FL protein as expected. In contrast, 5-Aza treatment led to a reduction in the degree of methylation at the 8 CpG sites examined within the CMV promoter (i.e., more thymines detected on DNA sequence after bisulfite treatment). These data suggest that 5-Aza acts by inhibiting DNA methyltransferase enzyme, which leads to more unmethylated CpG sites to allow better access of transcriptional factors to the CMV promoter, resulting in higher FL mRNA, protein, and enzyme activity.

Bioluminescence imaging of H9c2 cells transplanted into skeletal muscles of rats

To demonstrate that the reversal of reporter gene silencing can be maintained *in vivo*, one million H9c2-*Fluc*.3 cells were treated with 50 μ M of 5-Aza for 48 hours and then implanted into the left thigh of Sprague Dawley rats (n=10). The right thigh was injected with one million untreated H9c2-*Fluc* cells as control. Animals were imaged repetitively using D-Luciferin as the reporter probe starting at 6 hours after transplant (Figure 5A). On day 1, the bioluminescence signal for treated cells was significantly higher compared to untreated cells ($5.6 \times 10^6 \pm 8.5 \times 10^5$ versus $9.7 \times 10^4 \pm 2.2 \times 10^4$ photons/sec/cm²/sr; $P < 0.05$). After 8 days, untreated cells implanted at the right thigh could not be readily distinguished from the background signal (~ 7000 photons/sec/cm²/sr). By contrast, treated cells implanted at the left thigh showed visible signal for up to 14 days ($1.1 \times 10^4 \pm 5.8 \times 10^3$ photons/sec/cm²/sr). Non-reporter transfected H9c2 cells (negative control) were also injected into the right arm and showed no signal as expected. Because these animals were not immunosuppressed, there was gradual donor cell death within the first 2 weeks after cell transplantation in both legs (Figure 5B). Postmortem histological analysis of both legs at 4 weeks did not identify any remaining cells (data not shown).

DISCUSSION

This study examines the role of epigenetic modulation on reporter gene silencing used for noninvasive molecular imaging of cell transplantation in living subjects. Our major findings are as follows: (1) rat H9c2 embryonic cardiomyoblasts stably transfected with *Fluc* gradually lost their transgene expression over a span of 8 months; (2) the silenced gene expression could be reversed most impressively by a DNA methyltransferase inhibitor (5-azacytidine; 5-Aza) and a histone deacetylase inhibitor (trichostatin A; TSA), and minimally with a transcriptional activator (retinoic acid; RA); (3) the molecular mechanism of DNA methylation was further validated by DNA methylation studies as well as RT-PCR, Western, and enzyme assays; finally, (4) noninvasive bioluminescence imaging of living rats confirmed that H9c2-*Fluc* cells treated with 5-Aza had significantly higher signal activity compared to untreated H9c2-*Fluc* cells over a span of 2 weeks. Taken together, the data suggest that cellular control of exogenous transgene expression by epigenetic modulation can be reversed *in vitro* and extended to *in vivo* imaging.

Encouragingly, our results are concordant with other studies that have shown gene silencing in neural progenitor cell lines carrying CMV promoter driving green fluorescent protein (GFP) (19) and in adenovirus expressing CMV driven β -galactosidase (LacZ) (20). Although the CMV promoter is a robust expression cassette, it is susceptible to transcriptional inactivation due to several mechanisms, including DNA methylation and histone deacetylation as shown here (21, 22). This was also confirmed in a recent study involving human neural stem (HB1.F3) cells stably transfected with CMV promoter driving human sodium/iodide symporter (hNIS) (23). However, the main limitation of that study is its short duration of analysis. The hNIS transgene activity was assayed for only 8 passages and imaging was performed for only one time point at 3 hours after cell transplantation. In contrast, we analyzed reporter gene silencing for over 60 passages and noninvasively imaged cell survival for 2 weeks. At present, the firefly luciferase reporter gene is one of the most commonly used reporter genes for *in vivo* imaging. Unlike the semi-quantitative nature of GFP, LacZ, and hNIS, the FL activity is highly quantitative (linear range up to 8 orders of magnitude), which allowed us to precisely measure FL activity loss over time (18).

DNA methylation and histone deacetylation are well documented to play critical roles in regulating endogenous gene expression during mammalian development, genomic imprinting, and tumorigenesis (10). For example, many primary neoplasms have abnormal hypermethylation of tumor suppressor genes, which are needed to check against proto-oncogenes (24, 25). Because epigenetic processes are potentially reversible, pharmacologic inhibitors of DNA methylation provide a conceptually attractive and rational approach for rescuing the functions of tumor suppressor genes that are abnormally silenced by hypermethylation (26). The best characterized drug to reactivate silenced genes is 5-Aza, which was originally developed as a nucleoside anti-metabolite for acute myelogenous leukemia (27). Biochemically, 5-Aza is a cytosine analogue that incorporates into newly synthesized DNA in place of deoxycytidine and forms a covalent links with DNMT (28). This interaction leads to progressive depletion of functional DNMTs in the cell, resulting in profound hypomethylation after several rounds of DNA replication. TSA is a fungal antibiotic that inhibits histone deacetylase (HDAC) enzymes. This causes a relaxation of the tight supercoiling of chromatin that enhances accessibility of DNA-binding transcriptional regulatory proteins to promoter regions (29). Therefore, histone acetylation is required for DNA opening and active transcription, whereas histone deacetylation causes gene silencing.

Current regimen of drugs such as 5-Aza and TSA are considered too unstable and toxic for oral usage. Newer demethylation agent such as 1-(β -D-ribofuranosyl)-1,2-dihydropyrimidin-2-one (zebularine) has recently been shown to be more chemically stable for *in vitro* cell culture and *in vivo* animal study (30). However, as a group, demethylation agents are known to exert a broad and profound impact on cellular traits, including proliferation, apoptosis, and differentiation (10–12). Thus, their usage for animal imaging is still limited unless these agents can be designed to specifically target the reversal of a given reporter gene without affecting other genes. In our study, H9c2-*Fluc* cells treated with 5-Aza could be followed for ~2 weeks. Although we can not completely rule out the possibility of *in vivo* gene silencing after transplantation, several factors point to acute donor cell death as the primary culprit. First, the H9c2-*Fluc*.3 we transplanted were at passage 60 and the level of FL had shown relatively stable albeit low activity ranging from 1–3 RLU/ μ g. Second, we could not identify any remaining H9c2 cells at 4 weeks from either thigh by histologic staining. Third, several other investigators have also shown that the majority of transplanted cells die within the first 3–4 weeks due to inflammation, ischemia, or apoptosis (31). Instead of reporter gene imaging, they employed serial TUNEL apoptosis assay, TaqMan PCR, and histology obtained from a large number of animals sacrificed at different time points (32–34). In a recent study in which male donor neonatal myoblasts were transplanted into female host mice, Lee-Pullen *et al.* showed that approximately 80% of cells were lost by 24 hours

and only 2% remained at 3 weeks (35). Indeed, acute donor cell death is the historic reason why myoblast transplantation for treatment of Duchenne muscular dystrophy has not yet succeeded (36).

Molecular imaging is a relatively new field. It combines the disciplines of cell biology, molecular biology, synthetic chemistry, medical physics, and translational sciences into a powerful research paradigm (9). In recent years, the major advances can be attributed to the multitude of reporter genes and reporter probes available and the corresponding development of miniaturized detection devices for small animal applications. Thus, bioluminescence imaging can capture photochemical signals emitted from the interaction of *Fluc* reporter gene and D-Luciferin reporter probe (37); micro PET can register positron annihilation events from the interaction of a herpes simplex virus type 1 mutant thymidine kinase (HSV1-sr39tk) reporter gene and a 9-(4-[¹⁸F]-fluoro-3-hydroxymethylbutyl)guanine ([¹⁸F]-FHBG) reporter probe (38); micro MRI can detect contrast enhancement from the interaction of a transferrin reporter gene and a receptor-conjugated iron oxide nanoparticles (39); and micro SPECT can image the interaction of a sodium iodine symporter reporter gene with sodium ¹²⁵I reporter probes (40). Thus, the vast array of reporter constructs developed and the rapid progress made so far validates the exciting enthusiasm of the molecular imaging field. Except for BLI, all of these imaging techniques have direct human application using available clinical PET, MRI, and SPECT scanners (9). Indeed, clinical PET imaging of thymidine kinase reporter gene expression are now being used for treatment and monitoring of patients with recurrent glioblastoma (41) and hepatocellular carcinomas (42).

In conclusion, molecular imaging of reporter genes will continue to play an increasingly important role for monitoring stem cells noninvasively, repetitively, and quantitatively (37, 43). Because the loss of reporter gene expression poses a difficult challenge for molecular imaging of stem cells, we believe our current *in vitro* and *in vivo* assay system provides a useful experimental platform to study the mechanisms underlying gene silencing. In this study, we validated that DNA methylation is involved in silencing of a *Fluc* reporter gene expressed in a cardiomyoblast cell line. Moreover, this phenomenon could be rescued by using an inhibitor of DNA methyltransferase enzymes (e.g., 5-azacytidine) that removes methyl groups bound to CpG islands, or by an inhibitor of histone deacetylase enzymes (e.g., trichostatin A) that converts chromatin to an open structure that is more accessible for gene transcription. Further studies will be needed to determine if similar processes are involved in other promoters/enhancers and reporter genes as listed above (37–40). Our ongoing efforts focus on using endogenous promoters such as β -actin or ubiquitin to circumvent this issue. The answers to these questions will be particularly relevant as the field of molecular imaging moves forward.

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Figure 1.

Isolation and characterization of H9c2 clones stably expressing firefly luciferase. **A** Brightfield microscopy of control H9c2 (top) versus H9c2-*Fluc* clone (bottom) showed no gross difference in cellular morphology (400x magnification). **B** Emission of bioluminescence signals from H9c2-*Fluc* cells. After plasmid transfection with pCMV-*Fluc*-SV40-*neof* five stable H9c2-*Fluc* colonies were isolated, propagated, and cultured at 1×10^5 cells per well density. Cellular FL enzyme activity was determined by adding the reporter probe D-Luciferin onto the 6-well plates. Bioluminescence imaging signals were acquired using the Xenogen IVIS 200 system. Clone number 3 (H9c2-*Fluc.3*) was found to have the most robust *Fluc* expression. Control H9c2 cells (mock) showed background bioluminescence signal only. **C** Effect of *Fluc* expression on the cellular physiology. The CyQuant assay showed no significant difference between control H9c2 and H9c2-*Fluc.3* cell proliferation rate at 24, 48, and 72 hours as determined by fluorescence spectrophotometer.

Figure 2.

Reversal of reporter gene silencing and toxicity effects of 5-azacytidine, trichostatin A, and retinoic acid. **A** Rescue of gene silencing in H9c2-*Fluc.3* cells at 8 months was performed by treatment with different concentrations of DNA methyltransferase inhibitor 5-Aza (μM), histone deacetylase inhibitor TSA (nM), and transcriptional activator RA (μM). The FL activity was expressed as relative light unit per microgram protein (RLU/μg). **B** Cell proliferation rate of H9c2-*Fluc.3* cells was assayed using a fluorescence spectrophotometer. The same condition was tested for all 3 agents. The cell proliferation rate of untreated H9c2-*Fluc.3* was used as the control (100%). Adverse effect was stringently defined as a proliferation rate below 80% of control. In all cases, increasing dosage of treatment resulted in decreased proliferation rate. Error bar represents the standard deviation of each treatment dosage. * $P < 0.05$ vs. untreated control.

Figure 3.

Synergistic effect of combined drug treatment. H9c2-*Fluc.3* cells treated with increasing dosages of 5-Aza, TSA, and RA showed increased FL induction. However, cell toxicity (<80% cell proliferation) was observed when drug dosages were above 10 μM of 5-Aza, 50 nM of TSA, and 10 μM of RA. Error bar represents the standard deviation of each treatment dosage. * $P < 0.05$ vs. untreated control.

A

B

Figure 4.

Post-transcriptional analysis of firefly luciferase expression. **A** Western blot of control H9c2 cells show absent FL as expected. H9c2-*Fluc*.3 at passage 60 showed progressive increases in FL levels with higher dosages of 5-Aza. The α -tubulin protein was used as the control. **B** RT-PCR of control H9c2 show absent *Fluc* mRNA as expected. H9c2-*Fluc*.3 at passage 60 showed progressive increases in *Fluc* levels with higher dosages of 5-Aza. The α -tubulin mRNA was used as the control. **C** Bisulfite genomic sequencing shows higher percentage of DNA methylation of the CMV promoter for untreated H9c2-*Fluc*.3 cells (0 μ M). Treatment with 5-Aza reduced the percentage of DNA methylation at each of the 8 CpG sites examined (20 and 100 μ M). * P <0.05 vs. untreated control.

A

B **Figure 5.**

Noninvasive imaging of H9c2-*Fluc.3* cells transplanted into the skeletal muscles of living rodents. **A** Optical bioluminescence imaging shows a representative rat transplanted with untreated H9c2-*Fluc.3* cells at the right leg (a) versus 5-Aza treated H9c2-*Fluc.3* cells at the left leg (b). FL signal was detected within the first 6 hours after transplant. Cell survival could be observed up to 14 days for 5-Aza treated cells compared to 8 days for untreated cells. Blank H9c2 cells injected into right arm (c) showed background signal as expected. **B** Cell survival rate after H9c2 transplantation as measured by *in vivo* optical bioluminescence imaging. In both groups, drastic reduction of cell signal intensity was observed within the first 2 weeks of transplant due to acute donor cell death.