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

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CHAPTER 5

Clinical Hurdles for the Transplantation of Cardiomyocytes derived from Human Embryonic Stem Cells: Role of Molecular Imaging

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ABSTRACT

Over the past few years, human embryonic stem cells (hESCs) have gained popularity as a potentially ideal cell candidate for tissue regeneration. In particular, hESCs are capable of cardiac lineage-specific differentiation and confer improvement of cardiac function following transplantation into animal models. Although such data are encouraging, there remain significant hurdles before safe and successful translation of hESC-based treatment into clinical therapy, including the inability to assess cells following transplant. To this end, *molecular imaging* has proven a reliable methodology for tracking the long-term fate of transplanted cells. Imaging reporter genes introduced into the cells prior to transplantation enable non-invasive and longitudinal studies of cell viability, location, and behavior *in vivo*. Therefore, molecular imaging is expected to play an increasing role in characterizing the biology and physiology of hESC-derived cardiac cells in living subjects.

INTRODUCTION

Coronary artery disease remains the leading cause of death in the Western world.[1] As the human heart is not capable of regenerating the great numbers of cardiac cells that are lost after myocardial infarction, impaired cardiac function is the inevitable result of ischemic disease. Recently, three randomized clinical trails reported either clinically marginal[2, 3] or no[4] significant benefit following adult bone marrow cell transplantation for patients suffering from acute and/or chronic ischemic heart disease. These reports add to a growing body of evidence that adult-derived stem cells have limited capacity to aid renewal and regeneration of damaged organs and structures. By contrast, hESCs show greater promise, as they are capable of self-renewal and differentiation. hESCs were first isolated by Thompson and colleagues in 1998.[5] They are derived from the inner cell mass of the human blastocyst and can be kept in an undifferentiated, self-renewing state when cultured in the presence of inhibitory compounds, such as mouse embryonic fibroblast feeder layer cells. Compared to adult stem cells harvested from the bone marrow, hESCs have the advantage of being pluripotent, which provides them with the ability to differentiate into virtually all cells of the human body. For cardiac applications, hESCs have the ability to differentiate into cardiac cell lineages.[6, 7] These hESC-derived cardiac cells have structural and functional properties of human cardiomyocytes and can integrate with host myocardium after transplantation into rats[8] and pigs.[9] However, in order to critically evaluate and optimize hESC-based therapy for the heart, new methodologies for assessing the viability, location, and behavior of transplanted cells are needed. This article aims to provide a concise overview of the major hurdles that need to be addressed before hESC-derived cardiac cell transplantation can become a clinical reality. This is followed by an outline of potential of molecular imaging tools that may help to overcome these challenges in the future.

HURDLES FOR CLINICAL TRANSLATION

Although substantial progress has been made in recent years towards improving culture conditions, differentiation strategies, and potential hESC-based cardiac regeneration, several unresolved issues exist between the laboratory and bedside that still need to be bridged **(figure 1)**. This article will cover some of the major areas of concern regarding hESC-derived cardiac cell transplantation, including: (1) optimization of *in vitro* differentiation into cardiac cells; (2) purification of cardiac cells to minimize post-transplant cellular misbehavior such as teratoma formation; (3) *in vivo* integration and function with host myocardium; and (4) evaluation of post-transplant immune rejection and cell death.

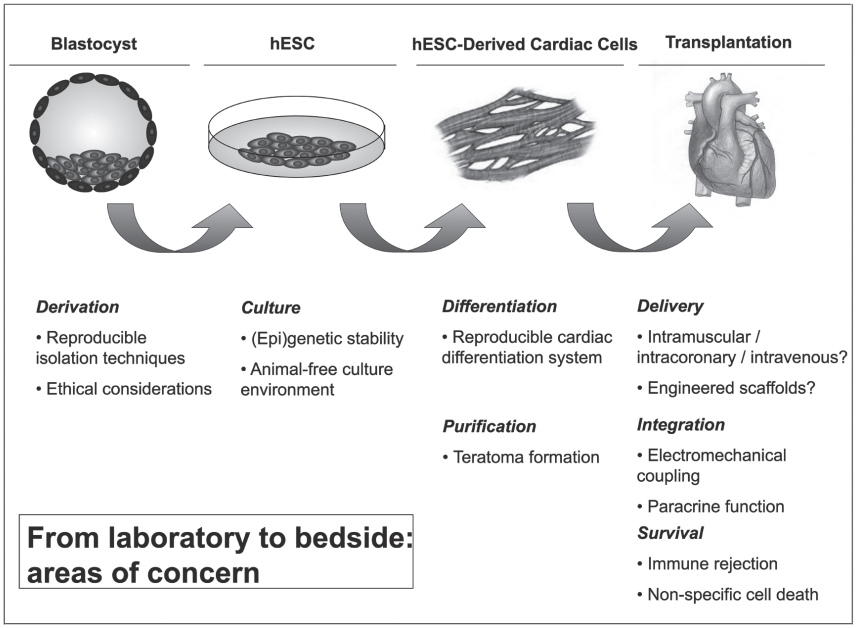

Figure 1. Human embryonic stem cells: from laboratory to bedside. Areas of concern in derivation, culture, differentiation, purification, delivery, integration, and survival are outlined.

In vitro differentiation to cardiomyocytes

Following removal of the inhibitory feeder layer cells, hESCs can aggregate into clusters of cells known as embryoid bodies (EBs). Within these EBs, various signals are activated to promote differentiation of cells into all three germ layers, including mesoderm-derived cardiac cells **(figure 2)**. Formation of cardiomyocytes usually starts 5 days after EB-formation, presenting as a beating area within the EB. Moreover, the hESC-derived cardiac cells within these beating areas actually resemble the structural and functional properties of early stage human cardiomyocytes.[6] Unfortunately, the rate of spontaneous differentiation of hESCs into cardiac cells is low. Typically ~8% of the EBs grown in suspension undergo differentiation into beating clusters, and ~30% of the cells contained in these clusters are actual cardiomyocytes.[6]

Mouse ESCs (mESCs) were originally isolated in 1981[10], and subsequent studies have focused on different strategies to induce cardiac-specific differentiation of mESCs *in vitro*. Retinoic acid was one of the first agents described that significantly increase the percentage of cardiomyocytes arising from ESCs.[11] Similar effects have been described for oxytocin[12], dynorphin B[13], nitric oxide[14], and ascorbic acid.[15] However, the efficacy of these compounds can be dose-dependent and bound to a specific time period in embryonic development.[11] Other groups have focused on the role of growth factors in mESC-derived cardiac cell differentiation, inclu-

ding transforming growth factor-β2[16], basic fibroblast growth factor, and bone-morphogenetic protein-2.[17] Interestingly, findings from mESC studies *do not* appear to translate to hESC research. Thus far, only 5-aza-2'-deoxycytidine[7], combination of activin A and BMP-4[18], and co-culture with murine END-2 visceral endoderm-like cells[19] have been shown to enhance cardiomyogenesis in hESC cultures.

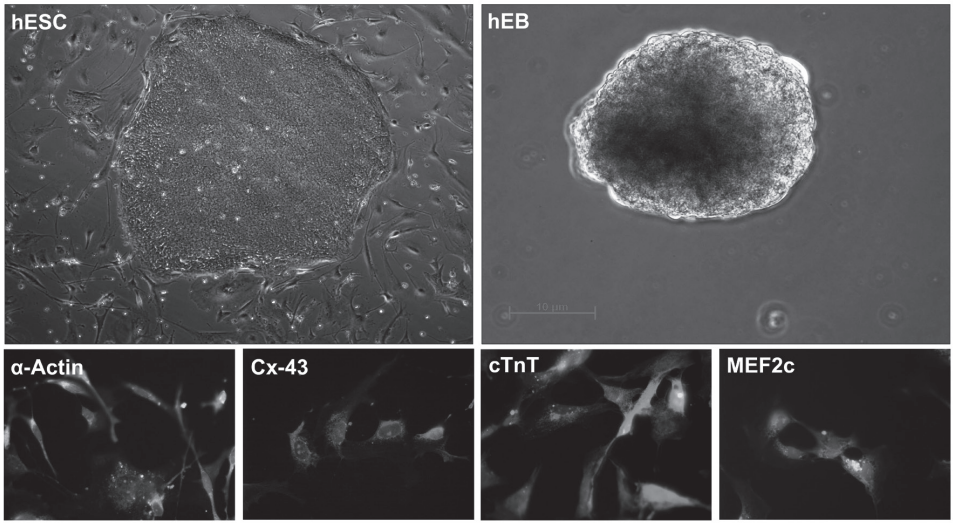

Figure 2. Undifferentiated hESCs (H9 cell line) grow indefinitely in culture on mouse embryonic fibroblast feeder layer cells (upper left panel). Following withdrawal of inhibitory feeder cells, hESCs can aggregate into EBs (upper right panel). Formation of cardiac cells usually starts 5 days after differentiation, initially presenting as a beating cluster within the EB. After isolation of EBs and further enrichment by Percoll gradient separation, these hESC-derived cardiac cells (lower panels) express cardiac lineage specific makers as shown by immunofluorescent staining of GFP-labeled cells with skeletal muscle alpha actin (α-Actin), connexin-43 (Cx-43), cardiac troponin T (cTnT), and Myosin Enhancer Factor 2c (MEF2c) (all in red; counterstaining with DAPI, blue).

Purification of hESC-derived cardiac cells

Once ESCs are successfully induced to adopt cardiac fate, it becomes yet another challenge to isolate and further purify such subpopulations while avoiding contamination by undifferentiated, pluripotent ESCs. Following transplantation, undifferentiated ESCs could cause teratoma formation, which are complex tumors comprised of cellular or oganoid components reminiscent of normal derivatives from the three germ layers.[20] This indicates the need to achieve a highly, if not completely, pure population of cardiomyocytes prior to transplantation. Currently, selection methods for ESC-derived cardiac cells include Percoll density gradient-based isolation, which can enrich up to ~70% pure cardiac cell population for hESC[7] and ~90% for mESC.[21] An alternative strategy for cardiac cell purification combines genetic engineering and

molecular biology techniques. Klug *et al.* utilized a fusion gene consisting of an alpha-cardiac myosin heavy chain (α-MHC) promoter that drives expression of aminoglycoside phosphotransferase, which is an enzyme that confers resistance to the antibiotic geneticin (G418). Once the transgenic mESCs differentiate into cardiac cells, activation of the cardiac specific α-MHC promoter leads to expression of aminoglycoside phosphotransferase and allows these cells to survive against treatment with G418. The resultant surviving cells represent 99% pure cardiomyocyte population[22]. Similarly, Muller *et al.* reported transfection of mESCs with a fusion gene of of myosin light chain-2v (MLC-2v) linked to enhanced green fluorescent protein (eGFP). In this case, mESC-derived cardiac cells expressed eGFP that enabled fluorescent-activated cell sorting (FACS) and collection of cardiomyocyte population (97% pure).[23]

In vivo integration and function of ESC-derived cardiac cells

Data from rodent models evaluating the fate of mESC transplantation into the heart have demonstrated mixed results. Early reports by Min *et al.* evaluating transplantation of mESC-derived beating cells into immunocompetent rat myocardium showed long-term (up to 32 weeks) cell survival, improvement of cardiac function, and improved angiogenesis in the infarct zone[24, 25]. Most notably, no adverse sequelae such as graft rejection, arrythmogenesis, or teratoma formation were observed. By contrast, two more recent studies demonstrated that mESCs transplanted into hearts of both immunocompetent mice[20] and athymic nude rats[26] formed teratomas by as early as 3 to 4 weeks following transplantation. At present, there are few published studies testing the efficacy of hESC-derived cardiac cell transplantation for cardiac repair. Kehat *et al.* showed promising results by injecting hESC-derived cardiac cells into swine heart with complete atrioventricular block.[9] They demonstrated electromechanical and structural coupling of the transplanted cells with the host myocardium. Xue *et al.* also showed functional integration and active pacing of hESC-derived cardiac cells following transfer into healthy myocardium of guinea pigs.[27] Furthermore, Laflamme *et al*. demonstrated that hESC-derived cardiac cells transplanted into athymic rat hearts successfully engrafted, proliferated, and expressed several cardiomyocyte markers.[8] Notably, none of these studies reported cellular misbehavior or teratoma formation. It is also not clear what percentage of these transplanted cells actually survived after transplantation

Immune rejection of allogeneic hESC transplantation

Several factors threaten hESC-derived cardiac cell survival following delivery into a new host, which, if properly modulated, might prevent the drastic post-transplant death of donor cells presently observed. One such major factor is cellular rejection based on immunological incompatibility. Theoretically, hESCs represent an immune-privileged cell population, since embryos consisting of 50% foreign material derived from the father are not rejected by the ma-

ternal host.[28] However, the understanding the immunogenicity of hESCs and their derivatives remains a challenge.

It has been shown that mESCs do not express major histocompatibility complex (MHC) antigens, the major system of alloantigens responsible for cell incompatibility.[29] Furthermore, mESCs can inhibit T-lymphocyte proliferation *in vitro* and establish multi-lineage mixed chimerism *in vivo*.[30] However, when allogeneic undifferentiated mESCs were transplanted into a murine model of myocardial infarction, our group found progressive intra-graft infiltration of inflammatory cells mediating both adaptive (T cells, B cells, and dendritic cells) and innate (macrophages and granulocytes) immunity, leading to rejection of the mESC allograft[20]. In contrast to mESCs, hESCs express low levels of MHC-I antigens.[31, 32] Drukker *et al.* observed that MHC-I expression increased two to four-fold when cells were induced to spontaneously differentiate to EBs[31], and eight to ten-fold when cells differentiated into teratomas. In contrast, Draper *et al.* reported MHC-I downregulation upon hESC differentiation towards EB.[32] Thus, questions regarding the character and intensity of immune responses towards allogeneic hESC-derived cardiac cells still remain. Solutions that reduce or eliminate the potential immunological response are needed, including: (1) forming MHC isotyped hESC-line banks; (2) creating a universal donor cell by genetic modification; (3) inducing tolerance by hematopoietic chimerism; (4) generating isogeneic hESC lines by somatic nuclear transfer; (5) and/or using immunosuppressive medication. Details of these strategies to minimize rejection of hESC-derived transplants have been extensively reviewed by others.[33, 34]

IMAGING HESC-DERIVED CARDIAC CELLS

Non-invasive cell tracking

As outlined earlier, hESC-derived cardiac cell transplantation is potentially feasible, but there are several aspects that require improvement. For clinical translation to occur, it is essential that tools be developed for longitudinal tracking and evaluation of transplanted cell viability and behavior. Traditionally, cell therapy studies have relied upon conventional reporter genes such as GFP and β-galactosidase (LacZ) to monitor cell survival and differentiation. However, visualizing GFP and LacZ cells requires postmortem tissue analysis, which provides only a single snapshot representation of cell fate, not a complete picture over time. Moreover, sampling error inherent in *ex vivo* analysis requires large numbers of animals be sacrificed to develop a realistic picture of longitudinal survival kinetics.

Another technique for measuring the efficacy of cell therapy is to assess secondary endpoints. Cardiac contractility can be monitored by conventional methodologies such as echocardiography or magnetic resonance imaging (MRI). Cardiac perfusion can be assessed using posi-

tron emission tomography (PET) or single-photon emission computed tomography (SPECT). However, these data cannot be correlated to the biological state of the cells, as the cells themselves can neither be visualized nor assayed in the living subject. Aiming to provide insight into the location and survival of transplanted cells, recent studies have reported labeling mESCs with magnetic iron particles and following them by MRI.[35] Although these iron particles are robust and facilitate repeated imaging over time, they do not reliably provide insight into cell *proliferation* and *viability*, due to the disparate passing of the particles from parent to daughter cells and the ability of non-specific immune cells (e.g., macrophages) to engulf particles, respectively.

Molecular imaging: direct vs. indirect approach

Ideally, a non-invasive method for *in vivo* tracking of hESC-derived cardiac cells should be capable of providing insight into the following processes: (1) localization and migration of the cells, (2) cell survival and proliferation kinetics, and (3) cell differentiation or de-differentiation patterns. Molecular imaging of reporter genes offers potential promise in meeting these goals. Molecular imaging can be broadly defined as the visualization of molecular and cellular processes in the living subject. For *in vivo* molecular imaging to work, two basic elements are required: a molecular probe that detects a quantifiable signal based on the presence of gene, RNA, or protein, and a method to monitor these probes *in vivo*.[36] In general, molecular imaging can be divided into two categories: (a) *direct* imaging of probe-target interaction or (b) *indirect* imaging based on reporter gene and reporter probe.

The most commonly used *direct* cardiac imaging modality utilizes ^{18}F-fluorodeoxyglucose ([^{18}F]-FDG), a glucose analog which can cross intact membranes into living cells and is phosphorylated by the endogenous enzyme, hexokinase, trapping the probe inside the cell. The phosphorylated [^{18}F] will undergo positron annihilation to give off two 511 keV photon signals that can be detected by PET, providing a measurement of cell or tissue viability[37] This approach has recently been shown feasible for imaging clinical cardiac cell therapy. Hofmann *et al.* labeled autologous bone marrow cells with [^{18}F]-FDG from nine patients suffering from acute myocardial infarction.[38] The [^{18}F]-FDG labeled cells were injected into either the infarct-related coronary artery or the antecubital vein five to ten days following coronary stenting. PET imaging was performed 50 to 75 minutes after the procedure and successfully detected the transplanted cell population in all patients, with higher signals in the intra-coronary group. Although PET is a valuable tool to monitor the location of cells shortly after transplant, the short half-life of the [^{18}F]-FDG radiotracer (~110 minutes) does not permit long-term follow-up of cell survival and/or migration. Furthermore, [^{18}F]-FDG is not passed on to daughter cells during cell division and therefore does not provide insight into cell proliferation.

The concept behind *indirect* molecular imaging is an expansion upon basic reporter gene technology whereby a promoter or enhancer region of interest is linked to the imaging reporter gene. The nature of the promoter can be inducible, constitutive, or tissue specific. The construct can be introduced into the target cell using either viral or non-viral techniques. Once incorporated, the reporter gene produces the reporter protein which then interacts with the introduced reporter probe, producing an analytic signal that can be detected by the detector system. Depending on the reporter gene used, available imaging modalities include PET, SPECT, MRI or a charged-coupled device (CCD) camera.[39] The two most widely used reporter gene imaging systems are firefly luciferase (Fluc)-based optical bioluminescence imaging and herpes simplex virus thymidine kinase (HSV-tk)-based PET imaging. For bioluminescence imaging, the Fluc reporter protein catalyzes the D-Luciferin reporter probe to produce low-energy photons (2-3 eV) that can be captured by an ultra-sensitive CCD camera. The reporter probe can be administered before every imaging session, allowing for multiple imaging acquisitions over time. For PET imaging, the HSV-tk reporter protein phosphorylates radiolabeled thymidine analogue 9-(4-[18F]fluoro-3-(hydroxymethylbutyl)guanine ([^{18}F]-FHBG) reporter probe, which emits high-energy photons (511 keV) that can be detected by PET. The reporter gene technique has been used to assay survival and localization of transplanted rat embryonic cardiomyoblasts[40] and more recently of mESCs.[26]

Reporter gene imaging of ESCs and ESC-derived cardiac cells

Regarding transplantation of hESC-derived cardiac cell transplantation, reporter gene imaging can be used to monitor critical events. First, since the reporter gene can be integrated into the DNA of transplanted cells, its expression is limited to only living cells, and thus facilitates assessment of cell survival. Second, the reporter gene can be passed onto daughter cells, thus providing insight into cell proliferation. This is an important feature given the tumorigenic potential of undifferentiated ESCs discussed earlier. Third, it is possible to introduce several reporter genes into the same cell, facilitating a multi-modality imaging approach. Recently, Cao *et al*. tested the efficacy of mESC with a self-inactivating lentiviral vector carrying the triple-fusion (TF) construct that consists of firefly luciferase (Fluc), red fluorescence protein (mRFP), and herpes simplex virus truncated thymidine kinase (HSV-ttk).[26] The mRFP facilitates imaging of single cells by fluorescence microscopy and allows for the isolation of a stable clone population by FACS. The Fluc allows for bioluminescence imaging for assessment of cell survival, proliferation, and migration in small animals. Finally, the HSV-ttk affords the ability to use PET imaging in small and large animals, as well as humans. Following transplantation into the hearts of athymic nude rats, mESC survival, migration, and proliferation was monitored for 4 weeks by bioluminescence and PET imaging. PET imaging, both with [^{18}F]-FHBG to image cells and [^{18}F]-FDG to image myocardial viability, proved to be a very sensitive tool to assess the tomographic

location of mESC engraftment **(figure 3)**. However, reporter gene signals increased rapidly within 4 weeks due to teratoma formation. Histologic samples obtained from both intra- and extra-cardiac sites revealed spontaneous differentiation of the mESC into all three germ layers. In a subsequent study, our group also demonstrated the ability of an anti-viral drug to selectively target teratomas expressing the HSV-ttk reporter gene.[41] Thus, in addition to its use for monitoring cell fate, the reporter gene also serves as an inducible suicide gene that facilitates selective cellular ablation. This could be an important tool in controlling cellular misbehavior and/or teratoma formation of transplanted hESC-derived cells.

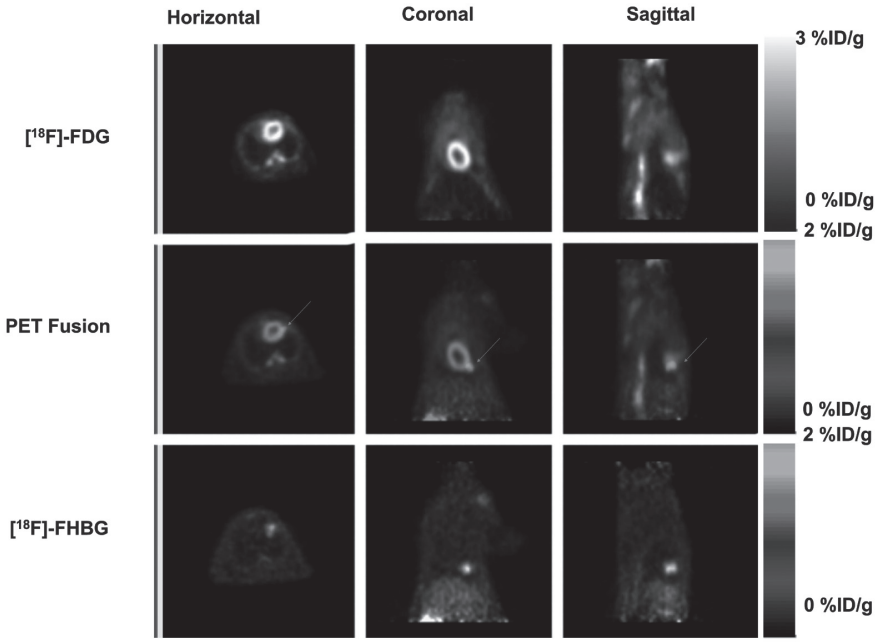

Figure 3. Positron emission tomography imaging of transplanted mESCs in the myocardium. Two weeks after mESC transplantation, nude rats underwent [^{18}F]-FHBG reporter probe imaging (top row) followed by [^{18}F]-FDG myocardial viability imaging (middle row). Fusion of [^{18}F]-FHBG and [^{18}F]-FDG images (bottom row) shows the exact anatomic location of transplanted mESC (arrow) at the anterolateral wall in horizontal, coronal, and sagittal views. (Reproduced with permission from Cao et al.[26])

Recently, our group has successfully transduced hESCs (H9 line) with a lentiviral vector containing a double fusion (DF) reporter gene that consists of Fluc and eGFP. Cardiac cells derived from hESCs using EB formation and Percoll gradient enrichment constitutively express Fluc and eGFP. Following transplantation into ischemic myocardium of severe combined immuno-

deficient (SCID) mice, these cells can be monitored by bioluminescence imaging for >3 months (Cao *et al.,* unpublished data). By contrast, injection of undifferentiated hESCs caused teratoma formation during the same period (**figure 4**). Taken together, these results highlight the valuable role of molecular imaging for following the developmental fate of transplanted hESCs and their derivates.

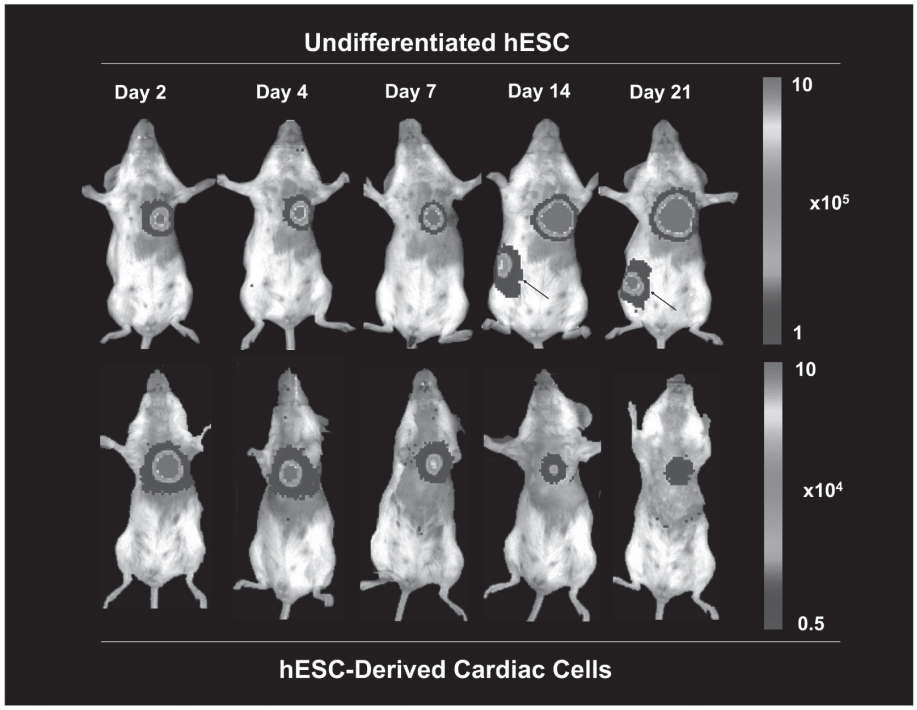

Figure 4. Comparison of undifferentiated hESCs versus hESC-derived cardiac cell survival and behavior *in vivo*. Both hESC (top row) and hESC-derived cardiac cells (bottom row) were transplantated into ischemic myocardium of SCID mice. Bioluminescence imaging during the first three weeks following transplantation reveals acute donor cell death followed by stable survival of hESC-derived cardiac cells. In contrast, undifferentiated hESCs proliferate uncontrollably in the heart as well as other seeded organs (arrows) to cause teratoma formation.

Finally, a critical question with regard to reporter genes is whether the might influence the biology and physiology of the stem cells. In the study by Cao *et al.,* the TF construct had no influence on mESC morphology, viability, proliferation, and differentiation capacity *in vitro.*[26] Likewise, both the bioluminescence (D-luciferin) and PET ([^{18}F]-FHBG) reporter probes had no adverse effects on mESC behavior. In two recent studies, the TF reporter genes affected <2% of the total mESC genome using transcriptional profiling analysis[42] and caused no significant differences in protein expression quantified by proteomic analysis.[43] Ongoing studies are also evaluating the effects of reporter gene and reporter probe on hESCs as well.

CONCLUSION

The last several years have produced revolutionary advancemences in exploring the capabilities of stem cells for treatment of cardiovascular disease. In particular, initial animal results involving hESC-derived cardiac cells appear promising for improving cardiac function after ischemic injury. Nonetheless, we are still years away from safely translating these initial observations into therapy for humans. There are several issues within the field that require improvement, especially those related to *in vitro* differentiation and purification, as well as *in vivo* integration and survival of the transplanted cells. As the field of hESC-derived cell transplantation emerges, there will be an urgent need for reliable methodologies to track and assess behavior of the cells following transfer into the injured heart. Molecular imaging serves these needs and will likely play a prominent role in future hESC research.

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