Stem Cell Monitoring with a Direct or Indirect Labeling Method

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Abstract The molecular imaging techniques allow monitoring of the transplanted cells in the same individuals over time, from early localization to the survival, migration, and differentiation. Generally, there are two methods of stem cell labeling: direct and indirect labeling methods. The direct labeling method introduces a labeling agent into the cell, which is stably incorporated or attached to the cells prior to transplantation. Direct labeling of cells with radionuclides is a simple method with relatively fewer adverse events related to genetic responses. However, it can only allow short-term distribution of transplanted cells because of the decreasing imaging signal with radiodecay, according to the physical half-lives, or the signal becomes more diffuse with cell division and dispersion. The indirect labeling method is based on the expression of a reporter gene transduced into the cell before transplantation, which is then visualized upon the injection of an appropriate probe or substrate. In this review, various imaging strategies to monitor the survival and behavior change of transplanted stem cells are covered. Taking these new approaches together, the direct and indirect labeling methods may provide new insights on the roles of in vivo stem cell monitoring, from bench to bedside.

Keywords Stem cell tracking · Radionuclide · Direct labeling method · Indirect labeling method

Introduction

Stem cell therapy applies stem cells to injured tissue to treat disease or injury. Stem cells have the ability to regenerate and differentiate, and they can potentially replace diseased or damaged cells. The current therapy for acute myocardial infarction (MI) is based on stem cells, presenting a significant advancement over many traditional approaches to disease treatment [1–3]. Experimental and clinical studies of stem cell transplantation in the heart revealed that stem cells can differentiate into cardiac myocytes, endothelial cells, and secrete paracrine factors that can reduce myocyte death, improve the microcirculation, attenuate adverse remodeling, and boost the regenerative capacity of MI. Stem cells can be isolated from various sources including bone marrow, skin, amniotic fluid, and adipose tissue [4–8]. The main goal of stem cell therapy is to repopulate the damaged tissue with functional cells. Over the last decade, progress in molecular imaging has allowed the investigators to answer some of these unsolved problems by providing a deep understanding of stem cell behavior in a living body [9, 10]. Particularly, some of the imaging strategies have the potential to be translated to patients, which makes them appropriate for use in clinical trials [11]. In vivo cell-tracking methods are able to monitor noninvasively and in real time at the injected site and to allow observation of the distribution and viability of the therapeutically administered cells. There are two main classes of cell labeling methods for molecular imaging: the direct and indirect labeling methods. For in vivo visualization, transplanted cells need to be directly labeled ex vivo with radiotracers [12–18], nanoparticles, or quantum dots [19–22], or they have to

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express a reporter gene by indirect labeling [23, 24]. Direct labeling was performed prior to cell transplantation with ^{99m}Tc -hexamethylpropylenamine oxime (^{99m}Tc -HMPAO) or ^{111}In -oxine [25]. It has been used in conjunction with single-photon emission computed tomography (SPECT), superparamagnetic iron oxide particles (SPIO) for magnetic resonance imaging (MRI), ^{64}Cu -pyruvaldehyde-bis(N4-methylthiosemicarbazone (^{64}Cu -PTSM) [26], and 2- ^{18}F -fluoro-2-deoxy-D-glucose (^{18}F -FDG) [27] with positron emission tomography (PET) for tracking various cell types following transplantation. Indirect labeling for nuclear medicine, including the herpes simplex virus type 1 thymidine kinase (HSV1-tk) [23] or human sodium iodide symporter (hNIS) [24], has been used for cell tracking in the heart. Direct and indirect labeling methods are powerful tools for molecular imaging, and they have advantages and disadvantages. Therefore, each labeling modality should be used properly according to their purposes.

Items Desired for Ideal Stem Cell Imaging Modalities

The ideal imaging agent/modality should provide the real-time visualization of distribution over time, quantification of viable transplanted stem cells, and long-term quantification of transplanted stem cell survival. Also, the applied labeling modality should not disturb the normal functions of stem cells.

The biocompatibility, toxicity, and safety should be considered seriously not only for the stem cells, but also for the stem cell recipient individuals, and they should be considered when choosing which modality to use for imaging. All imaging modalities have a certain degree of background/nonspecific signal, which may be confused with the signal interpretation under study. The preferred imaging modality should be able to provide a good contrast between the target and background signal by achieving a large signal-to-noise ratio. Additionally, it should have good specificity. Table 1 introduces different imaging modalities for the assessment of delivery and short- and long-term monitoring of stem cell viability and biology.

Direct Labeling

One of the most commonly used methods for the labeling of stem cells in molecular imaging is direct labeling [28–31]. In a direct labeling strategy, labeling agents are introduced into the cells prior to transplantation. Depending on the imaging modality to be used, stem cells can be labeled with radionuclides for SPECT or PET [32–35] and SPIO for MRI (Fig. 1) [36, 37]. Direct cell labeling is fairly simple and does not involve genetic modification of the cell. For example, cells are actively labeled with the appropriate method *in vitro* and are administered to a recipient. One disadvantage of direct cell labeling methods is that the label is diluted when cells divide, resulting in decreased amounts of labeled agent per individual cell. Furthermore, the label can be distributed asymmetrically to the progeny cells according to cell division or can be lost by the cells. These factors act as a limitation for long-term imaging. Additionally, iron-based agents for MRI require long incubation periods for cell labeling and are not suitable for patients with an intracardiac defibrillators or pacemakers. PET and SPECT imaging using a radionuclide has the high sensitivity and spatial resolution. Therefore, it can provide dynamic tracking imaging and the tissue distribution of transplanted stem cells injected as a therapeutic agent. Direct labeling agents for cell tracking have been developed for *in vivo* imaging [38, 39], such as ^{18}F -FDG, N-succinimidyl-4- ^{18}F -fluorobenzoate (^{18}F -FSB), hexadecyl-4- ^{18}F -fluorobenzoate (^{18}F -HFB), ^{99m}Tc -HMPAO, and ^{111}In -oxine [40–42]. Most radionuclides used for direct labeling have a limitation for long-term monitoring due to their short half-lives (^{18}F , $t_{1/2}=110$ min; ^{99m}Tc , $t_{1/2}=6$ h; ^{111}In , $t_{1/2}=2.8$ d). ^{18}F -FDG is taken up and retained in cells with glucose transporter and hexokinase, respectively. ^{99m}Tc -HMPAO and ^{111}In -oxine are nonspecific cell-labeling agents and are retained in cells by penetration through the cell membrane [43]. Hexadecyl-4- ^{124}I -iodobenzoate (^{124}I -HIB) is a radiotracer synthesized from a lipophilic long-chain ester, which is easily and efficiently anchored into cellular membranes by simply incubating with target cells. HIB is a versatile labeling agent with any radioiodine such as ^{123}I , ^{124}I , and ^{131}I (^{123}I , $t_{1/2}=13.2$ h; ^{124}I , $t_{1/2}=4.2$ d; ^{131}I , $t_{1/2}=8.1$ d) according to the user's purpose. Kim et al. monitored the change in cell survival of transplanted adipose-

Table 1 Different imaging modalities for the assessment of delivery and short- and long-term monitoring of stem cell viability and biology

Purpose	Cell delivery	Early cell localization and homing	Long-term monitoring of cell viability & stem cell biology
Strategies	Direct visualization	Direct labeling	Indirect labeling
Imaging modalities	Ultrasound MR fluoroscopy	Fluorescence (e.g., quantum dots fluorophores) MRI (e.g., SPIO) SPECT (e.g., ^{111}In -oxine, ^{99m}Tc -HMPAO) PET (e.g., ^{18}F -FDG, ^{64}Cu -ATSM)	Fluorescence (RFP, GFP) Bioluminescence (fLuc, rLuc) SPECT (NIS) PET (HSV1-tk, D2R, CD) MRI (Transferrin, Ferritin, Tyrosine)

Fig. 1 Direct cell labeling methods. In a direct labeling strategy, various labeling agents are for radionuclide imaging (**a**, **b**) or MRI (**c**) and are introduced into the inner part of stem cells (**a**, **c**, e.g., ^{18}F -FDG, ^{111}In -oxine, $^{99\text{m}}\text{Tc}$ -HMPAO, or SPIO) or anchored to the cell membrane (**b**, e.g., ^{64}Cu -DOTA-HB, $^{123/124}\text{I}$ -HIB). To track the transplanted stem cells, first, the direct-labeled stem cells are transplanted to the target tissue/organ. Then, noninvasive imaging including MRI or radionuclide imaging using PET or SPECT is performed at the target site for detection of transplanted stem cells

derived stem cells (ADSCs) responding to myocardial infarction (MI) via the hexadecyl-4- ^{124}I -iodobenzoate (^{124}I -HIB)-mediated direct-labeling method in vivo. They reported that in vivo tracking of the ^{124}I -HIB-labeled ADSCs was possible for 9 and 3 days in the normal and MI model, respectively,

Fig. 3 Small-animal PET/CT image of intra-myocardially injected-labeled ADSCs (1.8–3.7 MBq, 100 μl) in normal heart for 18 h. Arrow indicates the site of ^{64}Cu -DOTA-HB-labeled ADSC injection

which was feasible only by an indirect labeling method previously using the reporter gene [29, 30] (Fig. 2). They showed that the radioactivity of the transplanted ^{124}I -HIB-labeled ADSCs remained at 24.67 ± 1.18 % of the day 1 value (100 %) ($P < 0.05$) 9 days after transplantation in the normal myocardium (Fig. 2). However, in the MI, the radioactivity of the transplanted ^{124}I -HIB-labeled ADSCs remained at 13.62 ± 7.66 % of the 0.08 day (2 h) value (100 %) ($P < 0.05$) 3 days after transplantation (Fig. 2). This difference of in vivo monitoring periods reflects the poor viability of transplanted ADSCs in host tissue due to harsh microenvironments such as ischemia, inflammation, and/or anoikis in the infarcted myocardium [44].

Fig. 2 Small-animal PET/CT imaging of transplanted ^{124}I -HIB-labeled ADSCs in the normal and MI model and longitudinal quantification of the relative percent of the injected dose (%ID). **a**, **b** Small-animal PET/CT images of transplanted ^{124}I -HIB-labeled ADSCs in the normal (**a**) and MI model (**b**) for 9 days. **c**, **d** Longitudinal quantification of the relative %ID

of transplanted ^{124}I -HIB-labeled ADSCs in the normal (**c**, $^*P < 0.05$ vs. day 1) and MI model (**d**, $^*P < 0.05$, $^{**}P < 0.001$ vs. 2 h) for 9 days. Arrows indicate the injection site of ^{124}I -HIB-labeled ADSCs. A: anterior, P: posterior, RV: right ventricle, LV: left ventricle

Fig. 4 Various types of reporter gene/reporter probe strategies. **a** Enzyme-based bioluminescence imaging. D-luciferin is a substrate molecular probe that is acted upon by the enzyme firefly luciferase (fLuc) to result in bioluminescence via a chemiluminescent reaction under physiological conditions only within living cells expressing the fLuc gene. **b** Enzyme-based PET imaging. 8- ^{18}F -fluoropenciclovir (^{18}F -FPCV) or other acycloguanosines are substrate molecular probes phosphorylated by the herpes simplex virus type 1 thymidine kinase (HSV1-tk) enzyme to result in the intracellular trapping of the probe in cells expressing the HSV1-tk gene. **c** Receptor-based PET imaging. 3-(2'- ^{18}F -fluoroethyl)sipiperone (^{18}F -FESP) is a ligand molecular probe interacting with the dopamine receptor D2 (D2R) to result in trapping of the probe on/in cells expressing the D2R gene

Fig. 5 Schematic presentation of NIS function. NIS transports two sodium ions and one iodide ion into the cytoplasm together. The electrochemical sodium gradient generated by the Na^+/K^+ ATPase pump provides energy for this transfer

^{64}Cu has a relatively long half-life (12.7 h), and its decay property [β^- , 0.573 MeV (38.4 %); β^+ , 0.655 MeV (17.8 %); γ , 0.511 MeV (35.6 %)] allows longitudinal PET imaging and tracking for transplanted cells [14, 45]. ^{64}Cu can react with a wide variety of chelator systems including 1,4,7,10-tetraazacyclododecane-tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1,4,7-triacetic acid because of its well-

established coordination chemistry [46, 47]. DOTA is currently used in ^{64}Cu -diacetyl-bis(N4-methylthiosemicarbazone (^{64}Cu -ATSM), approved by the US Food and Drug Administration (FDA) in the USA. Therefore, the DOTA chelator is better clinically available and has a simple synthetic process [48]. ^{64}Cu can potentially be linked to the target, including peptides, antibodies, or nanoparticles, via a specified chelator system. Hexadecyl benzoate (HB) has a lipophilic property, so it easily and tightly anchors with the cell membrane, similar to HIB. Moreover, it is free from an effect mediated by other receptors existing in the cell. Kim et al. reported that ^{64}Cu -labeled hexadecyl-1,4,7,10-tetraazacyclododecane-tetraacetic acid-benzoate (^{64}Cu -DOTA-HB)-labeled ADSCs were monitored for 18 h in normal rat heart by PET imaging (Fig. 3). Adonai et al. reported that the lipophilic agent, ^{64}Cu -PTSM, was rapidly effluxed from C6 rat glioma cells; radioactivity was decreased by $\approx 80\%$ at 24 h [14]. Also, Park et al. reported that the radioactivity of intravenously injected ^{64}Cu -PTSM-labeled K562 (human erythromyeloblastoid leukemia cell line) cells were rapidly decreased in the lungs from 2 h to 24 h [49]. However, the intramuscularly injected ^{64}Cu -DOTA-HB-labeled ADSCs were clearly visualized at the injected site for 18 h. These results indicate that HIB or HB can utilize promising radiotracers for monitoring of transplanted stem cells [50].

Fig. 6 In vivo cell fate in a canine MI model. **a** Representative transaxial $^{99\text{m}}\text{TcO}_4^-$ SPECT images of the saline vehicle control group and experimental group. When compared to the saline vehicle control group, the experimental group showed distinguishable $^{99\text{m}}\text{Tc}$ accumulation until day 9, which reflects the viability of injected stem cells in the left ventricular wall. **b** Time course of myocardial accumulation of $^{99\text{m}}\text{Tc}$ - TcO_4^- counted from serial $^{99\text{m}}\text{Tc}$ - TcO_4^- SPECT images in the saline vehicle control group and experimental group

Indirect Labeling

There is an increasing need for the understanding of the post-transplantation behavior of stem cells. Reporter genes for imaging can precisely monitor stem cell behavior longitudinally, unlike direct labeling. Target cells can be genetically modified to be labeled by the introduction of a reporter gene. Reporter gene-based imaging offers unique advantages of long-term imaging for cell survival and longitudinal measurements. Although indirect labeling using reporter genes has disadvantages including the cost, cellular dysfunction, cell death, immunogenicity of the gene product, and potential risk for uncontrolled growth or malignancy, the advanced development in molecular biology and imaging modalities has produced a reporter gene system that allows the evaluation of transgene expression in various diseases. From these advancements, various types of reporter genes or probe systems have developed. (Fig. 4) [19, 51–59]. Reporter genes consist of gene regulatory elements, such as promoters and enhancers, which drive the reporter gene DNA sequence and poly A sequence providing stabilization on the final product. Initially, reporter genes were mainly used in histology and ex-vivo studies (beta-galactosidase and luciferase) to assess the transgene expression in many different tissues and organs [60–62]. The reporter gene needs to include cells before transplantation for stem cell imaging. The incorporated reporter gene will be expressed only in the case that stem cells are viable. However, in the case of cell death, the signal of the reporter gene becomes extinct. The substrate can be externally administered at the interested point of imaging time, and the specific signal was expressed by the interaction between the substrate and encoded reporter protein. Through bioluminescence imaging or radionuclide imaging, the specific signal of the reporter gene can be detected noninvasively. The reporter gene was commonly used for monitoring survival in the transplanted stem cells with the reporter gene controlled by a cytomegalovirus (CMV) based on the constitutive promoter [63]. When the cell is viable and transcriptional machinery intact, it will result in the production of a reporter protein. Additionally, particular biological pathways can be investigated using the reporter gene.

NIS is an intrinsic membrane protein in thyroid cells with 13 transmembrane domains, which mediates the active transport of iodine into thyroid follicular cells (Fig. 5). NIS genes can be used as imaging reporter genes in PET as well as SPECT imaging, because they concentrate radionuclides such as ^{124}I , ^{131}I , and $^{99\text{m}}\text{Tc}$ -pertechnetate, which can be visualized by conventional γ -camera or PET [64–66]. Terrovitis et al. showed that NIS-expressing (NIS⁺) rat cardiac-derived stem cells (rCDCs) were visualized in vivo up to 6 days post-injection in a rat MI model by SPECT [67]. Lee et al. showed that intramyocardially injected rats with NIS-expressing adenovirus-transfected canine stem cells (Ad-hNIS-canine ADSCs) were monitored for 9 days in the canine MI model by $^{99\text{m}}\text{Tc}$ -pertechnetate ($^{99\text{m}}\text{TcO}_4^-$) SPECT imaging (Fig. 6) [68].

A specific promoter is used for the investigation of certain pathways, and the target reporter protein is made only when the intracellular signal is active for protein production. The activation of that specific promoter will drive the expression of the reporter gene, and this could be visualized noninvasively by using the proper imaging modalities. Kang et al. developed transgenic mice using NIS expression driven by the α -MHC promoter for cardiomyocyte-specific expression, in which NIS is constitutively expressed as an imaging reporter gene only in cardiomyocytes. They showed that the myocardium of transgenic mice showed rapid and intense uptake of $^{99\text{m}}\text{Tc}$ -pertechnetate and also showed long retention by γ -camera imaging [24]. Kim et al. reported that bone marrow-derived mesenchymal stem cells (BMSCs) from transgenic mice [24], using NIS expression driven by the α -MHC promoter, expressed upregulated NIS gene activity according to in vitro cardiomyogenic differentiation (Fig. 7) [69]. These results will likely lead to future

Fig. 7 Expression of cardiac-specific genes and stem cell-specific markers according to cardiomyogenic differentiation of BMSCs. **a** Expression of cardiac-specific genes (α -MHC, MLC-2V) and a stem cell-specific marker (Sca-1) in cardiomyogenic differentiated BMSCs treated with ATRA. **b** Relative quantification of gene expression normalized against GAPDH. **c** Radioiodine (^{125}I) uptake of cultured BMSCs with or without ATRA in vitro. Cardiomyogenic differentiated BMSCs treated with ATRA showed higher ^{125}I uptake than nontreated BMSCs

applications of cell survival and differentiation monitoring in vivo using the NIS reporter gene.

Summary

In this review, transplanted stem cell survival and differentiation were monitored using direct and indirect labeling. Generally, direct imaging techniques only enable the short-term monitoring of stem cells because of the physical half-life of the labeled radionuclide.

However, in the current study, transplanted ADSCs were longitudinally monitored by PET in the normal and MI model using ^{124}I -HIB, which has a relatively long half-life, which reflects cell survival change against the harsh microenvironment of MI. Survival of transplanted stem cells was longitudinally monitored by using NIS reporter genes. Also, cardiomyogenic differentiation of stem cells in vitro was monitored using a tissue-specific promoter, an α -MHC promoter-driven NIS reporter gene. These new approaches including direct and indirect imaging may provide new insights into the roles of stem cell monitoring in the living body for stem cell therapy, from pre-clinical studies to clinical trials.

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Compliance with Ethical Standards

Conflict of Interest Min Hwan Kim, Yong Jin Lee, and Joo Hyun Kang declare that they have no conflict of interest, etc.

Ethical Statement The care, maintenance, and treatment of animals in these studies followed protocols approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences (KIRAMS).

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