Mechanistic Molecular Imaging of Cardiac Cell Therapy for Ischemic Heart Disease

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ABSTRACT

Cell-based myocardial regeneration has emerged as a promising therapeutic option for ischemic heart disease, though not yet at the level of routine clinical utility. Despite the encouraging results from initial preclinical studies that have demonstrated improved function and reduced infarct size of the ischemic myocardium following several candidate cell transplantation, the beneficial effects and molecular mechanisms of cardiac cell therapy are still unclear in clinical applications to date, and much remains to be optimized. In order to improve engraftment, accurate methods are required for tracking cell fate and quantifying functional outcome. In the present review, we summarized the current status and challenges of cardiac cell therapy for ischemic heart disease, and discussed the strengths and limitations of currently available in vivo imaging techniques with special focus on the newly developed multimodality approaches for assessing the efficacy of engrafted donor cells. We also addressed the hurdles these imaging modalities are facing, issues regarding immunogenicity and tumorigenicity of transplanted stem cells, and provided some future perspectives on stem cell imaging.

Keywords: molecular imaging; stem cell therapy; ischemic heart disease
Introduction

Ischemic heart disease (IHD) is the predominant contributor to cardiovascular morbidity and mortality. After myocardial infarction (MI), the limited survival of cardiac cells renders the injured heart susceptible to unfavorable remodeling and functional failure. For now, heart transplantation is the only viable treatment option for patients with end-stage heart failure. Due to the persistent shortage of donor heart organs, stem cell therapy has emerged as a promising therapeutic strategy for acute and chronic ischemic heart disease. Several candidate cell types have been used in the preclinical and clinical trials for myocardial repair either directly through tissue regeneration or indirectly through paracrine action. Representative seed cells are adult stem cells (ASCs), embryonic stem cells (ESCs) (14), and induced pluripotent stem cells (iPSCs)(70). At present, the most clinically applicable cell type is ASCs, which include skeletal myoblasts (SKMs) (1, 51), bone marrow derived cells (15, 49, 61), and most recently cardiac stem cells (CSCs) (43, 53).

Various cell types exhibit potential for cardiovascular repair, and numerous animal studies in recent years have shown that stem cell therapy administered after MI can improve function and limit infarction. Despite the initial encouraging results, the functional benefits are modest and inconsistent in clinical applications to date, and much remains to be optimized. Several fundamental questions remain unanswered regarding to the long-term fate of the transplanted cells; do they survive and integrate? How to control their proliferation, differentiation and migration? What are their transcriptional and functional profiles? This review will start with a brief discussion of currently available in vivo imaging modalities which hold potential to non-invasively provide quantitative information and longitudinal assessment of cell fate. We will then focus on molecular imaging of the efficacy and safety issues regarding stem cell therapy in ischemic heart disease.

Molecular Imaging for the Survival and Kinetics of Engrafted Cardiac Stem Cells

It is a prerequisite that the transplanted stem cells reach the injury site and survive so as to function well in the target tissue. Before the advent of molecular imaging, determination of cell fate mainly relies on post-mortem histological analysis, which is performed at pre-determined time points following cell transplantation and requires animal sacrifice, precluding longitudinal cell tracking. Molecular imaging enables in vivo tracking of the distribution and long-term viability of stem cells by labeling cells with specific markers, including iron particles, radionuclides, and reporter genes. Labeled stem cells can be visualized non-invasively using
multiple imaging modalities, such as MRI (17), single photon emission tomography (SPECT), positron emission tomography (PET) (16) and bioluminescent imaging (BLI) (22).

**Iron Particle Labeling** Labeling cells with iron particles for MRI visualization is one of the most frequently applied methods for cell tracking. The most commonly used iron formulation is super paramagnetic iron oxide (SPIO) particles because of their potent negative contrast effects and inherent lack of cell toxicity (56). The labeling of iron particles has to be completed *ex vivo* prior to transplantation, because most stem cells must be induced to take up these MRI contrast agents (28). The feasibility of tracking SPIO-labeled stem cells has been demonstrated previously in animal models of myocardial infarction (33). With high spatial resolution and without ionizing radiation, MRI represents an attractive nontoxic way to produce anatomic information of highest quality for cell localization. In addition, the relatively low sensitivity has been recently demonstrated to be enhanced to single cell level using higher magnetic fields >3 Tesla (85), though exposure to such high magnetic fields is not recommended in humans. However, limitations exist regarding this technique. Labels, via taken up by macrophages, could persist in the myocardium for up to 5 weeks after cell death, generating false-positive signals that could be misinterpreted as robust cell survival (73). In addition, the labels may no longer be detectable once the cells divide, and MRI is contraindicated in IHD patients with implantable devices (e.g., pacemakers and defibrillators), who are often in greater need of stem cell therapy. Further, despite minimal effect of iron particles on in vitro proliferative capacity and cell viability, there are recent data raising concern about the impact of chondrogenic differentiation of mesenchymal stem cells (40).

**Radionuclide Labeling** Recent improvements in spatial resolution of small animal PET and SPECT cameras have enabled the implementation of specialized systems for *in vivo* tracking of radionuclide-labeled cells (46). Direct radionuclide labeling of stem cells has been used to quantify the retention and bio-distribution of transplanted cells in experimental and clinical studies. For example, *ex vivo* cell labeling with $^{18}$FDG is mediated via glucose transporters on cell surface (27). Following uptake into cells, $^{18}$FDG is phosphorylated by hexokinase, thus trapped within the labeled cells, permitting the detection using PET. Similarly, direct cell labeling with $^{111}$indium-oxine (3) or $[^{99m}Tc]$ hexamethylpropyleneamine oxime ($^{99m}Tc$-HMPAO) (52), has been used in conjunction with SPECT, and $^{64}$Cu-PTSM (copper-64-pyruvaldehyde-bis (N$^4$-methylthiosemicarbazone ($^{64}$Cu-PTSM)) (2) or $^{18}$F-fluoro-deoxy-glucose ($^{18}$FDG) (8) with PET for tracking various cell types following transplantation. Compared with MRI, PET and SPECT have significantly higher sensitivity despite a lower spatial resolution. However, a major concern
of using radioactive labeling is radiation exposure (16), which may inhibit cell viability and
differentiation. A recent study in a rat model of MI demonstrated significant reduced proliferation
and function of $^{99m}$Tc-HMPAO labeled human hematopoetic progenitor cells despite accurate
homing to the infarcted myocardium (52). Another problem lies in the short half-life of the
radiotracer, which may limit the duration for long-term cell tracking (a couple of hours in the case
of $^{18}$FDG and a few days in the case of $^{111}$In and $^{64}$Cu). Additionally, a potential limitation is the
label loss by diffusion or cell death with uptake of the label by neighboring viable cells (16).

In order to overcome the high toxicity of most radiopaque contrast agents, new emerging
strategies have exploited microencapsulation techniques that provide immunoprotection of
transplanted donor cells. High concentrations of radiopaque agents can be incorporated into the
microcapsule to enable visualization by x-ray fluoroscopic and CT imaging (5). Such technique
can be used in combination with reporter gene transfection so as to deliver stem cells using
conventional x-ray imaging platforms with follow-up examination by PET/CT or SPECT/CT.

**Reporter Gene Labeling** Reporter genes labeling offers the possibility to circumvent the
aforementioned limitations of direct radiolabeling (63). The reporter gene encodes a membrane
receptor, transporter or enzyme that is not normally expressed in the target cell, and is linked to
an inducible, constitutive, or tissue specific promoter/enhancer. Therefore, the resultant reporter
construct enables the enrichment of a systemically injected probe exclusively in the genetically-
modified cells, and more importantly, the signal is specific, and dependent on the viability of the
transplanted cells to maintain the transcription and translation of reporter proteins. If the cells are
apoptotic or dead, they will in most cases cease to emit signals. Therefore, the imaging signal by
reporter gene labeling is able to determine cell viability, preclude the risk of probe dilution or
leakage following cell death, and allow monitoring of cell proliferation and long-term repeated
imaging (66). Another obvious advantage of using report gene constructs is their adaptability to
multiple imaging modalities. For example, fluorescent reporter proteins (e.g., monomeric red
fluorescent protein, enhanced green fluorescent protein, and enhanced cyan fluorescent protein)
allow imaging at the single-cell level by fluorescence microscopy as well as isolation of stable
cell populations by flow cytometry cell sorting. Luciferase reporters (e.g., firefly luciferase,
renilla luciferase, and click beetle luciferase), on the other hand, can be used for BLI, a high-
throughput and low-signal-to-noise strategy for cell tracking in small animals (22). Fluorescence
and BLI, however, rely on low energy photons that become attenuated within deeper tissues. To
overcome poor tissue penetration and high background signal, imaging of large animals and
humans requires PET/SPECT imaging. For instance, the herpes simplex virus thymidine kinase
(HSV-tk) reporter gene labeled with $^{124}$I or $^{18}$F is used for PET imaging (26, 48) (Fig.1), and the human sodium-iodide symporter (hNIS) reporter gene is used for PET imaging with $^{124}$I as tracer or for SPECT imaging with $^{99m}$Tc (72).

Despite the above advantages, several hurdles prevent reporter imaging from being applied in routine clinical practice. Transgene expression in the cell progeny is not guaranteed even when viral vectors are used, thus the signal will decrease when cells divide. Genetic modification may alter stem cell properties and compromise the functional benefit from cell transplantation. Transcriptional and proteomic analysis have showed changes in cell cycling, cell death, and metabolic gene expressions by reporter genes in mESCs, though no significant interference of function and differentiation was observed (82). Other issues include tissue spatial resolution, immunogenicity of reporter proteins, mutagenesis and oncogenicity of viral vectors.

Contrast-enhanced Ultrasound and 3D optical imaging Echocardiography has been traditionally used as an inexpensive and noninvasive method to evaluate cardiac structure and function in clinical practice. Molecular imaging using contrast-specific ultrasound (CEU) has recently become possible with the development of novel “site-targeted” microbubbles (45). This technique relies on the ultrasonic detection of custom-designed microbubble contrast agents that are retained in area of interest via special shell composition or the conjugation of specific targeting ligands. A recent study by Kuliszewski et al. demonstrated in vivo CEU imaging of EPCs using EPC-targeted microbubbles loaded with monoclonal antibody against H-2Kk, a unique marker protein expressed by EPCs (55). The efficacy of CEU imaging of cell therapy was further confirmed by Cui et al.(20), which reported significantly increased signal from microbubble-filled MSCs than unlabelled MSCs. These studies are the first to describe CEU imaging of progenitor cells and demonstrate the potential of this technique for tracking engraftment of progenitor cells.

Recently, investigators have developed approaches that allow for 3D optical imaging. Fluorescence molecular tomography (FMT) and bioluminescence tomography (BLT) collects photons that have propagated through tissue at multiple views and combines these signals tomographically to obtain the distribution of luminous source in deep tissues. Our group recently developed a dual BLT/microCT imaging system for visualizing transplanted stem cells in ischemic tissue (46). BLT with microCT co-registration can improve reconstruction accuracy and benefit 3D volumetric data.

Multimodality Molecular Imaging In order to combine the strengths of different imaging system, multimodality imaging have been recently explored to monitor the kinetics of stem cells
survival, proliferation and migration in vivo. For example, we recently established a versatile imaging platform using ASCs with constitutive expression of dual-reporter gene firefly luciferase and enhanced green fluorescent protein (Fluc-eGFP), which enabled quantitative 3-dimensional (3D) imaging of the cells’ distribution and kinetics in vivo via BLI/FRI/BLT/micro-CT (24). Recently, Templin et al. demonstrated the feasibility of detecting $^{123}$I labeled and sodium–iodine symporter (NIS) transfected human iPSCs in border zones of infarcted territory by using cardiac hybrid imaging (71). This new technique utilized 3-dimensional NOGA mapping to guide catheter-based intramyocardial injection of NIS$^{\text{pos}}$-hiPSCs, and applied dual-isotope single photon emission computed tomographic/computed tomographic (SPECT/CT) imaging with the use of $^{123}$I SPECT to follow donor cell survival and distribution, and $^{99m}$Tc-tetrofosmin SPECT for perfusion imaging (Fig.2). This technique was later optimized by contrast-enhanced coronary computed tomography angiography to reveal the exact anatomic location of the injected stem cells with the corresponding perfusion defect together with the related coronary vessels (25) (Fig.3).

**Molecular Imaging the Efficacy of Donor Stem Cells**

**Differentiation and Incorporation of Stem Cells**

In vivo imaging offers the opportunity to answer one fundamental question whether the transplanted stem cells could effectively differentiate into cardiomyocytes and incorporate to the host environment within infarcted myocardium. To this end, reporter gene imaging modalities are most appropriate for assessing cell differentiation and incorporation, whereas direct imaging is more suitable for high resolution detection of cell location. Cardiac-specific promoters have been used as sensors of the cell differentiation state. In this approach, α-myosin heavy-chain (α-MHC) promoter driving the expression of an antibiotic resistance gene can be used for in vitro cardiomyocytes selection (37). A similar system using firefly luciferase or HSV-tk reporter gene can offer in vivo quantitative monitoring of stem cell differentiation via BLI or PET. A previous study using murine ESCs which was engineered to express the enhanced cyan fluorescent protein (ECFP) under the control of the cardiac-specific α-actin promoter, demonstrated cardiomyocyte-directed differentiation of stem cells via ECFP fluorescence (7).

Previously, the integration and synchronization of differentiated stem cells with the host tissue was confirmed by positive staining for MLC2v, a specific ventricular sarcomeric protein, and the gap junction protein connexin 43. Using molecular imaging techniques, noninvasive tracking of acute cardiac retention and long-term survival is now feasible. A previous study using
In-radiotracer observed that only 4.7% of injected human EPCs were retained in the infarcted myocardium of athymic nude rats (49). Similar findings were seen in a human study using [18F]-FDG PET tracer to follow the intracoronary delivery of bone marrow cells in MI patients (83). In addition, a recent study in a rat MI model showed less than 0.5% of transplanted CSCs remained alive 8 weeks after transplantation (44). A recent study using mechanistic BLI demonstrated that MSCs survived for up to 50 days, differentiated into endothelial cells and integrated into capillary network in vivo after experimental myocardial infarction (Fig. 4), and increased angiogenesis and decreased fibrosis were associated with cardiac functional improvement after MSC transplantation (78). Another study using BLI for in vivo analysis of biodistribution showed that a significant number of BMMNCs injected into the heart actually migrated to the femur, liver, and spleen (75).

Paracrine Action of Stem Cells

Myocardial and vascular regeneration have been initially proposed as mechanisms underlying the improved cardiac function after cell therapy in MI. However, in many cases, the number of differentiated cardiomyocytes and vascular cells derived from transplanted stem cells, especially the adult stem cells appear too small to account for the observed significant cardiac improvement. Thus, the prevailing concept of stem cell efficacy has shifted towards an alternative hypothesis, the “paracrine mechanism”, according to which the transplanted cells are proposed to release soluble factors that contribute to cardiac repair and regeneration (30). This notion is further supported by studies showing that the administration of conditioned medium is able to recapitulate, at least partly, the beneficial effects of stem cell therapy (29). Indeed, various implanted stem cells, including skeletal myoblasts (59), bone-marrow-derived cells (39, 50) and cardiac-derived cell (18) have demonstrated the ability to produce and secrete a wide range of cytokines, chemokines and growth factors that are known to be involved in cardiac repair.

The paracrine factors may influence adjacent cells and exert their actions via several mechanisms. They can induce cytoprotection of resident myocytes, mediate neovascularization, modulate the post-infarction inflammatory and fibrogenic processes, and stimulate endogenous regeneration and recruitment of endogenous stem cells (19, 30). Furthermore, it is noteworthy that the production of these paracrine factors could be further increased by ischemic insult (36). Under hypoxic stress in ischemic myocardium, the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), hypoxia inducible factor-1 (HIF-1) and stromal cell-derived factor-1 (SDF-1) are significantly increased in injured hearts treated with MSCs or multipotent stromal cells (30). With multimodality BLI/BLT/microCT
imaging, we recently demonstrated multipotent adipose stromal cells therapy promoted recovery from hindlimb ischemia through enhancing pro-angiogenic signal in vivo, though the cells failed to incorporate into the host microvasculature as functional components (24). Using in vivo BLI, a recent study reported that porcine pluripotent stem cells derived endothelial cells (piPSC-ECs) could also improve cardiac function after myocardial infarction via release of proangiogenic and antiapoptotic factors in the ischemic microenvironment (31) (Fig. 5). Thus, the paracrine hypothesis extends the traditional concept to include the influence of stem cell released factors on the post-MI microenvironment, and rationalizes the persistence of benefit despite the poor survival of transplanted cell.

As the survival of transplanted stem cell is challenged by hypoxia, inflammation and anoikis, it is necessary to enhance the cell resistance to ischemic niche, for promoting the paracrine or differentiation potential of donor cells (79). The current strategies include pharmacological pre-/post-conditioning, genetic modification, combined cell transplantation and biomaterial engineering (81). Using BLI, a recent study from our group demonstrated that rosuvastatin could improve the survival of engrafted multipotent adipose stromal cells in infarcted myocardium involving PI3K/Akt and MEK/ERK1/2 signaling pathways, which significantly promoted post-MI function (unpublished data) (Fig. 6). The emerging epigenetics and post-transcription processing strategies potentiate the DNA-free modification of stem cells, which may promote the efficacy of cell-based IHD therapy to translate in to clinical setting. Moreover, biochemical engineering tools can also be designed to promote the survival and paracrine manner (e.g. growth factors) of engrafted stem cells (21, 80). For example, our latest efforts has been focused on the synthesis of a multifunctional VEGF-loaded IR800-conjugated nanocarrier, which targets VEGF receptors, maintains an elevated level of VEGF in ischemic tissues for a prolonged time (67). The dynamic accumulation of these nanocarriers in the ischemic muscle and the resultant increase in blood perfusion, oxygen saturation and angiogenesis could be monitored by multi-modality imaging combining fluorescence imaging, laser Doppler, photoacoustic imaging and 18F-alfatide PET imaging.

**Functional Outcomes of Ischemic Myocardium**

The goal of stem cell therapy is to replace injured tissue with new cells to restore heart function. Thus precise evaluation of the functional outcome of cell therapy is of primary importance. Clinical studies as well as animal studies have mainly focused on detection of differences in left ventricular (LV) function, infarct size, myocardial perfusion, and myocardial
viability, and these parameters can be achieved non-invasively by various imaging modalities, including echocardiography, MRI, CT and nuclear techniques.

For LV size and systolic function measurement, the most accurate assessment is contrast-enhanced MRI, which is often selected over echocardiography because of its high spatial resolution, whereas CT and PET/SPECT are not preferred due to their lower temporal resolution and exposure to radiation. Previous clinical trials, which investigated the cardiac benefit of adult progenitor cell therapy in acute MI and chronic IHD, have shown increase in LV ejection fraction (LVEF) varying from 3% to 18% (6). The most preferred technique for infarct size measurement is again MRI, which allows precise detection of infarction and currently the only technique discriminating between subendocardial and transmural infarction (77). Reduction in infarct size has been reported in patients undergoing cell therapy, however, control patients have also exhibited a comparable reduction in infarct size (54). Therefore, randomized controlled trials are needed to evaluate changes in infarct size after cell therapy as compared with the natural evolution after reperfusion.

Most of the currently available studies used SPECT for evaluating myocardial perfusion, and have shown a decrease in myocardial perfusion defect size after adult progenitor cells therapy, suggesting increased growth of new small vasculature after stem cell engraftment (6). Only Janssens et al. (35) used PET to evaluate the effect of cell therapy on perfusion, and failed to observe increased myocardial perfusion. It should be emphasized that only PET permits absolute quantification of myocardial perfusion, whereas SPECT provides information on relative changes in tracer uptake. Therefore, future investigation with the use of PET is guaranteed to assess both rest and stress perfusion in patients receiving stem cell therapy. In addition, coronary blood flow can be invasively assessed using Doppler flow wire at rest and during pharmacologic stress. Subsequent calculation of the coronary flow reserve provides insight into the integrity of both the epicardial conduit arteries and the distal microvascular bed.

The final measure of functional outcome is myocardial viability, which can be assessed by either nuclear imaging with PET (mainly using $^{18}$F-FDG) and SPECT (using $^{18}$F-FDG or $^{99}$Tc-labeled agents) to evaluate glucose utilization, or low-dose dobutamine echocardiography and MRI to assess contractile reserve. We have previously performed 4 years follow-up study evaluating the safety and efficacy of intracoronary delivery of autologous BMMNC in STEMI patients who received PCI, and found no significant improvement in myocardial viability of the infarcted area assessed by SPECT, though overall LV function increased (13, 65). Clinical studies using nuclear imaging techniques have shown increased tracer uptake ranging from 15–55% post-
therapy with adult progenitor cells (23, 64). Contractile reserve as measured by echocardiography, however, has not shown significant enhancement (84). Additional studies, evaluating different features of viable myocardium in the same patients, are needed to elucidate changes in myocardial viability after cell therapy. In addition, non-fluoroscopic catheter-based electromechanical mapping enables identification and localization of viable myocardial tissue by simultaneous assessment of electrical activation and local mechanical response.

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**Molecular Imaging in Safety Issue of Stem Cells**

Unlike adult stem cells that are generally limited by their plasticity, the capacity of ESCs and iPSCs to differentiate into almost all human cell types highlights their promising role in regenerative therapy for IHD. However, there are several critical issues unresolved before clinical translation. Two major concerns are the ability of undifferentiated ESCs and iPSCs to form teratomas and the possibility to trigger host immune response after allogeneical transplantation. Therefore, it is imperative to develop noninvasive imaging modalities that allow for longitudinal, repetitive, and quantitative assessment of transplanted cell survival, proliferation, and migration in vivo, which enable early detection of teratoma formation and immunological rejection.

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**Imaging of Immunogenicity of Transplanted Stem Cells**

Although the ESCs was initially expected to possess “immune privilege” for low expression of MHC-I and no MHC-II, previous studies showed that transplanted ESCs could trigger intense host immunologic rejection (62). This immunogenicity will further increases upon ESCs differentiation and teratoma formation. This notion is supported by a study using murine model of MI which demonstrated intra-graft infiltration of inflammatory cells following allogeneic injection of undifferentiated mouse ESCs and accelerated immune response against ESCs that had differentiated in vivo for two weeks (69). Not only are ESCs immunogenic, even iPSCs (86) and MSCs (60) that are previously believed to express lower levels of alloantigens and other co-stimulatory molecules, have been recently reported to be visible to the recipient's immune system. However, the immunogenicity of iPS is still controversial, as a latest study suggested limited immunogenicity of transplanted cells differentiated from iPSCs and ES cells (4). Therefore, the immunogenicity of different donor cell types for IHD treatment should be rigorously investigated in preclinical studies.

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Monitoring cell viability is a critical requirement to assess immunogenicity, as a provoked immune reaction can kill transplanted cells. In small animals, molecular imaging has not only
been used to confirm the fact that stem cell rejection occurs in immunocompetent recipients, but has also provided insight into therapeutic strategies to prevent rejection [possible strategies to minimize rejection of stem cell transplants have been extensively reviewed elsewhere (9)]. One study used BLI to show that transplanted xenogeneic human ESCs in immunocompetent mice survived only 7 to 10 days after primary injection, and only 3 days after repeat transplantation (68). And another study used BLI to demonstrate that short-term immunosuppression with leukocyte costimulatory blockade agents allowed long-term engraftment of xenogeneic human ESCs and iPSCs (58).

**Imaging of Tumorigenicity of Transplanted Stem Cells**

Although the pluripotent character of ESCs and iPSCs make them extremely suitable for regeneration therapy, they can also exert unintended tumorigenic adverse effects. We have previously observed the formation of intracardiac and extracardiac teratoma 4 weeks after injection of murine ESC into the myocardium of adult nude rats (12), suggesting a major hurdle that we have to overcome before taking pluripotent cells from bench to bedside for myocardial repair.

Although it is not fully understood now how teratoma formation occurs within differentiated ESC- or iPSC-derived cell lines, it is believed that the presence of remnant, undifferentiated cells present within the transplanted cell population may, at least in part, contribute to teratoma formation. The molecular basis of the tumorigenicity of pluripotent cells lies in their cancer-resembling properties, including self-renewal, rapid proliferation, lack of contact inhibition and telomerase activity, which are promoted by several molecular processes and have been described in detail elsewhere (38). Clear insights into the gene expression of pluripotent cells will allow researchers to select a pluripotent cell population with a reduced tumorigenic signature. It should be noted that no selection method is available at present to yield 100% pure cell population, which is a major obstacle for in vitro manipulation of ESCs and iPSCs. Interestingly, transplantation of selected hESC-derived cells in a more developed phase into immunosuppressed rats did not result in teratoma formation, even when this population is not 100% pure (82.6 ± 6.6%, range 71–95%) (41). Nevertheless, intracardiac injection of undifferentiated mESCs leads to teratoma formation inevitably in all recipients after only 3-4 weeks (12, 57). Clinical translation of pluripotent cells will therefore partly depend on the ability to purify the cell population and to detect teratoma formation early. Nongenetic method using a mitochondria fluorescent dye, tetramethylrhodamine methyl ester perchlorateselectively, has been recently reported to selectively mark iPSC-derived cardiomyocytes, and purify cardiomyocyte by fluorescence-
activated cell sorting (32), Biochemical differences in glucose and lactate metabolism between cardiomyocytes and noncardiomyocytes, including undifferentiated cells als enabled mass-production of iPSC-derived cardiomyocytes(74). Both methods obtained cardiomyocytes of up to 99% purity that did not form tumors after transplantation.

Tumor detection can be achieved by several non-invasive imaging modalities to track the survival and proliferation of pluripotent cells in vivo. The most commonly used technique BLI using Fluc. BLI is easy to use and very sensitive to detect teratoma formation even before a palpable tumour is formed (42, 76). It has been shown that no teratoma formation is observed following intramyocardial injection of a maximum of $1 \times 10^4$ undifferentiated hESCs into immunodeficient mice (42). These findings provided important pre-clinical insights into the effects of hESC numbers and local niches on teratoma development as well as the kinetics of teratoma formation.

However, BLI is not suited for clinical application because of its limited penetration of the signal through tissue in larger animals, and the lack of spatial resolution. In contrast, MRI and PET are superior but have significantly lower detection threshold. Therefore, the use of multimodality imaging may provide a better solution. We have tested BLI combined with PET imaging to monitor transplanted ES cell survival and proliferation in vivo and assess the efficacy of suicide gene therapy as a backup safety measure against teratoma formation (10). In specific, teratoma ablation was achieved via a reporter-suicide gene construct. mESCs were stably transduced with a triple-fusion (ESC-TF) reporter gene that consisted of mRFP, Fluc and HSV-ttk transplanted into adult nude mice. BLI and PET imaging were performed using D-luciferinand $[^{18}\text{F}]$-FHBG reporter probes, respectively. ESCs were completely ablated by Ganciclovir treatment 3 weeks after transplantation.

Although this study showed ablation of teratomas formed from ESCs, the construct is still based on genetically modifying the pluripotent cells by viral transduction, which has tumorigenic potential as well. Future approaches therefore should focus on site-specific genomic integration approaches such as zinc finger nuclease (34) or phiC31 integrase (47) to minimize potential adverse effects to the cells. Alternatively, one could bypass the reporter gene technique by designing molecular probes that target cell surface receptors of teratoma, as was recently demonstrated using $^{64}\text{Cu}$-labelled RGD tetramer that targets $\alpha\beta3$ integrin receptors on hESC-derived teratomas (11).

**Conclusion and Future Perspectives**
Imaging cell fate after transplantation is a high priority in both basic research and clinical translation. For cell-based therapy to truly succeed, we must be able to track the locations of delivered cells, the duration of cell survival, and any potential adverse effects. The ideal imaging platform should be biocompatible, safe and nontoxic; noninvasive in living subjects; capable of detecting single cell and quantifying cell number; and have no dilution with cell proliferation. The advances in the field of molecular imaging hold promise for answering various questions about the optimal cell types, cell dosage, timing of administration, as well as cell location and viability over time. Future efforts should continue focusing on (i) labelling cells without adverse effects on cellular function, (ii) tracking them in vivo with high sensitivity, spatial resolution, and over long periods of time without loss of signal, (iii) demonstrating differentiation of cardiomyocytes function in a normal physiological way in vivo, (iv) targeting cells upon tumor formation and preventing immunological rejection. These goals are laden with obstacles that will ultimately require integration of molecular biology, cell biology, immunology, tissue engineering, transplantation biology, and clinical expertise. The development of multimodality imaging approaches will pave the way for safe myocardial regeneration in IHD.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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**FIGURE LEGENDS**

**Fig.1** Temporal survival kinetics of thymidine kinase (TK)-expressing hCPC with differential uptake of [18F]-FHBG in living animals.

(A) MicroPET qualitative assessment of [18F]-FHBG uptake in TK-expressing hCPCs shows uptake of [18F]-FHBG was highest in hCPCs stably expressing the A168H and sr39-tk variants. 

(B) Representative images of adult SCID beige mice 24 hours after intramyocardial transplantation of 1×10^6 TK-expressing hCPCs. (C) Longitudinal PET imaging of a representative adult female SCID mouse injected with 1×10^6 hCPCs stably expressing the A168H variant of TK. (D) Progressive decrease in both PET and BLI signal intensity was observed over the 28-day time course (n=37 includes both A168H and sr39-tk groups). hCPC indicates human cardiac progenitor cell; PET, positron emission tomography; TK, thymidine kinase; BLI, bioluminescence imaging. Reprinted with permission from Liu et al. *Circ Cardiovasc Imaging*. 2012.

**Fig.2** *In vivo* cardiac hybrid single photon emission computed tomography/computed tomography (SPECT/CT) demonstrates successful induction of myocardial infarction and suggests long-term survival of sodium iodide symporter transgenic human induced pluripotent stem cell (NISpos-hiPSC) grafts.

Representative images of a heart of 1 of 3 recipient animals that were euthanized 6 hours after cell injection are shown. Line 1: SPECT/CT *in vivo* imaging of the left ventricle demonstrates a loss of myocardial perfusion (blue) in the anteroapical and septal walls after occlusion of the left anterior descending coronary artery. Noninfarcted myocardium appears orange colored, indicating normal 99mTc-tetrofosmin uptake. Line 2: Three-dimensional NOGA mapping of the left ventricle recorded during cell injection. NOGA colors represent unipolar voltage values: red=scar, green to blue=viable tissue. Cell injection sites in the lateral (NISpos-hiPSCs + mesenchymal stem cells), septal (NISpos-hiPSCs), and anterior (nontransgenic mesenchymal stem cells) walls are shown as brown dots. Line 3: SPECT/CT imaging of left ventricle 1 hour after catheter-based intramyocardial cell injection demonstrating intense 123I signals (yellow) in the lateral and septal walls that correspond exactly to the injection sites (each injection area 5×10^7 hiPSCs), as recorded by NOGA mapping; control cells did not result in a detectable radiotracer signal (anterior wall). Coadministration of mesenchymal stem cells markedly increased signal intensity (lateral wall). Immunohistochemistry: Depicted are immunohistological sections of the lateral ventricle wall showing a cell injection channel 6 hours after cell application filled with
cotransplanted human mesenchymal stem cells and Venus\textsuperscript{pos} NIS\textsuperscript{pos}-hiPSCs stained for Venus and OCT4 (each with brown color). Reprinted with permission from Templin et al. Circulation 2012.

Fig. 3 Multidimensional imaging in a porcine model of myocardial infarction injected with human induced pluripotent stem cells (hiPSCs) transfected with sodium–iodine symporter (NIS).

(A) Cardiac 3D fusion of coronary computed tomography angiography (CCTA) with $^{99m}$Tc-tetrofosmin single photon emission computed tomography (SPECT) revealing anterior myocardial perfusion defect at rest (red area, normal myocardium depicted in yellow) of the left ventricle. Aortic arch together with adjacent coronary tree clearly identifies cardiac anatomy. Right cardiac chambers are visualized transparently. (B) Cardiac 3D fusion of CCTA and SPECT locating $^{123}$I labeled NIS-transfected hiPSCs (depicted as white hot spots with red border zones) in the anterior wall (myocardium shown in yellow) of the left ventricle. (C) Cardiac 3D triple fusion of CCTA, $^{99m}$Tc-tetrofosmin SPECT and $^{123}$I SPECT clearly visualizing myocardial perfusion defect (shown in red/pink) and myocardial regions containing NIS-transfected hiPSCs (white spots) demonstrating accurate localization of stem cells in the border zone of the infarcted myocardial tissue. (D) Polar plots of NOGA unipolar voltage mapping and linear local shortening (LLS) together with 3D rendered volume of LLS (E) with NOGA-guided intramyocardial injected stem cells (brown spheres). Red areas indicate a loss of electrical activity (perfusion defect), blue a normal voltage signal (normal myocardium), and yellow and green areas of decreased perfusion (border zone of the infarcted myocardial tissue). (F) Overlay of NOGA-guided intramyocardial injected NIS-transfected hiPSCs on cardiac triple fusion hybrid image resulting in 3D Quadruple-Fusion volume revealing accurate positioning of intramyocardial sites of injection to myocardial areas containing $^{123}$I labeled NIS-transfected hiPSCs. Note sites of injection of control cells lacking $^{123}$I which are not co-localized with $^{123}$I hot spots. Reprinted with permission from Fiechter et al. Int J Cardiol 2012.

Fig. 4 Mechanistic molecular imaging of human mesenchymal stem cells (hMSCs) for murine myocardial infarction.

(A) Validation of endothelial expression of the reporter gene in target cells in vitro. A, Schematic representation of lentivector, the dual reporter construct. The mCherry-Renilla luciferase ($C/r$-Luc) fusion reporter gene is driven by the murine stem cell virus (MSCV) constitutive promoter. The eGFP-firefly luciferase ($G/f$-Luc) fusion reporter gene is driven by the endothelial cell–specific promoter, Tie-2. The transcriptional activity of juxtaposed promoters is oppositely directed. (B)
Representative fluorescent microscopy images illustrate the specific and constitutive expression of reporter genes in hMSCs transduced with lentivirus. Tie-2-driven expression of G/f-Luc in hMSCs was not detected by fluorescence microscopy. Scale bars in B, 200 um. (C) In vitro assessment of bioluminescence imaging (BLI) signal in mouse endothelial cells (mETCs; positive control) and hMSCs is shown 72 hours after lentivirus transduction. The BLI signal is detected in transduced mETCs with coelenterazine (CLTZ) and D-luciferin (D-Luc), indicating expression of both Tie-2–driven r-Luc and constitutive f-Luc. In contrast, only r-Luc–induced BLI signal is detected in transduced hMSCs. Quantification of the BLI signals (photons/s) is illustrated as a bar graph (bottom). (D) BLI of graded numbers of hMSCs 48 hours after lentivirus transduction was performed to correlate signal intensity and cell number. Quantification of BLI signal intensity shows a robust correlation between r-Luc activity and the number of transduced hMSCs (bottom). (E) The BLI signals in the heart of a representative mouse were superimposed on photographs of a representative SCID mouse for the indicated time point after injection of hMSCs/medium after AMI. The signals were probed by intraperitoneal injection of CLTZ (top) or of D-Luc (bottom). (F) Quantitative BLI intensity in SCID mice after hMSC/medium injection. BLI intensity was assessed by measuring the photon flux from the region of interest (ROI) drawn over the precordium. Immunostaining for CD31 and GFP showed the presence of endothelial differentiated hMSCs (GFP+) in the vessels of the border zone at 2 weeks after hMSC injection. Scale bar, 200 um. Reprinted with permission from Zhang et al. Circ Cardiovasc Imaging 2012.

Fig.5 Molecular imaging for tracking functional survival of the porcine pluripotent stem cells derived endothelial cells (piPSC-ECs).

(A) Representative M-mode echocardiographic views of infarcted hearts receiving PBS, pASCs, pAorta-ECs, and piPSC-ECs (n=20 per group). (B) Quantification of fractional shortening (FS) reveals significant improvement in systolic function of animals receiving piPSC-ECs at week 2 and week 4 after-MI compared with animals receiving PBS. Greater improvement was also seen in animals receiving piPSCs compared with pASCs, although this did not reach statistical significance. Comparable improvement is observed between animals receiving piPSC-ECs and their endogenous counterpart, pAorta-ECs. (C) Representative BLI of an animal receiving 1x10^6 piPSC-ECs demonstrated robust cell engraftment at day 2 after injection. Progressive decrease in signal was observed over the next several weeks, but persistent cell engraftment is still noted at week 4. (D) Representative axial non-enhanced PET-CT fusion image of piPSCs (left) with coronal reconstruction of PET data set (right) one hour after delivery of cells into the peri-infarct area. A strong signal (0.05±0.2 %ID/g) can be seen at the sites of the injection in the distal left
ventricular wall (noted by single arrow). (E) Representative T2 weighted GRE images by MRI shows iron-labeled cells as hypointense signals (dark areas noted by the single arrow) in the apical lateral wall (left), which corresponds to areas of strong signal noted on PET imaging. Cells were injected near the region of infarct, noted by areas of hyperenhancement on gadolinium enhanced MRI (bright white areas shown on right). Reprinted with permission from Gu et al. Circ Res 2012.

**Fig.6 Rosuvastatin promoted the engraftment and therapeutic effect of transplanted mADSCsFluc+GFP+ in infarcted myocardium.**

(A) Representative BLI images of mADSCFluc+GFP+ administered with or without rosuvastatin. To assess longitudinal cell survival, animals were imaged for 3 weeks. The bioluminescence signal decreased gradually from day 1 to day 21 after transplantation in both groups. But at day 7 and 14, signals in the AD-MSCs+R-tin group (bottom row) were significantly higher than those in the AD-MSC group (top row). Color scale bar values are in P·s⁻¹·cm⁻²·sr⁻¹. (B) Quantitative analysis of imaging showed a drastic decrease in Fluc activity in the AD-MSC group and significantly higher activity in animals with combined transplantation of AD-MSCs and rosuvastatin at day 7 and 14. (C) Representative illustrations of Masson’s trichrome staining. Red indicates viable myocardium; blue indicates fibrosis due to infarction damage. (D) Quantitative analysis of the fibrotic area showed that the fibrotic area in the AD-MSCs+R-tin group was significantly decreased compared with the MI group, *p<0.05 vs. MI. The magnification for (A) is 4X. Reprinted with permission from Zhang et al. Basic Res Cardiol 2013.