Is Reliable *in vivo* Detection of Stem Cell Viability Possible in a Large Animal Model of Myocardial Injury?

**Running title:** Yang; *Long term in vivo tracking of iPSCs*

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**Journal Subject Code:** [32] Nuclear cardiology and PET

**Key words:** Editorials; imaging; stem cells
In vivo cellular and molecular imaging of the stem cells has been developed to characterize the biological processes at the most fundamental level in an intact, living organism. With the emergence of cell-based therapy in a failing heart, the capability to evaluate engraftment and survival of the transplanted stem cells in vivo represents a critical measure of therapeutic efficacy. The stem cells at the very least must survive to restore the injured myocardium. Cell viability signal holds physiologic relevance as it may correlate with the resultant myocardial restoration. There are 2 primary considerations for in vivo imaging of stem cell viability in the heart: 1) amplification of molecular and cellular signals and 2) high spatial and temporal resolution imaging of the myocardium. It is widely acknowledged that there is no single imaging modality that will fulfill all needs of in vivo stem cell imaging. However, an imaging modality that optimizes the technical objectives may extract meaningful information on cellular and molecular events of the transplanted cells. The predominant imaging modalities to assess stem cell survival in vivo in pre-clinical models consist of radionuclide (RN), optical (OI), and magnetic resonance imaging (MRI). These modalities are commonly employed in small animal models. Direct transfer of in vivo cellular and molecular imaging techniques from small to large animal models, however, has not been straightforward.

The report by Templin et al in this issue of Circulation addresses the critical issue of in vivo imaging of stem cell viability in a porcine myocardial injury model. The study evaluated sodium iodide symporter (NIS) transgene to follow in vivo survival of human induced pluripotent stem cells (hiPSCs) and their derivatives. A stable transgenic hiPSC line expressing a fluorescent reporter and NIS (NIS\textsuperscript{pos}-hiPSCs) was employed. Longitudinal dual isotope SPECT-CT imaging was applied using \textsuperscript{123}I to follow donor cell survival and distribution. \textsuperscript{99m}TC-
tetrofosmin was subsequently utilized for myocardial perfusion imaging. In vivo, viable NIS\textsuperscript{pos}-hiPSCs were visualized near the injection sites for up to 15 weeks. The in vivo data were validated by immunohistochemistry, which demonstrated that the hiPSC-derived endothelial cells contributed to intramyocardial revascularisation. Up to 12-15 weeks after transplantation, no undifferentiated hiPSCs or teratoma was detected.

Translation of stem cell technology requires a systematic validation, progressing from a small to large animal model. In order to conduct these pre-clinical studies, different imaging modalities are employed to assess stem cell engraftment and survival. First, PET and SPECT are highly sensitive systems, which detect trace amounts of γ- and β-emitting radionuclide well-suited for tracking systemic biodistribution of cellular and molecular signals. The sensitivity of PET is very high in the range of 10^{-11}-10^{-12} mole/L and is at least 1-2 orders of magnitude better than SPECT\textsuperscript{2}. Advanced molecular imaging applications utilizing various techniques including radiolabeled ligand, antibody, and reporter gene have been developed\textsuperscript{4,5}. Second, the most successful optical method for imaging small numbers of transplanted cells in experimental models is bioluminescence imaging (BLI). The sensitivity reaches 10^{-15}-10^{-17} mole/L\textsuperscript{5}. This modality utilizes an internal biological light source, such as luciferase, which can be detected within the tissues of small animals using sensitive low-light imaging systems and has allowed localization and tracking of as few as 10\textsuperscript{3} cells\textsuperscript{5,7-9}. While optical imaging detects signals from near-cellular level, this technique is limited to small animal imaging due to depth penetration of 1-2 cm\textsuperscript{8}. Third, MRI combines chemical sensitivity of nuclear magnetic resonance with high spatial and temporal resolution. It routinely offers sub-millimeter resolution with temporal resolution in the millisecond range and intrinsically superior contrast mechanism. These specifications provide optimal technical characteristics to assess myocardial physiology.
However, MRI is several magnitudes less sensitive than RN or OI, only capable of detecting molecular signal in the range of $10^{-3}-10^{-5}$ mole/L presenting limited options for MRI molecular probes\(^6\). \textit{Ex-vivo} cell labeling with iron oxide, nevertheless, has improved the MRI sensitivity several fold to $10^{-9}-10^{-11}$ mole/L range approaching the sensitivity of SPECT\(^10\). Novel imaging sequences, higher magnetic fields, hardware development, and targeted contrast agents have enabled \textit{in vivo} MRI to combine molecular and cellular signals with precise 3-D and 4-D characterization of the target organ\(^11\).

While Templin \textit{et al} demonstrated encouraging long-term data on \textit{in vivo} evaluation of stem cell viability, several questions remain. First, there is no clear explanation as to why the combined delivery of hiPSCs and mesenchymal stem cells (MSCs) enabled robust survival for 15 weeks. Although anti-inflammatory and immunosuppressive effects of MSCs have been reported, there is no clear explanation for this robust survival. Second, NIS expression or radiolabeling of NIS+-hiPSCs appeared not to have affected the cell viability in vitro. Nevertheless, the quantification of the \textit{in vivo} signal related to the degree of stem cell engraftment and survival has not been well described. Third, there are number of questions regarding stem cell biology. Does the absence of teratoma and robust endothelial differentiation of the hiPSCs indicate 1:1 differentiation? Even with \textit{ex vivo} commitment to vascular lineage, this is an extremely rare phenomenon. Was there other lineage commitment? If not, why did the hiPSCs differentiate only along the endothelial and not along cardiac or mesenchymal lineage? Finally, critical data on myocardial function following engraftment of the transplanted hiPSCs are missing.

In order to answer these questions, other available imaging options need to be explored. While the investigators are commended for employing SPECT, which is more accessible to the
cardiology community, a more definitive alternative is PET. $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) pre-labeled cells will allow a robust quantification of immediate stem cell engraftment in vivo in a porcine model$^{12}$. However, the approach is limited by the short half-life of only 110 minutes. For longitudinal monitoring, $^{111}$In-labeled SPECT imaging of cells has a half-life of 2.8 days, enabling a follow-up of 2 weeks after cell delivery$^{13}$. However, $^{111}$In-labeling is known to be toxic to cells, allowing labeling of only a limited percentage of the delivered cells. A more long-term strategy, allowing serial monitoring in a large animal model, is the PET herpes simplex virus-thymidine kinase reporter gene system (HSV-tk)$^5$. In this system, the HSV-tk reporter gene is integrated into the cell genome. Although HSV-tk has been demonstrated robustly in small animals, only recently, longitudinal expression (up to 5 months) of HSV-tk has been reported following transplantation of HSV-tk transduced MSCs in a porcine myocardial injury model$^{14}$. A feasible alternative to HSV-tk is the NIS system reported in this issue. One of the advantages of NIS is that it employs an endogenous mammalian gene generating minimal immunogenic response in comparison to HSV-tk$^5$. Prior to this study by Templin et al, successful demonstration of NIS was also limited to small animals.

Another commonly utilized imaging option to localize stem cells in a large animal model is MRI. Earlier techniques required ex vivo labeling of the stem cells to detect successful delivery and subsequent migration using iron-oxide based agents$^{15}$. The robust negative contrast generated by iron oxide agents has enabled reliable cell labeling. Utilization of nanoparticles of iron oxide covalently bound to HIV-1 tat-peptide, anti-transferrin receptor, and transfection agents such as poly-L-lysine and protamine sulfate have demonstrated efficient intracellular labeling$^{15-17}$. Studies have also reported successful tracking at near single-cell resolution$^{18}$. Although iron-oxide nanoparticles have been used to localize the transplanted stem cells reliably,
ex vivo cell labeling with these agents do not detect stem cell viability. In order to assess their survival, several approaches have been proposed in MR molecular imaging. The first approach employs a reporter gene mechanism which utilizes promoter genes to induce specific gene expression. This system exploited conformational change of a Gd-containing compound to be induced by enzymatic cleavage to interact with water protons to generate T1 shortening effects. The second approach integrates reporter system consisting of genetically engineered cells to over-express transferrin receptors. The receptors accumulate the iron particles intracellularly to generate T2-weighted signal. Third approach utilizes iron-oxide tagged monoclonal antibodies to detect a certain end-product of molecular expression. This targeted, antibody-mediated approach to molecular MRI has been demonstrated successfully in a murine myocardial injury model. Finally, non-genomic technique has been developed using manganese-enhanced MRI (MEMRI). MEMRI utilizes an essential heavy metal ion, Mn2+, which enters the viable cells via voltage-gated calcium channels. Intracellular accumulation of Mn2+ shortens T1 relaxation time to generate positive signal. MEMRI combines the high spatial and temporal resolution of MRI with cell viability specific signal to enable dual assessment of myocardial restoration and stem cell viability. Successful demonstration of this technique has been reported in small and large animal studies.

Many in vivo imaging studies have reported limited engraftment of stem cells, mostly dying after transplantation. However, other studies have demonstrated that the stem cells delivered into the myocardium do survive and demonstrate robust viability in both murine and porcine myocardium. Mouse ESCs (mESCs) delivered into 3 regions (remote, peri-, and intra-infarct) of myocardial injury exhibited differential survival and restorative potential. Furthermore, improved survival of mESCs compared to mouse embryonic fibroblasts (mEFs)
shown by in vivo BLI and validated by polymerase chain reaction analysis for the male Sry gene, correlated increased survival of mESCs with enhanced myocardial restoration\textsuperscript{31}. In a porcine model, enhanced survival of MSCs demonstrated by MEMRI and PET correlated with improved myocardial function\textsuperscript{14,28}. Furthermore, this study by Templin \textit{et al} confirms survival of transplanted hiPSCs when optimized by the MSCs. While there are conflicting data on \textit{in vivo} stem cell survival, these studies offer promise to cell therapy in cardiovascular diseases.

Stem cell research stands at a critical juncture where systematic translation of basic discoveries will be crucial. Recent development in nuclear reprogramming of human skin cells to generate pluripotent stem cells and directed differentiation of human embryonic stem cells to cardiomyocytes may lead to significant clinical breakthrough\textsuperscript{32,33}. These advances, however, require rigorous \textit{in vivo} validation. \textit{In vivo} imaging represents a dedicated platform to evaluate and quantitate molecular and cellular events related to cellular engraftment, a critical step in regenerative medicine. This integrative approach should enable systematic translation of basic stem cell science to clinical application.

\textbf{Conflict of Interest Disclosures:} None.

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Circulation. published online July 5, 2012;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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