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Stem cell therapy for cardiovascular disease : answering basic questions regarding cell behavior

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CHAPTER 3

Spatial and Temporal Kinetics of Teratoma Formation from Murine Embryonic Stem Cell Transplantation

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ABSTRACT

Pluripotent embryonic stem cells (ESCs) have the potential to form teratomas composed of derivatives from all three germ layers in animal models. This tumorigenic potential prevents clinical translation of ESC research. In order to understand the biology and physiology of teratoma formation, we investigated the influence of undifferentiated ESC number, migration, and long-term follow up after transplantation. Murine ESCs were stably transduced with a self-inactivating (SIN) lentiviral vector with a constitutive ubiquitin promoter driving a double fusion (DF) reporter gene that consists of firefly luciferase and enhanced green fluorescent protein (Fluc-eGFP). To assess effects of cell numbers, varying numbers of ES-DF cells (1, 10, 100, 1000, and 10000) were injected subcutaneously into the dorsal regions of adult nude mice. To assess cell migration, 1×10^6 ES-DF cells were injected intramyocardially into adult Sv129 mice and leakage to other extra-cardiac sites was monitored. To assess effects of long-term engraftment, 1×10^4 ES-DF cells were injected intramyocardially into adult nude rats and cell survival response was monitored for 10 months. Our results show that ES-DF cells caused extra-cardiac teratoma in both immunocompetent and immunodeficient hosts; the lowest number of undifferentiated ESCs capable of causing teratoma was 500 to 1000; and long-term engraftment could be visualized for >300 days. Collectively, these results illustrate the potent tumorigenic potential of ESCs that present an enormous obstacle for future clinical studies.

INTRODUCTION

Embryonic stem cells (ESCs) can divide for unlimited generations without losing their ability to differentiate into cells of all three germ layers: ectoderm, mesoderm, and endoderm.¹ This pluripotency has given ESCs a major advantage over adult stem cells, which are generally tissue-specific and whose plasticity is more limited. Therefore, ESCs have great potential as future treatments for a wide variety of diseases, including cardiovascular², neurodegenerative³, and endocrine⁴ disorders. Ironically, the pluripotency and fast growth kinetics of ESCs also underlie their major disadvantage, as these characteristics can easily turn into undefined growth *in vivo*, giving rise to teratoma. Teratoma is a complex tumor consisting of cell lines from different germ layers which are known to develop after ESC transplantation in animal models.^{5,6} Therefore, prior to future clinical translation of ESC-based therapy, basic characteristics of teratoma formation must be elucidated.

Previous studies have shown that the tumorigenic potential of ESCs seems to decrease with increasing purity of pre-differentiated target cell populations, such as cardiomyocytes⁷ or oligodendrocyte progenitor cells.³ However, the maximum number of contaminant undifferentiated ESCs in these pre-differentiated populations for safe non-tumorigenic application remains unknown. Secondly, it has been shown recently that the tumorigenic potential of ESCs is dependent upon the site of transplantation.⁸ Whether it is also possible that transplanted ESCs can form teratoma in distant graft sites need to be examined. Lastly, as teratoma formation usually presents around two to four weeks after transplantation^{2,8}, the long-term fate of transplanted, undifferentiated cells has never been investigated. By studying these issues, this study aims to evaluate the *in vivo* tumorigenic characteristics of ESCs.

METHODS

Culture of undifferentiated ESCs. The murine ES-D3 cell line was derived originally from Sv129 mice and obtained commercially from ATCC (Manassas, VA). Undifferentiated cells were maintained with 1000 IU/mL leukemia inhibitory factor (Chemicon, ESGRO, ESG1107) and murine embryonic fibroblasts (MEFs) feeder layer inactivated by 10 µg/mL mitomycin C (Sigma). Murine ESCs were cultured on 0.1% gelatin-coated plastic dishes in ES medium containing Dulbecco Modified Eagle Medium (DMEM) supplemented with 15% fetal calf serum, 0.1 mmol/L β-mercaptoethanol, 2 mmol/L glutamine, and 0.1 mmol/L nonessential amino acids (Gibco) as described previously.⁹ Culture medium was changed every day. Murine ESCs were passaged every 1 or 2 days. Images were obtained using a ZEISS Axiovert microscopy (Sutter Instrument).

Lentiviral transduction of ESCs with a double fusion (DF) reporter gene. The self-inactivating (SIN) lentiviral vector was constructed by deleting 133 bp in the U3 region of the 3' LTR,

including the TATA box and binding sites for transcription factors Sp1 and NF-kappaB as first described by Miyoshi *et al.*¹⁰ The deletion is transferred to the 5' LTR after reverse transcription and integration in infected cells, resulting in the transcriptional inactivation of the LTR in the proviruses. For the DF construct, pFUG-DF containing Fluc-eGFP was co-transfected into 293T cells with HIV-1 packaging vector ($\delta 8.9$) and vesicular stomatitis virus G glycoprotein-pseudotyped envelop vector (pVSVG) as described.¹¹ Lentivirus supernatant was concentrated by sediment centrifugation using a SW29 rotor at 50,000g for two hours. The concentrated virus was titered on 293T cells. ESCs were transduced at a multiplicity of infection (MOI) of 10. The infectivity was determined by eGFP expression as analyzed on FACScan (Becton Dickinson, San Jose, CA). These ESCs underwent a total of 3 rounds of FACS to increase the percentage of GFP+ to >95%. Note the sorter cannot guarantee 100% positive cells because of the setting, compensation, autofluorescence, and false positives. These stably transduced ESCs (termed ES-DF cells) are then used for subsequent *in vitro* studies and *in vivo* imaging as described next.

Effect of reporter genes on ESC proliferation. The control non-transduced ESCs and stably transduced ESCs (ES-DF cells) were plated uniformly in 96-well plates at a density of 5,000 cells per well. The CyQuant cell proliferation assay (Molecular Probes, Eugene, OR) was measured using a microplate spectrofluorometer (Gemini EM, Sunnyvale, CA) at 24, 48, and 72 hour time points. Eight samples were assayed and averaged.

Intramyocardial transplantation of ESCs into mice and rats. All animal study protocols were approved by the Stanford Animal Research Committee. Adult immunocompetent Sv129 mice (20-25g) and nude athymic rats (200-250g) underwent aseptic lateral thoracotomy. Briefly, animals received isoflurane (2% for mice, 3% for rats) for general anesthesia, banamine (2.5 mg/kg) for pain relief, and normal saline (0.2-2 ml) for volume replacement. Harvested ES-DF cells (after 3 rounds of FACS to increase eGFP+ cells to ~95-98%) were kept on ice for <30 minutes for optimal viability prior to injection into the mouse or rat myocardium. Sv129 mice (n=10) were injected intramyocardially with 1×10^6 ES-DF cells in 50 μ l PBS. Nude athymic rats (n=5) were injected intramyocardially with lower numbers of cells (1×10^4 ES-DF cells in 50 μ l PBS) purposely to allow long-term study. Control animals (n=5 in each group) received non-transduced ESCs in equal numbers. All animals recovered uneventfully and underwent subsequent BLI.

Subcutaneous transplantation of varying numbers of ES-DF cells into mice. Adult nude mice (20-25g) received isoflurane (2%) for general anesthesia. Harvested ES-DF cells were kept on ice for <30 minutes for optimal viability. Mice (n=10) were injected subcutaneously within dorsal regions with 1, 10, 100, 1000 or 10,000 of ES-DF cells accompanied by 99999, 99990, 99900, 99000, and 90000 of irradiated MEFs, respectively, in 20 μ l of Matrigel (BD Biosciences,

San Jose, CA). The final total cell number was 100,000 in each site. Afterwards, animals underwent longitudinal BLI as described below.

Optical bioluminescence imaging (BLI) of mESC transplantation. BLI was performed using the Xenogen *In Vivo* Imaging System (IVIS, Xenogen, Alameda, CA), which is an ultra-sensitive charged coupled device (CCD) camera.¹² Animals received isoflurane (2% for mice, 3% for rats) for general anesthesia. After intraperitoneal injection of the reporter probe D-Luciferin (250 mg/kg body weight), animals were imaged for 30 min using 1-min acquisition intervals repetitively for the study period. The gray-scale photographic images and BLI color images were analyzed using the Igor image analysis software (Wavemetrics, Lake Oswego, OR) and overlaid using the LivingImage software (Xenogen, Alameda, CA). Region of interest (ROI) were drawn over the signals and BLI was quantified in units of maximum photons per second per centimeter square per steradian (p/s/cm²/sr) as described.²

Postmortem immunohistochemical staining. After completion of study periods, animals were sacrificed according to protocols approved by the Stanford Animal Research Committee. Explanted subcutaneous teratomas were routinely processed for hematoxylin and eosin (H&E). Slides were interpreted by an expert pathologist (A.J.C) blinded to the study.

Data analysis. Data are given as mean \pm SD. For statistical analysis, the two-tailed Student's t-test was used. Differences were considered significant at $P < 0.05$.

RESULTS

Lentiviral transduction and characterization of murine ES-DF cells. The DF construct consists of Fluc and eGFP reporter genes linked by a 14-amino acid long linker (LENSHASAGYQAST) as shown in **figure 1a**. After 48 hours of transduction, there was no change in morphology of ESCs (**figure 1b**) and the percentage of eGFP was quantified as 21 ± 4 based on the average of three FACS scans (**figure 1c**). In order to obtain more purified GFP⁽⁺⁾ cells, the cells were passaged 4 to 5 times and sorted three times during a 2 week period. After the third sort, the percentage of ESCs expressing eGFP was typically 95 ± 3 . These stably transduced ESCs (ES-DF) were used for further *in vitro* and *in vivo* analysis. To ensure that the construct and the lentiviral transduction did not favorably influence tumorigenic potential, we examined the growth kinetics of control non-transduced ESCs and ES-DF *in vitro*. Both control ESCs and ES-DF cells showed no significant differences in proliferation rate ($P = NS$) (**figure 1d**). Taken together, these data suggest that lentiviral transduction is a robust method to introduce reporter genes into ESCs without significantly affecting their self-renewal and pluripotency characteristics.

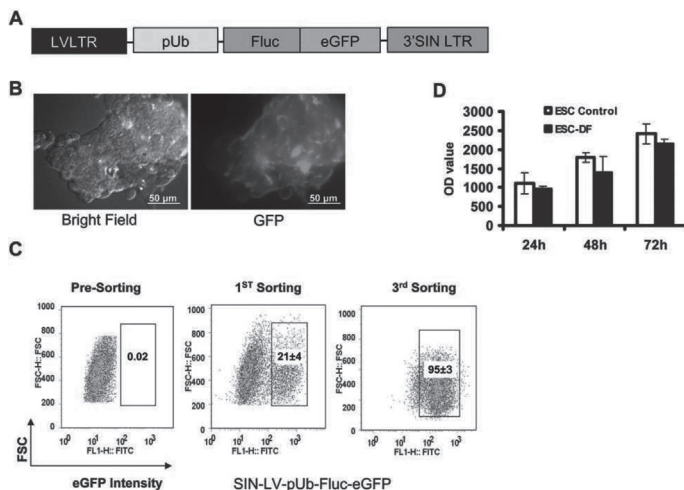


Figure 1. Stable lentiviral transduction of murine embryonic stem cells (ESCs) with a double fusion (DF) reporter gene. (a) Schematic overview of the lentiviral DF reporter construct with firefly luciferase (Fluc) and enhanced green fluorescent protein (eGFP). These reporter genes were cloned into a self-inactivating (SIN) lentiviral vector (LV) driven by an ubiquitin promoter (pUb). (b) Morphology of the transduced ES-DF cells, which clearly showed green fluorescence (left: brightfield, right: fluorescence). (c) Flow cytometry plots showing non-transduced control ESCs do not express GFP (left). After lentiviral transduction, the transduction efficiency is $\sim 21 \pm 4\%$ after the first round (middle) and $\sim 95 \pm 3\%$ after the third round of sorting (right). (d) CyQuant cell proliferation assay showing that lentiviral transduction and DF reporter gene expression did not significantly affect growth of ES-DF cells compared to control non-transduced ESCs.

Minimal number of undifferentiated murine ESCs causing teratoma formation. At present, it is not feasible to select or grow 100% pure ESC-derived progenitor cells.^{13, 14} Thus, it is likely that at the time of therapeutic transplantation, mixed cell populations may still contain some undifferentiated ESCs. Therefore, we set out to determine the lowest threshold of undifferentiated ESC contaminant that can still cause teratoma formation. In order to do so, we first investigated the minimal reproducible cell number that can be imaged *in vitro*. The results from *in vitro* BLI indicated that 10,000 ES-DF cells were easily noticeable (**figure 2a**). Marginal signal from 1000 ES-DF cells led us to hypothesize that it is possible to image as low as 500 to 1000 cells *in vivo* if they are located within a compact region. Indeed, 7 days after subcutaneous transplantation of 500 to 1000 ES-DF cells, which were kept localized to the same region by Matrigel, faint signals were noticeable on BLI *in vivo* (**figure 2b**). Moreover, these 500 to 1000 ES-DF cells induced tumor growth over a time period of 3 months, which was confirmed by histology (**figure 2c**). By contrast, no such tumorigenic transformation was observed after transplantation of 1, 10, or 100 ES-DF cells.

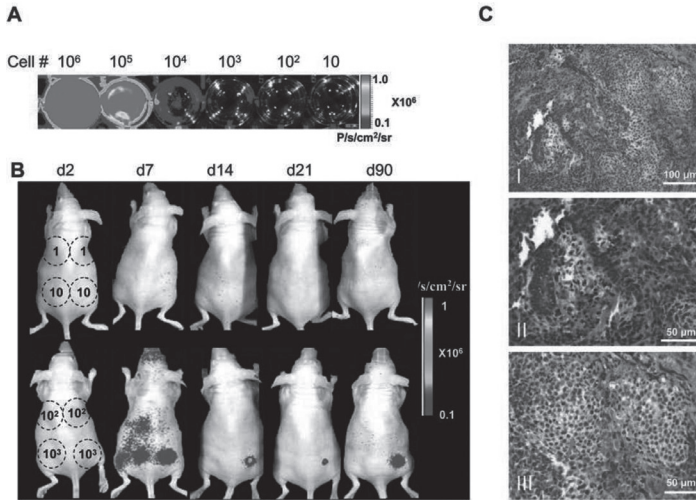


Figure 2. Potential for different numbers of undifferentiated ESCs to form teratomas *in vivo*. (a) *In vitro* BLI shows the ability to image different numbers of ESCs, with a lower threshold of around 1000 ESCs. (b) Different numbers of undifferentiated ESCs, as marked on the picture, were then subcutaneously transplanted into nude mice and accompanied by irradiated non-proliferating mouse embryonic fibroblasts to achieve a total cell number of 100,000 in 20 μ l Matrigel. BLI imaging for 3 months revealed teratoma formation with only 500 to 1000 ESCs. (c) Postmortem H&E staining confirmed teratoma formation in the 500 to 1000 ESCs group: (I) lower power field of mesenchymal and gland cell differentiation; (II) gland cells; and (III) cartilage formation (arrow).

ESC survival and migration after intramyocardial injection. Previous studies have shown that transplantation of undifferentiated murine ESCs into immunodeficient mice leads to teratoma formation using conventional histological techniques.¹⁵ However, these histological techniques do not allow for *in vivo* longitudinal tracking of tumorigenicity. To demonstrate the importance of following cell fate in a whole-body living animal system, we injected 1×10^6 ES-DF cells intramyocardially into immunocompetent syngeneic Sv129 mice. Cell survival was monitored longitudinally for about 35 days. **Figure 3a** shows a representative Sv129 mouse whereby BLI revealed teratoma formation. Quantitative analysis showed cardiac bioluminescence signals increased from $1.3 \times 10^6 \pm 1.0 \times 10^5$ at day 2 to $1.9 \times 10^7 \pm 1.3 \times 10^6$ at day 7 to $5.4 \times 10^7 \pm 4.7 \times 10^6$ at day 14 and to $4.2 \times 10^8 \pm 3.7 \times 10^7$ p/s/cm²/sr at day 35. As early as 4 days after intramyocardial delivery, a strong BLI signal was present within the chest region. Moreover, a separate focal signal within the long femur bone marrow was noticeable, which is likely due to leakage from the intramyocardial injection and seeding peripherally. At day 35, all mice were too weak for further follow-up and had to be euthanized. During necropsy, the organs were explanted and imaged immediately. *Ex vivo* bioluminescence signals were found in the heart, spine, humerus, femur, submandible (**figure 3b**).

Animals injected with control non-transduced ESCs showed no bioluminescence signals as expected (data not shown). Finally, histological examination with H&E staining also confirmed presence of teratoma formation in the heart, femur, and submandible (**figure 4**). Interestingly, only a small amount of intramyocardially transplanted ES-DF cells expressed the cardiac-specific marker troponin T, suggesting that the *in vivo* differentiation of ESCs into cardiomyocytes also occurred at a low frequency (**figure 4a**).

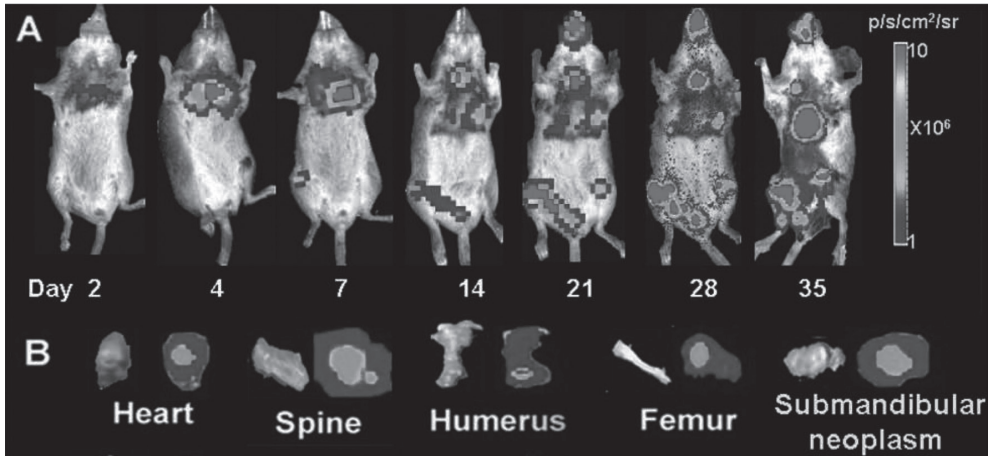


Figure 3. Noninvasive bioluminescence imaging of ESC survival, proliferation, and teratoma formation in Sv129 mice. (a) One million ES-DF cells were injected intramyocardially into immunocompetent syngeneic Sv129 mice hearts. Imaging of the same representative animal over 5 weeks showed a progressive increase in intra-cardiac and extra-cardiac signals. (b) Ex vivo imaging of explanted organs from the same sacrificed animal at week 5. Robust signals were seen in the heart, spine, humerus, femur, and submandible.

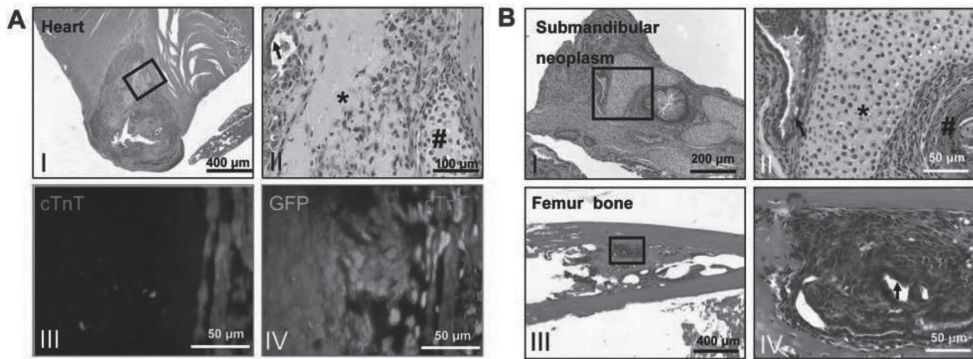


Figure 4. Postmortem histology confirms teratoma formation. (a) Representative H&E staining of a section of heart showing (I) teratoma (lower power field) with (II) epithelium (arrow), cartilage (#), and osteoid (*) formation inside (higher power field). Few numbers of the transplanted ES-DF cell population were found to differentiate into cardiomyocytes as assessed by double-staining of GFP and cardiac troponin T (cTnT) (III, IV). (b) Teratoma formation was also observed in extra-cardiac organs: (I) H&E staining showing submandibular teratoma formation (lower power field) and (II) typical gland (arrow), cartilage (*), and epidermis (#) development (high power field of I). Concordantly, teratoma formation was observed (III) in the femur bone with multiple cell lineages including (IV) gland cells (arrow).

Long-term tracking of ESC fate by BLI. In order to investigate whether lower cell number would have a different outcome, we decided to inject a smaller number (~10,000) of ES-DF cells and follow the animals for a longer period. Since the smaller size of mice was only able to bear teratoma formation for up to 5 weeks, we switched to the larger size rats. This also allowed us to test the influence of different animal species. After intramyocardial injection of 10,000 ES-DF cells, nude rats were followed by BLI longitudinally (**figure 5a, 5b**). After 10 months, animals were sacrificed and hearts were explanted for visual inspection of gross morphology. Histological sectioning of all hearts confirmed differentiation into all three germ layers (**figure 5c**). These results indicate that, even after deliberate local delivery into the left ventricular mass, the process is still associated with extra-cardiac leakage and seeding to other organs.

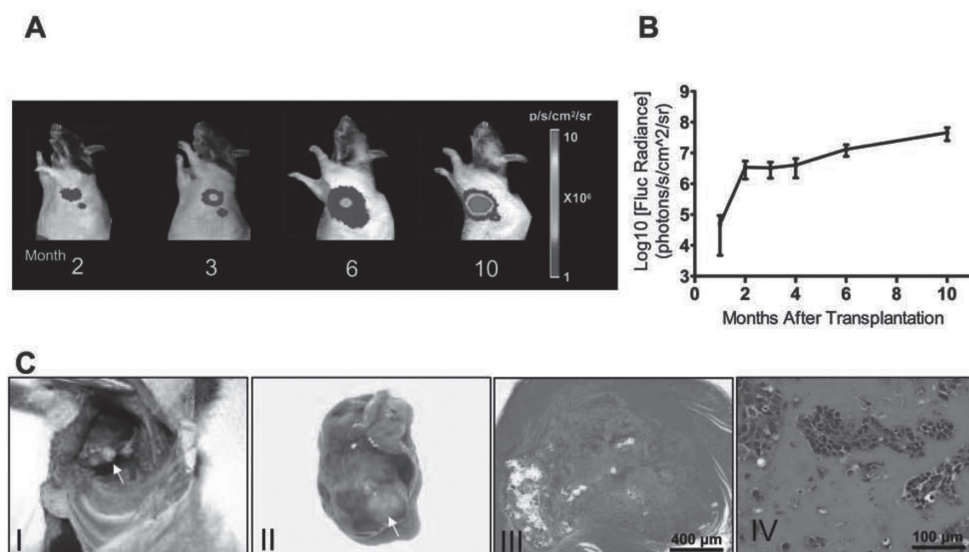


Figure 5. Long term noninvasive tracking of ESC survival kinetics in nude rats. (a) *In vivo* visualization of ESC growth and migration until 10 months after transplantation into the hearts of nude rats. (b) Growth curve of transplanted ES-DF cells *in vivo*. Y-axis shows BLI signal from a fixed region of interest (p/s/cm²/sr) over the heart, while the X-axis represents time after transplantation (months). (c) Gross (I) *in situ* and (II) *ex vivo* picture of the heart from figure 5a containing a visible teratoma. H&E staining of slides from the explanted heart confirms (III) clear-edged teratoma formation within the cardiac tissue (lower magnification), including (IV) bone formation.

DISCUSSION

ESCs hold great promise in regenerative medicine. However, several critical obstacles need to be resolved before ESCs can play a role in clinical medicine. Among these are the immunogenicity of ESCs, and perhaps more importantly from the safety standpoint, the tumorigenic ability of ESCs.^{6, 16} This study has shown that molecular imaging is a reliable tool for long-term *in vivo* imaging of ESCs. Our major findings are as follows: (a) reporter genes do not affect ES cell viability and proliferation; (b) as low as 500-1000 undifferentiated ESCs are capable of inducing teratoma formation; and (c) ESCs are capable of causing teratomas in both intra-cardiac and extra-cardiac sites after local intramyocardial delivery.

Although the risk of teratoma formation may be reduced by prolonged cell differentiation before its engraftment, in reality pre-differentiated ESCs or ESC-derived precursor cells might still form teratoma after transplantation into animals.¹⁷ Recently, Harkany *et al.* observed teratoma formation after transplantation of 400 murine ESCs in the brain of naive mice.¹⁸ Here we pre-

sent further evidence that even 500 to 1000 murine ESCs were sufficient to induce teratoma formation, while less than 100 ESCs were not. In contrast, Nussbaum *et al.* have shown that 50,000 intramyocardially injected murine ESCs were not noticeable in the heart and spleen and did not form teratoma after 3 weeks.¹⁶ Several factors might underlie the discrepancy between the findings. First of all, to mimic cell contamination, we have transplanted different numbers of ESCs accompanied by MEFs to achieve a total cell number of 100,000 cells. The MEFs might have contributed to better ESC engraftment, survival, and subsequent teratoma formation. Secondly, using conventional histological staining, Nussbaum *et al.* investigated teratoma formation at the site of transplantation (myocardium) and at one distant location (spleen), with both locations free of ESCs.¹⁶ This was in concordance with a recent report, whereby teratomas were induced in the kidney capsule of immunodeficient mice but without any human ESC activity in the spleen after 8 weeks as well.¹⁹ By contrast, using more sensitive *in vivo* whole body molecular imaging, which was validated by immediate *ex vivo* imaging of explanted organs, we did observe profound activity at other distant locations, such as the submandible and femur, suggesting ESC leakage during transplantation and subsequent engraftment and proliferation into teratoma. Taken together, these findings suggest longitudinal noninvasive imaging may give more valuable insight into teratoma formation and migration compared to conventional histology.

Previously, we had demonstrated the feasibility of using triple fusion reporter gene to track the fate of transplanted murine ESCs in living subjects by fluorescence, bioluminescence and positron emission tomography (PET) modalities.² This was followed by another study showing that not only does the herpes simplex virus thymidine kinase (HSV-tk) PET reporter gene can be used to monitor ESC engraftment, it also serves useful purpose as a suicide gene to ablate ESC misbehavior.²⁰ In the current study, we further expanded on these initial observations by using a SIN lentiviral vector with ubiquitin promoter driving the bioluminescence (Fluc) and fluorescence (eGFP) to address the influence of undifferentiated ESC number, migration, and long-term follow up after transplantation. The human immunodeficiency virus type 1 (HIV-1)-based lentiviral vector has been shown to be an excellent tool for stable and efficient gene transfer to mouse²¹ and human ESCs.²² The enhanced safety of the self-inactivating (SIN) lentivirus is achieved by (a) using a three-plasmid expression system which consists of packaging, envelop, and vector constructs²³, (b) by eliminating accessory genes (*vif*, *vpr*, *vpu*, and *nef*) from the packaging construct without losing the ability to transduce nondividing cells²⁴, and (c) by deleting the viral enhancer and promoter sequences from the long terminal repeat (LTR) sequence¹⁰. The double fusion construct we employed consists of two optical reporter genes (Fluc-eGFP) separated by a 14-amino acid linker. The Fluc enzyme catalyzes the oxidation of the D-luciferin (ATP and oxygen dependent) reporter probe into oxyluciferin, which emits low energy photons (2-3 eV) at 560 nm wavelength that can

be detected by an ultra-sensitive CCD camera¹². By contrast, GFP does not require a substrate (or reporter probe) such as D-luciferin but rather relies on excitation wavelength in the visible light range of 395-600 nm. Wild-type GFP emits green (509 nm) light when excited by violet (395 nm) light. The variant eGFP has a shifted excitation spectrum to longer wavelength (488 nm) and has increased brightness (35-fold).²⁵ Thus, by using the double fusion reporter gene construct, we were able to perform FACS to isolate stably transduced ESCs (via eGFP) and track their fate in living animals (via Fluc) at a rapid and high-throughput fashion.²⁶

In this study, we were able to reliably monitor the fate of 10,000 transplanted ESCs in the heart for a period as long as 10 months in living animals. The clinical relevance of these findings is profound. As an example, current clinical trials involving bone marrow stem cells for the infarcted heart report injecting a mean cell number of 6.8×10^7 to 2.36×10^8 .²⁷⁻²⁹ Assuming that the same cell numbers of human ESC-derived cardiac cells are used as treatment in the future, then ~500 undifferentiated ESCs (enough to give rise to teratomas in this study) as contaminant would represent a total population of less than 0.001%. Yet current selection methods for pre-differentiated ESC-derived cardiomyocytes typically yield a maximum purity of 70% for human ESCs³⁰ and 90% for murine ESCs.¹⁴ This scenario highlights the tedious and careful clinical protocols that need to be devised to avoid contaminants.

Several limitations of the study need to be raised. First, we have focused on murine rather than human ESCs. While murine and human ESCs have been compared for their gene expression pattern³¹, there is a lack of comparison studies on teratoma formation. In the future, the same issue of minimal human ESCs that can cause teratoma formation will need to be addressed. Second, ESCs tend to differentiate at an earlier stage when implanted subcutaneously (compared to intra-hepatic transplantation) as described by Cooke and colleagues⁸. In this respect, the well vascularized, growth factor-rich, and perhaps immune-privileged porous structure in the liver may help to maintain ESCs in undifferentiated state.⁸ Thus, it is plausible that when transplanted in the liver, the minimal cell number needed for teratoma formation might be even lower than the 500 to 1,000 cells found on subcutaneous study in the present study. Third, we used nude mice while others have relied on severe combined immunodeficient (SCID) mice. Nude mice (lacking furs) are easier to image but have more natural killer (NK) cell activity as compared to SCID mice³², which could also contribute to transplanted cell death by NK cell-mediated cytotoxicity.³³ Moreover, decreased tumor engraftment and higher regression rates of several tumor cell lines have been observed in nude mice as compared to increased tumor growth and larger tumor mass in SCID mice.^{34,35} These observations suggest that the actual cell number needed for teratoma formation could be even lower in the case of SCID mice.

In summary, we have provided important insight into the influence of ESC number, cell migration, and engraftment period on teratoma formation. These findings are an additional stimulus for further research on this topic. Therefore, our ongoing work is focusing on optimizing *in vitro* differentiating systems to acquire purer populations for transplantation, *in vivo* imaging of stem cell behavior, and how to control teratoma formation after transplantation. All these issues need thorough understanding in order for ESCs to play the prominent role they are expected to play in clinical treatments.

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