Cellular Bioenergetics Is an Important Determinant of the Molecular Imaging Signal Derived From Luciferase and the Sodium-Iodide Symporter

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Rationale: Molecular imaging is useful for longitudinal assessment of engraftment. However, it is not known which factors, other than cell number, can influence the molecular imaging signal obtained from reporter genes.

Objective: The effects of cell dissociation/suspension on cellular bioenergetics and the signal obtained by firefly luciferase and human sodium-iodide symporter labeling of cardiosphere-derived cells were investigated.

Methods and Results: 18Fluorodeoxyglucose uptake, ATP levels, 99mTc-pertechnetate uptake, and bioluminescence were measured in vitro in adherent and suspended cardiosphere-derived cells. In vivo dual-isotope single-photon emission computed tomography/computed tomography imaging or bioluminescence imaging (BLI) was performed 1 hour and 24 hours after cardiosphere-derived cell transplantation. Single-photon emission computed tomography quantification was performed using a phantom for signal calibration. Cell loss between 1 hour and 24 hours after transplantation was quantified by quantitative polymerase chain reaction and ex vivo luciferase assay. Cell dissociation followed by suspension for 1 hour resulted in decreased glucose uptake, cellular ATP, 99mTc uptake, and BLI signal by 82%, 43%, 42%, and 44%, respectively, compared with adherent cells, in vitro. In vivo 99mTc uptake was significantly lower at 1 hour compared with 24 hours after cell transplantation in the noninfarct (P<0.001; n=3) and infarct (P<0.001; n=4) models, despite significant cell loss during this period. The in vivo BLI signal was significantly higher at 1 hour than at 24 hours (P<0.01), with the BLI signal being higher when cardiosphere-derived cells were suspended in glucose-containing medium compared with saline (PBS).

Conclusions: Adhesion is an important determinant of cellular bioenergetics, 99mTc-pertechnetate uptake, and BLI signal. BLI and sodium-iodide symporter imaging may be useful for in vivo optimization of bioenergetics in transplanted cells. (Circ Res. 2013;112:441-450.)

Key Words: metabolism ▪ molecular imaging ▪ stem cell

Cellular cardiomyoplasty is plagued by low engraftment and small functional benefit. Molecular imaging facilitates the study of in vivo stem cell biology and optimization of cell therapies by permitting in vivo quantification of stem cell engraftment. For in vivo visualization, transplanted cells need to express a reporter gene or they must be labeled ex vivo.1 Reporter gene strategies (eg, bioluminescence imaging [BLI] of firefly luciferase [luc] gene expression2 and positron emission tomography/single-photon emission computed tomography [SPECT] imaging of herpes simplex virus thymidine kinase3 or human sodium-iodide symporter [hNIS]4 gene expression) are superior to ex vivo labeling of cells with tracers (eg, 18fluorodeoxyglucose [FDG]5 or indium6) or nanoparticles for longitudinal assessment of engraftment,8 because transplanted cell viability is a prerequisite for reporter gene expression. However, it is not known which factors, other than cell number, can influence the molecular imaging signal obtained from reporter genes.

Cell metabolism is an important determinant of cell survival, proliferation, and function.9–11 Studies in cancer cells reveal that they primarily use glucose via glycolysis, rather than oxidative phosphorylation for ATP generation, despite the availability of O2; this phenomenon is referred to as the Warburg effect.12 However, very little is known about the determinants of metabolism and its relationship to function in stem cells.

In this study, we examined the effect of cell dissociation/suspension on cellular bioenergetics and the molecular imaging...
signal obtained by hNIS (SPECT) and fluc labeling (BLI) of cardiosphere-derived cells (CDCs). CDCs are adherent cells composed of a mixture of cardiac-derived progenitor and supporting cells and have recently completed a phase 1 clinical trial. Sodium-iodide symporter (NIS) promotes cellular uptake of iodide or 99mTc-pertechnetate, driven by the transmembrane sodium gradient, which is maintained by Na+–K+ ATPase, whereas BLI is based on oxidation of the injected substrate d-luciferin by luciferase, a reaction that requires O2, Mg2+, and sodium gradient, which is maintained by Na+–K+ ATPase, whereas BLI is based on oxidation of the injected substrate d-luciferin by luciferase, a reaction that requires O2, Mg2+, and ATP, and results in emission of photons. Because signal generation by luciferase and NIS is linked to cellular ATP, we hypothesized that cellular bioenergetics would influence the molecular imaging signal obtained from hNIS and fluc-labeled cells. We studied hNIS labeling because it is a human gene that could be useful for longitudinal follow-up of cell engraftment in small and large animal models and patients, and we studied luciferase labeling because it is widely used for quantification of engraftment in rodent models of cell transplantation. Our results illustrate that cell dissociation, which is an essential first step in most clinical and experimental studies of cell transplantation, impairs cellular bioenergetics and the molecular imaging signal derived from NIS and luciferase.

Methods

Cell Isolation and Culture

Rat cardiosphere-derived cells (rCDCs) were isolated from syngeneic, male Wistar Kyoto rat hearts, as previously described. rCDCs were cultured in Iscove's Modified Dulbecco's Media (IMDM) medium (Invitrogen) containing 10% FBS, 10% glutamine, and 0.1 mmol/L mercaptoethanol and expanded to 3 to 5 passages before experiments. Please see Online Data Supplement for details.

Lentivirus Synthesis

A third-generation lentiviral vector system (kindly supplied by Professor Inder Verma, Salk Institute, La Jolla, CA) was used to label rCDCs. Please see Online Data Supplement for details.

In Vitro Glucose Uptake (18FDG)

One day before the experiment, rCDCs were plated at a density of 1×10⁵ cells/well. Before labeling, cells were washed twice with PBS and the medium was changed to glucose-free DMEM for 1 hour. 18FDG (74 kBq/mL) was added to half the plated cells for 30 minutes to measure glucose uptake in adherent cells; the remainder of the cells were trypsinized and suspended in medium containing 18FDG (74 kBq/mL) for 30 minutes to measure glucose uptake in cell suspension. Subsequently, cells were washed twice with cold PBS to remove any remaining free 18FDG, suspended in 1 mL 20% sodium dodecyl sulfate to lyse the cells, and transferred to 5 mL vials. Counts were recorded in a γ-counter (LKB Wallac, Turku, Finland). After the addition of change of dissolved O2 in each well (termed as O2 consumption rate). ATP estimation was performed using the ATP Determination Kit (A22066, Molecular Probes) using a luminometer (Turner BioSystem Veritas, Madison, WI). All experiments were performed using 1×10⁴ rCDCs (nontransduced) per well in a 96-well plate after 18 to 24 hours of culture for the adherent condition and after trypsinization and 1 hour of suspension for the suspension condition. For suspension conditions, the wells were coated with poly-2-hydroxyethyl methacrylate, overnight, before cell plating. Poly-2-hydroxyethyl methacrylate was chosen because it is known to prevent cell attachment and spreading. Cell lysis buffer (Cell Signaling Technology) was used to lyze the cells in each well for 20 minutes; standard reaction solution was injected through the automated injector. The signal was normalized to protein content using the Bradford assay. ADP/ATP ratio was assessed using the ApoSENSOR ADP/ATP Ratio Assay Kit.

Flow Cytometry

Annexin V and propidium iodide (Invitrogen) were used to quantify apoptotic and dead cells, respectively, immediately after cell dissociation and after suspension in cell culture medium for 1 hour and 6 hours. Please see Online Data Supplement for detailed methods.

Measurements of Cellular Metabolism

Seahorse Bioscience XF96 instrument was used to measure the rate of change of dissolved O2 in each well (termed as O2 consumption rate). The respiratory rates in each well were normalized to protein content using trophotransferase uptake was measured by incubating hNIS+ rCDCs cultured for 18 to 24 hours in a 6-well plate at a density of 10⁵ cells/well for the adherent condition or trypsinized and suspended in PBS or IMDM medium (Invitrogen). 99mTc-pertechnetate (11.1 kBq/mL) was added for 1 hour, immediately after generation of cell suspensions. The effect of oligomycin (2 µmol/L), an inhibitor of the mitochondrial ATP synthase, was used to assess dependence of CDCs on oxidative phosphorylation for ATP synthesis. Iodoacetate (600 µmol/L), an inhibitor of the glycolytic enzyme GAPDH, was used to assess the contribution of glycolysis to ATP synthesis. Cells were treated with these compounds for 30 minutes before measurements.

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In Vitro 99mTc-Pertechnetate Uptake

rCDCs were transduced with a third-generation lentivirus expressing the hNIS (Lv-CMV-hNIS) at a multiplicity of infection of 20. In vitro 99mTc-pertechnetate uptake was measured by incubating hNIS+ rCDCs cultured for 18 to 24 hours in a 6-well plate at a density of 10⁵ cells/well for the adherent condition or trypsinized and suspended in PBS or IMDM medium (Invitrogen). 99mTc-pertechnetate (11.1 kBq/mL) was added for 1 hour, immediately after generation of cell suspensions. The effect of perchlorate (100 µmol/L), a specific NIS blocker or 99mTc-pertechnetate uptake, was measured by adding perchlorate to some wells before the addition of 99mTc-pertechnetate. At the end of 1 hour, cells were rinsed twice with ice cold PBS and lysed with 20% sodium dodecyl sulfate. Counts were recorded in a gamma counter (LKB Wallac, Turku, Finland), and the Bradford protein assay was performed to normalize 99mTc uptake by protein content. We investigated cell suspension in PBS and IMDM medium because PBS/saline, which lacks substrates, has been extensively used in experimental and clinical studies of CDC transplantation; IMDM medium (Invitrogen) contains metabolic substrates, such as D-glucose, Ca2+, and Mg2+, and is used for CDC culture.

ATP Measurements

ATP estimation was performed using the ATP Determination Kit (A22066, Molecular Probes) using a luminometer (Turner BioSystem Veritas, Madison, WI). All experiments were performed using 1×10⁴ rCDCs (nontransduced) per well in a 96-well plate after 18 to 24 hours of culture for the adherent condition and after trypsinization and 1 hour of suspension for the suspension condition. For suspension conditions, the wells were coated with poly-2-hydroxyethyl methacrylate, overnight, before cell plating. Poly-2-hydroxyethyl methacrylate was chosen because it is known to prevent cell attachment and spreading. Cell lysis buffer (Cell Signaling Technology) was used to lyze the cells in each well for 20 minutes; standard reaction solution was injected through the automated injector. The signal was normalized to protein content using the Bradford assay. ADP/ATP ratio was assessed using the ApoSENSOR ADP/ATP Ratio Assay Kit.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BLI</td>
<td>bioluminescence imaging</td>
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<tr>
<td>CDCs</td>
<td>cardiosphere-derived cells</td>
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<tr>
<td>CF</td>
<td>calibration factor</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>fluc</td>
<td>firefly luciferase</td>
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<td>hNIS</td>
<td>human sodium-iodide symporter</td>
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<td>OCR</td>
<td>O2 consumption rate</td>
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<td>SPECT</td>
<td>single-photon emission computed tomography</td>
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signal obtained by hNIS (SPECT) and fluc labeling (BLI) of cardiosphere-derived cells (CDCs). CDCs are adherent cells composed of a mixture of cardiac-derived progenitor and supporting cells and have recently completed a phase 1 clinical trial. Sodium-iodide symporter (NIS) promotes cellular uptake of iodide or 99mTc-pertechnetate, driven by the transmembrane sodium gradient, which is maintained by Na+–K+ ATPase, whereas BLI is based on oxidation of the injected substrate d-luciferin by luciferase, a reaction that requires O2, Mg2+, and ATP, and results in emission of photons. Because signal generation by luciferase and NIS is linked to cellular ATP, we hypothesized that cellular bioenergetics would influence the molecular imaging signal obtained from hNIS and fluc-labeled cells. We studied hNIS labeling because it is a human gene that could be useful for longitudinal follow-up of cell engraftment in small and large animal models and patients, and we studied luciferase labeling because it is widely used for quantification of engraftment in rodent models of cell transplantation. Our results illustrate that cell dissociation, which is an essential first step in most clinical and experimental studies of cell transplantation, impairs cellular bioenergetics and the molecular imaging signal derived from NIS and luciferase.
To investigate the influence of trypsin on \(^{99m}\text{Tc}\)-pertechnetate uptake, \(r\)NIS\(^+\) rCDCs were dissociated using either 0.05% trypsin (Invitrogen), which usually takes \(\approx 2\) minutes, or nonenzymatic cell dissociation solution (Sigma-Aldrich; contains EDTA and other proprietary reagents), which takes \(\approx 20\) to 30 minutes (for complete dissociation into single cells), after which they were suspended in IMDM medium containing \(^{99m}\text{Tc}\)-pertechnetate (11.1 kBq/mL) for 1 hour. Subsequently, cells were rinsed twice using ice cold PBS, lyzed, and analyzed using the gamma counter; Bradford assay was performed to normalize uptake results to protein content. These experiments were performed in triplicate and repeated twice. All in vitro studies were performed at 37°C.

### In Vivo \(^{99m}\text{Tc}\) Uptake (SPECT/CT Imaging)

Dual-isotope imaging was performed. Data were acquired in list mode and postprocessed by applying 2 energy windows (75 keV +10% ‐10% and 140 keV +10% ‐10%) to obtain \(^{201}\text{Tl}\) and \(^{99m}\text{Tc}\) projections separately. Please see Online Data Supplement Methods for details of animal surgery and imaging.

Three million NIS\(^+\) rCDCs suspended in 100 µL of IMDM (Invitrogen) were injected directly into the myocardium at 3 sites in the anterior wall of the left ventricle using a 30-G needle. In vivo dual-isotope SPECT imaging was performed 1 hour after injection of \(^{99m}\text{Tc}\)-pertechnetate (555–740 MBq) and \(^{201}\text{Tl}\) (37–74 MBq); computed tomography (CT) imaging was performed before SPECT imaging. Animals were allowed to recover in their cages after completion of imaging on day 0. After 24 hours, the same rats were re-injected with \(^{99m}\text{Tc}\)-pertechnetate (555–740 MBq) and \(^{201}\text{Tl}\) (37–74 MBq) via the tail vein, and in vivo dual-isotope SPECT-CT imaging was performed. The rats were euthanized after completing the 24-hour imaging protocol.

### Ex Vivo SPECT Imaging

To validate the results obtained by in vivo imaging and to confirm the origin of the in vivo signal, a high-resolution ex vivo SPECT scan was performed in hearts excised from a separate set of animals at 1 hour (n=2) and 24 hours (n=2) after transplantation with NIS\(^+\) rCDCs. Please see Online Data Supplement for details.

### Image Quantification

For absolute quantification of in vivo \(^{99m}\text{Tc}\)-pertechnetate uptake, we generated a dose–response plot by dual-isotope SPECT imaging of a rat size phantom containing several concentrations of \(^{99m}\text{Tc}\)-pertechnetate and \(^{201}\text{Tl}\). Calibration factor (MBq/i.i.) was defined as the average intensity (i.i/cm\(^3\)) within regions of interest drawn over the small radioactive sphere in the phantom divided by the measured mean intensity within the regions of interest was multiplied by the calibration factor to give the radioactivity (MBq) and further divided by the regions of interest volume to obtain the uptake concentration (MBq/mL). Please see Online Data Supplement for details.

### In Vivo BLI

In vivo BLI was performed in a 96-well plate with a luminometer (Turner VERTITAS Microplate; Promega, Madison, WI) using \(\beta\)-luc-transduced rCDCs. Ten thousand cells (10° cells/well) were plated for 18 to 24 hours for the adherent cell condition and for 1 hour on poly-2-hydroxyethyl methacrylate–coated wells (condition 3) in PBS/saline or PBS/saline containing \(\text{Ca}^{2+}/\text{Mg}^{2+}\) (1 mMol/L) and \(\text{n-glucose}\) (5.6 mmol/L). We used \(\text{Ca}^{2+}/\text{Mg}^{2+}/\text{glucose}\)-containing PBS rather than IMDM to avoid the possible effects of phenol red, a component of IMDM medium, on the bioluminescence signal. The signal (relative light units [RLU]) was measured in suspended and adherent cells after injection of 30 µg/mL of \(\beta\)-luciferin (sodium salt; Gold Biotechnology, Saint Louis, MO) into each well using the automated injector. Cells were lysed using the lysis buffer contained in the Dual-Luciferase Reporter Assay System (E1910). The signal was normalized by protein content using the Bradford assay. All experiments were performed at least in triplicate and repeated twice.

### Quantification of Engraftment

We used the ex vivo luciferase assay to quantify in vivo engraftment of \(\beta\)-luc rCDCs and quantitative polymerase chain reaction for the male rat–specific SRY gene to quantify engraftment of NIS\(^+\) rCDCs in the first 24 hours after transplantation. Please see Online Data Supplement for detailed methods and rationale for the 2 methods.

### Statistical Analysis

For matched comparisons of continuous variables at different time points, the paired \(t\) test was used (or repeated measures ANOVA, in the case of >2 groups). For comparisons of continuous variables between 2 independent groups, the Student \(t\) test was used. A \(P<0.05\) was chosen for statistical significance. Values are reported as mean±SD.

### Results

### Bioenergetics in CDCs

Because cell trypsinization/dissociation is an important step in most experimental and clinical studies of cell transplantation in the heart, we initially compared glucose uptake, ATP levels, and cellular metabolism in nontransduced rCDCs that were trypsinized and suspended in culture media for 1 hour and in CDCs that were plated on an adherent surface (tissue culture treated wells) for 18 to 24 hours.

Cell viability was consistently >96% immediately postdissociation, when assayed by trypan blue staining and flow cytometry (Figure 1A and 1B). However, CDC viability was reduced to 83±4% and 74±3% (n=3), respectively, after 1 hour and 6 hours of suspension (Figure 1C).

Cellular glucose (%\(^{18}\text{FDG}\)) uptake and ATP levels were decreased by 82% and 43% respectively, after 1 hour of cell suspension compared with adherent cells. (Figure 2A: %\(^{18}\text{FDG}\), 3.03±1.06×10\(^4\) versus 1.74±0.23×10\(^4\) counts/mg protein in suspended and adherent cells respectively, \(P<0.001\); Figure 2B: ATP, 2.19±0.27 versus 3.87±0.65 nmol/mg protein in suspended and adherent cells, respectively; \(P<0.005\)). The ADP/ATP ratio increased from 0.27 in adherent rCDCs to 1.1 and 1.8 in suspended rCDCs at 1 hour and 3 hours, respectively, indicating progressive bioenergetic deficits with increasing periods of cell suspension.

OCR that reflects oxidative phosphorylation was significantly reduced after 1 hour of cell suspension (2.5±3 pmol/min in adherent rCDCs versus 12±5 pmol/min in suspended rCDCs; n=3; \(P<0.01\)). Extracellular acidification rate, which reflects glycolysis, was also significantly reduced on 1 hour of cell suspension (16±2 mHP in adherent rCDCs versus 10±3 mHP in suspended rCDCs; n=3; \(P<0.05\)), indicating that cell suspension impairs cellular bioenergetics (Figure 2C).
OCR and extracellular acidification rate returned to normal at 24 hours after replating of viable cells suspended for 3 and 6 hours, indicating that the metabolic derangements after cell suspension are reversible and linked to cell adhesion (Figure 2D).

Oligomycin, an inhibitor of mitochondrial F1-F0 ATP synthase, reduced OCR by 59±2% (P<0.001; n=3) in adherent CDCs (Figure 2E), without affecting cellular ATP levels (Figure 2F); in contrast, iodoacetate, an inhibitor of glycolysis reduced ATP levels by 88±3% (P<0.001; n=6), despite the presence of glucose (Figure 2F), indicating dependence of CDCs on glycolysis for ATP synthesis, evidence for the Warburg effect.

In Vitro Studies
We measured the effect of cell dissociation/suspension on the signal derived from hNIS and fluc labeling of rCDCs in vitro in the presence of either IMDM medium (Invitrogen) or PBS containing glucose/Ca2+/Mg2+ and PBS/saline.

Figure 2. Cell dissociation impairs bioenergetics. A, 18FDG uptake and (B) ATP levels in 18 to 24 hours of adherent and 1-hour suspended cardiosphere-derived cells (CDCs) after dissociation using trypsin. C, O2 consumption rate (OCR) and extracellular acidification rate (ECAR) are significantly reduced in suspended CDCs. D, Replotting after suspension for 1 hour and 6 hours results in restoration of cellular metabolism, when assessed at 24 hours (n=6). E, Oligomycin reduces OCR without affecting ATP levels in adherent CDCs (n=3). F, Iodoacetate but not oligomycin reduced ATP levels in adherent CDCs, indicating dependence on glycolysis for ATP generation (n=6).
Trypsinization followed by suspension of NIS+ rCDCs for 1 hour in regular culture medium (IMDM) decreased 99mTc-pertechnetate uptake by 42% (1.10±0.21×10⁴ versus 1.89±0.15×10⁴ counts/mg protein in suspended and adherent rCDCs, respectively; *P*<0.001; Figure 3A); suspension in PBS resulted in an additional decrease in 99mTc-pertechnetate uptake by 43% (6.23±3.14×10³ counts/mg protein; *P*<0.001 versus rCDCs suspended in IMDM; Figure 3A). Because hNIS is a surface protein that may be degraded by trypsin, we measured 99mTc-pertechnetate uptake in suspended hNIS+ rCDCs dissociated using trypsin or nonenzymatic dissociation solution that contains EDTA, but no enzymes. We found that 99mTc-pertechnetate in suspended rCDCs dissociated using trypsin was similar to that obtained by nonenzymatic dissociation (1.08±0.14×10⁴ versus 1.07±0.14×10⁴ counts/mg protein; *P*=0.45), indicating that trypsin-mediated NIS protein degradation does not underlie the decreased 99mTc-pertechnetate uptake observed in NIS+ rCDCs after suspension (Figure 3B).

In vitro BLI revealed a 44% reduction in signal (compared with adherent rCDCs) after suspension of luc+ rCDCs for 1 hour in saline/PBS containing glucose, Ca²⁺, and Mg²⁺ (1.35±0.008×10⁴ versus 2.41±0.16×10⁴ RLU/mg protein in suspended and adherent cells respectively; *P*<0.01) and further reduction by 95% when suspended in PBS/saline (6.21±0.23×10² RLU/mg protein; *P*<0.01; Figure 3C).

**In Vivo Studies**

Because cell dissociation/suspension decreased 99mTc uptake and BLI in vitro, we investigated the effect of cell dissociation/suspension on in vivo 99mTc uptake and BLI acutely after cell transplantation, when the transplanted cells are likely to still be in suspension, and at 24 hours after transplantation, when live cells are expected to have attached to the cardiac extracellular matrix and nonadherent cells have undergone anokis (and hence are unable to generate a molecular imaging signal).

The in vivo BLI signal was higher at 1 hour than at 24 hours. Importantly, at both time points, the in vivo BLI signal was lower when rCDCs were suspended in PBS/saline compared with the glucose-containing medium IMDM (4.09±1.73×10⁵ versus 1.05±0.34×10⁶ RLU at 1 hour for PBS and IMDM, respectively; *P*<0.05, and 5.04±2.40×10⁴ versus 1.73±0.37×10⁵ RLU for PBS and IMDM, respectively, at 24 hours; *P*<0.05; Figure 4A–4C), indicating that presence of substrates in the cell suspension medium improves the sensitivity of in vivo BLI.

NIS+ rCDCs were suspended in IMDM to optimize their metabolism in suspension. In vivo dual-isotope SPECT-CT imaging of intramyocardially injected NIS+ rCDCs suspended in IMDM revealed that NIS+ rCDCs were clearly visible 24 hours after transplantation, but not 1 hour after transplantation in infarct and noninfarct models of cell transplantation (Figure 5A); perfusion imaging confirmed good perfusion at the site of cell injection in the noninfarcted animals (Figure 5B), and ex vivo SPECT imaging confirmed the presence of cells at the 1-hour time point (Figure 5C). Calibration factor was calculated to be 3.37×10⁻⁵ MBq/i.i. from the dose–response plot (Figure 5D). Signal quantification revealed that the in vivo ⁹⁹mTc-pertechnetate uptake ratio was significantly greater at 24 hours compared with 1 hour in both noninfarct (0.05±0.01 MBq at 1 hour versus 0.23±0.05 MBq at 24 hours) and infarct (0.09±0.02 MBq at 1 hour versus 0.33±0.12 MBq at 24 hours) rat models (*P*<0.05; Figure 5E). Similarly, the ex vivo SPECT-CT imaging of noninfarct and infarct models showed that NIS+ rCDCs were clearly visible 24 hours after transplantation, but not 1 hour after transplantation (Figure 5F).
vivo $^{99m}$Tc-pertechnetate uptake ratio was higher at 24 hours (0.01 MBq at 1 hour versus 0.12±0.001 MBq at 24 hours).

Longitudinal, in vivo SPECT/CT imaging of NIS+ rCDCs and BLI of fluc+ rCDCs over 7 days revealed progressive cell loss from day 1 to 7 after transplantation (Online Figure I). Based on our in vitro studies which reveal that cellular metabolism is stable after adhesion for 24 hours (Online Figure II), we believe that factors other than cell adhesion play a role in cell loss at later time points after transplantation.

at 24 hours (0.07±0.03 MBq at 1 hour vs 0.28±0.18 MBq at 24 hours). Cell-derived signal at 1 hour is similar to the background signal from myocardium (0.05±0.02 MBq).
Confirmation of Engraftment
Ex vivo luciferase assay (n=12) using fluc her CDCs and quantitative polymerase chain reaction for the male-specific SRY gene using NIS+ CDCs (n=8) in separate sets of animals revealed a significant reduction in cell number (−72.39±14.10% for fluc+ CDCs and −74.77±13.13% for NIS+ rCDCs) between 1 hour and 24 hours after transplantation (Figure 6A and B), confirming similar amounts of in vivo cell loss in both groups in the first 24 hours.

Discussion
This is the first study to report a link among stem cell adhesion, bioenergetics, and the molecular imaging signal. The novel results of this study are as follows: (1) CDCs exhibit the Warburg effect (aerobic glycolysis); (2) cell adhesion is an important regulator of cellular metabolism and the molecular imaging signal derived from hNIS and luciferase; (3) in vivo 99mTc-pertechnetate uptake is higher at 24 hours after transplantation of cell suspensions (compared with 1 hour), despite fewer cells at this time point.

NIS Imaging
Reporter gene strategies are useful in the longitudinal assessment of engraftment because transplanted cell viability is required for gene expression. We used a self-inactivating, third-generation lentivirus and the constitutively active cytomegalovirus virus promoter, which results in integration of the expression cassette into the host genome and high levels of transgene expression. Previous studies by our group have shown that expression of fluc or the human NIS gene in CDCs at a multiplicity of infection of 20 did not affect CDC viability or function. NIS promotes cellular uptake of iodide or 99mTc-pertechnetate and Na+, driven by the transmembrane sodium gradient, which is generated by the Na−/K+ ATPase. Remarkably, after ectopic NIS expression, only cells overexpressing NIS will transport 99mTc-pertechnetate or iodine-124 after intravenous injection of these tracers, permitting noninvasive, longitudinal monitoring of stem cell engraftment by SPECT and positron emission tomography, respectively.

In vivo 99mTc-pertechnetate uptake was low at 1 hour after cell transplantation and increased at 24 hours, despite significant cell loss during this period. This finding is not a result of impaired perfusion at the injection site at 1 hour or a result of cell proliferation, based on our perfusion results in noninfarcted animals at 1 hour after transplantation (Figure 5B) and quantitative polymerase chain reaction for the SRY gene using NIS+ CDCs (Figure 6). We attribute this result to impairment of bioenergetics in suspended CDCs based on our in vitro studies in adherent and suspended cells.

For in vivo studies, hNIS+ rCDCs were dissociated using trypsin, suspended in IMDM, and injected intramyocardially. We used IMDM rather than PBS/saline as the vehicle for our in vivo experiments because in vitro studies revealed that 99mTc-pertechnetate uptake was higher when cells are suspended in IMDM compared with PBS/saline (Figure 3A). Our in vitro studies also confirmed that 99mTc-pertechnetate uptake was similar in CDCs dissociated using trypsin or nonenzymatic dissociation solution (Figure 3B), indicating that impairment of cellular bioenergetics, rather than trypsin-mediated degradation of NIS, is the dominant mechanism underlying this phenomenon.

Cell dissociation and suspension resulted in significant downregulation of glucose uptake, cellular metabolism, and ATP levels (Figure 2A and 2B). Our in vitro studies also indicate a progressive increase in the cellular ADP/ATP ratio from 1 hour to 3 hours in suspension, which would result in a lower Gibbs free energy availability from ATP hydrolysis, limiting Na−/K+ ATPase function, which in turn reduces transmembrane Na+ gradient and consequently 99mTc-pertechnetate uptake (Online Figure III); this could explain the very low in vivo 99mTc-uptake in the immediate posttransplantation period (the time interval between cell dissociation and completion of in vivo SPECT/CT [NIS] imaging was ≈2–3 hours). Transplanted cells would be expected to adhere to the cardiac extracellular matrix during the 24-hour period after transplantation, resulting in improved bioenergetics that would translate into improved 99mTc-pertechnetate uptake. In vitro studies confirmed increased 99mTc-pertechnetate uptake in NIS+ CDCs, cultured on adhesive surfaces for 18 to 24 hours, compared with suspended cells. Cells that do not attach in vivo would undergo anoikis, which would explain some of the cell loss in the first 24 hours. Other mechanisms underlying cell loss could be progressive cell egress from the transplantation site via lymphatics and coronary veins (an effect that is more pronounced when cells are injected into contractile, noninfarcted myocardium) and cell death because of oxidative stress, inflammation, or other mechanisms.

![Figure 6. Confirmation of engraftment after intramyocardial transplantation of firefly luciferase (fluc) and sodium-iodide symporter (NIS) rat cardiosphere-derived cells (rCDCs).](http://circres.ahajournals.org/Downloaded_from http://circres.ahajournals.org by guest on July 27, 2017)

**A**, Similar amounts of cell loss were observed between 1 hour and 24 hours after intramyocardial transplantation of fluc+ and NIS+ rCDCs (P<0.005). **B**, Standard curve correlating ex vivo luciferase activity in rat heart homogenates with known numbers of fluc+ rCDCs. RLU indicates relative light units.
Increase in the in vivo SPECT signal, despite significant cell loss, probably occurred because the magnitude of the increase in $^{99m}$Tc-pertechnetate uptake of CDCs surviving at 24 hours greatly exceeded the effects of cell loss over the same time period.

**Bioluminescence Imaging**

BLI-fluc imaging is based on the oxidation of the substrate $\beta$-luciferin by luciferase, a reaction that requires $O_2$, $\text{Mg}^{2+}$, and ATP, and results in a red-shifted light emission (wavelength, 500–700 nm), which can be detected using a camera. Because the luciferase reaction is directly dependent on ATP and $\text{Mg}^{2+}$, composition of the cell suspension solution had a profound influence on the BLI signal. We found that the BLI signal generated by cells suspended in IMDM was higher than when suspended in PBS/saline and 24 hours after transplantation. These differences could be related to differences in the in vivo cell survival and metabolism between the 2 groups. Further studies are needed to test whether cell suspension in solutions that contain substrates, $\text{Mg}^{2+}$, and $\text{Ca}^{2+}$ are superior to PBS/saline with respect to cell engraftment.

As in the case of $^{99m}$Tc-pertechnetate uptake, the in vitro BLI signal was also higher in $\text{fluc}^+$ rCDCs that were adherent for 18 to 24 hours compared with suspended cells in our in vitro studies. Hence, transplanted $\text{fluc}^+$ rCDCs would also be expected to attach to cardiac extracellular matrix and improve cellular bioenergetics and light generation by luciferase during the first 24 hours after transplantation. However, the BLI signal was higher at 1 hour compared with 24 hours after transplantation. This discrepancy in the in vivo molecular imaging signal at 24 hours between in vivo SPECT and BLI is not as a result of differences in the in vivo survival of $\text{fluc}^+$ CDCs and NIS+ CDCs because cell loss in the first 24 hours was similar in the 2 groups (Figure 6A). We attribute the different results at 24 hours, using NIS and luciferase, to the dependence on $O_2$ for generation of the luciferase signal, but not the NIS signal. Our in vitro metabolism studies reveal that stem cells rely primarily on glycolysis for ATP generation, a phenomenon referred to as the Warburg effect. Furthermore, inhibition of the mitochondrial $F_1$-$F_0$ ATP synthase did not affect ATP levels (Figure 2F) in contrast to cell dissociation that rapidly depressed ATP levels (Figure 2B). Because signal derived from NIS relies on the transmembrane $\text{Na}^+$ gradient, which is dependent on cellular ATP levels, it would be expected to increase with cell adhesion at 24 hours after in vivo cell transplantation. However, the luciferase reaction is dependent on $O_2$, as well as ATP. Low $O_2$ tension at the transplantation site because of tissue damage from injection and combined with cell loss during this interval would be expected to impair recovery of the BLI signal but not the NIS signal at 24 hours.

**Future Implications for Stem Cell Imaging**

We chose 1 hour as the first time point for our in vitro and in vivo studies because 1 hour after transplantation is often chosen as the baseline time point for longitudinal assessment of engraftment in experimental models of cell transplantation. We picked 24 hours as the second time point because it is unclear from the literature whether the signal at 1 hour or 24 hours should be used as the baseline signal for longitudinal follow-up of engraftment using molecular imaging.

With in vivo BLI, the BLI signal was higher at 1 hour compared with 24 hours after transplantation and hence, more accurately represented cell fate in the first 24 hours after transplantation. However, the NIS-derived signal using suspended cells was higher at 24 hours compared with 1 hour after cell transplantation, despite significant cell loss. This result has important implications if NIS is used for longitudinal assessment of engraftment. Based on our results, the NIS-derived signal immediately after transplantation, using suspended cells, is more reflective of cellular bioenergetics than actual cell number compared with the signal at 24 hours. Hence, it could confound quantification of engraftment in the first 24 hours using SPECT-CT imaging of NIS $^+$ rCDCs. This phenomenon may be harnessed to monitor the effects of small molecules and tissue-engineered scaffolds on transplanted cell bioenergetics in the immediate posttransplantation period.

**Study Limitations**

This is the first report of impaired bioenergetics by cell dissociation/suspension; further in-depth studies are needed to understand the signaling pathways underpinning this phenomenon. Tissue-engineered matrices containing Arg-Gly-Asp (RGD) motifs that promote encapsulated cell attachment to the matrix or cell–cell adhesion by generation of cell aggregates may abrogate the downregulation of cellular metabolism in stem cell suspensions, and they need to be tested.

We limited this study to rat CDCs because we have extensive experience with stem cell isolation, gene transduction, and molecular imaging using this cell type. However, we believe that these results are not specific to CDCs and will be reproducible in other adherent cell types (eg, mesenchymal stem cells, embryonic stem cells, and endothelial progenitor cells). Unfortunately, we were unable to transduce commercially available human mesenchymal stem cells using this third-generation lentivirus and hence were unable to test this clinically important cell type.

Technical limitations precluded cell sorting to exclude dead cells, in cell suspensions, before in vitro experiments at 1 hour and measurement of transplanted cell bioenergetics in vivo to verify our in vitro results. However, using flow cytometry, we consistently observed an $\approx 15\%$ reduction in cell viability, which is much less than the reduction of cellular ATP levels, respiratory measurements, $^{99m}$Tc uptake, and in vitro BLI, indicating that cell suspension rather than decrease in cell viability is the primary cause of the observed depression of cellular bioenergetics.

**Conclusions**

Cell dissociation impairs cellular bioenergetics, resulting in reduced $^{99m}$Tc-pertechnetate uptake and BLI signal. Because cell survival, proliferation, and function are intimately linked to metabolism, BLI and NIS imaging could be useful tools for in vivo optimization of bioenergetics and engraftment of transplanted cells.
Acknowledgments
We are grateful to Jianhua Yu, Catherine Foss, PhD, and Miguel Aon, PhD, for helpful advice and to James Fox and Gilbert Green for technical assistance.

Sources of Funding
This study was funded by American Heart Association-Beginning Grant-in-Aid and National Institutes of Health (NIH) ROI HL092985. Dr Chan was supported by NIH T32HL07227 Training Grant. Dr Lin was partially supported by a China Scholarship Grant. Dr Vakrou was supported by a grant from the Hellenic Society of Cardiology. K. Woldemichael was supported by an NIH diversity fellowship.

Disclosures
None.

References
Cell transplantation in the heart is limited by low engraftment. We used in vivo molecular imaging by SPECT and BLI to examine engraftment of cardiosphere-derived cells in the first 24 hours after transplant. Using a combination of in vitro studies of cell viability, metabolism and function, and in vivo longitudinal imaging, we demonstrate a link among cardiosphere-derived cell dissociation/suspension, bioenergetics, and the molecular imaging signal obtained by NIS (SPECT imaging) and firefly luciferase labeling (BLI) of transplanted cells. Specifically, we found that cell dissociation/suspension reversibly impairs cellular metabolism and ATP levels, resulting in decreased 99mTc-pertechnetate uptake and BLI signal in suspended cells compared with adherent cells. This translated into increased 99mTc-pertechnetate uptake (SPECT signal) at 24 hours compared with 1 hour after transplantation, despite considerable cell loss during this interval. However, the BLI signal decreased between 1 hour and 24 hours after transplantation and was reflective of the cell loss during this period. Because the BLI signal depends on ATP, Mg²⁺, and O₂, while the NIS-derived signal depends only on ATP, we propose that SPECT imaging of NIS⁺ cells may be useful for in vivo optimization of cellular bioenergetics and engraftment in small/large animal models and humans.

Novelty and Significance

What Is Known?

- Stem cell engraftment in the heart is low and results in small functional benefit.
- Molecular imaging is useful to study in vivo stem cell biology and optimize engraftment.
- Cell survival, proliferation, and function are intimately linked to metabolism.

What New Information Does This Article Contribute?

- Stem cell dissociation and suspension impair cellular bioenergetics, resulting in reduction of the molecular imaging signal obtained by single-photon emission computer tomography (SPECT) and bioluminescence imaging (BLI) of cardiosphere-derived cells labeled with the sodium-iodide symporter (NIS) and luciferase, respectively.
- Impairment of cellular bioenergetics in dissociated NIS⁺ cells can confound quantification of engraftment by in vivo SPECT imaging in the first 24 hours after transplantation.
- SPECT imaging of NIS-labeled cells may be useful for in vivo optimization of bioenergetics and engraftment in transplanted stem cells.

Cellular Bioenergetics Is an Important Determinant of the Molecular Imaging Signal Derived From Luciferase and the Sodium-Iodide Symporter
Connie Chang, Angel Chan, Xiaoping Lin, Takahiro Higuchi, John Terrovitis, Junaid M. Afzal, Andrew Rittenbach, Dongdong Sun, Styliani Vakrou, Kirubel Woldemichael, Brian O'Rourke, Richard Wahl, Martin Pomper, Benjamin Tsui and M. Roselle Abraham

*Circ Res.* 2013;112:441-450; originally published online December 19, 2012; doi: 10.1161/CIRCRESAHA.112.273375
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplemental Material

Detailed Methods

Cell isolation and culture
Briefly, small pieces of myocardial tissue (explants) derived from male rats were placed on fibronectin-coated dishes. In the following days, cells exited the explants and formed an adherent monolayer on the dish surface with phase bright cells on top. These cells are harvested using mild enzymatic digestion and transferred to D-poly-lysine coated dishes, where they form three dimensional structures called cardiospheres which are enriched in cardiac progenitors. Cardiospheres are subsequently harvested and grown as monolayers in fibronectin-coated flasks – these cells are called cardiosphere-derived cells (CDCs). CDCs were cultured in IMDM medium (Invitrogen) containing 10% FBS, 10% glutamine and 0.1mM mercaptoethanol, and expanded to 3-5 passages prior to lentiviral transduction.

Lentivirus synthesis
The cDNA encoding the hNIS gene or the cDNA pGL4.10[luc2] encoding firefly luciferase (Promega, Madison, WI, USA) was sub-cloned in place of eGFP into the vector RRLsin18.cPPT.CMV.eGFP.Wpre, resulting in plasmids designated cpPPT.CMV.hNIS or pPPT.CMV.fluc. Viral vectors were produced by Lipofectamine 2000 (Invitrogen) transfection of 4 lentiviral vector plasmids into HEK293T cells (ATCC, Manassas, VA, USA). Vector-containing supernatant was collected 48 and 72 hours after transfection, filtered, and concentrated (Centricon Plus-70, Millipore, Billerica, MA, USA). Viral titer was assigned on concentrated supernatant by HIV-1 p24 ELISA (Dupont, Wilmington, DE, USA). For genetic labeling, rCDCs were transduced at a multiplicity of infection of 20 yielding transduction efficiencies of >70% for hNIS expression and >90% for fluc expression. NIS expression was confirmed by immunostaining using a monoclonal mouse anti-hNIS antibody (Abcam, Cambridge, MA, USA) and by in-vitro $^{99}$Tc-pertechnetate uptake, while luciferase expression was examined by immunostaining using a polyclonal goat anti-luciferase antibody (Promega) and by an in vitro bioluminescence assay. We have previously demonstrated that transduction of CDCs with firefly luciferase or hNIS at an MOI (multiplicity of infection) of 20 does not affect cell proliferation, using in vitro studies.\textsuperscript{1, 2} Percentage of transduced cells was calculated by immunostaining prior to cell transplantation.

Flow Cytometry
Annexin V and Propidium iodide were used to identify apoptotic and dead cells, respectively. Annexin V was diluted at a concentration of 1 mg/ml in binding buffer and cells (1x10\textsuperscript{6} cells) were re-suspended in 1ml of this freshly made solution. Cells were incubated for 10 min in the dark at room temperature then PI solution (0.1ml to give final conc. of 1mg/ml) was added to this solution 5 minutes prior to the analysis with flow cytometry equipment (with 10,000 events collected per sample). Cell debris was excluded by scatter gating and 10,000 gated events were collected per sample using BD Accuri C6 flow cytometer. For measurements of cell viability after dissociation, CDCs were trypsinized, counted and used immediately after suspension in media. Trypan blue measurements were compared to measurements by flow cytometry. For measurements of cell viability following suspension for 1hr and 6hrs, CDCs were trypsinized, counted and suspended in cell culture medium for 1hrs and 6hrs respectively, in the incubator at 37\degree C prior to measurements by flow cytometry.

In vivo SPECT/CT imaging
Male WKY rats underwent left thoracotomy in the 4th or 5th intercostal space under general anesthesia (isoflurane inhalation, 4% for induction and 2% for maintenance). In the myocardial infarction group, the heart was exposed and the left anterior descending coronary artery was
ligated using a 5-0 silk suture. Three million NIS\(^{+}\) rCDCs suspended in 100µl of IMDM (Invitrogen) were injected directly into the myocardium at three sites in the anterior wall of the left ventricle using a 30G needle. In the non-infarct group, the same procedure was followed as the myocardial infarction group with the exception of the ligation of the left anterior descending coronary artery. Subsequently, the chest was closed with a 3-0 silk suture. \(^{99m}\)TcO\(_{4}^{-}\) (\(^{99m}\)Tc labeled technetium-pertechnetate; 555-740 MBq) and \(^{201}\)TlCl (\(^{201}\)Tl labeled thallous chloride; 37-74 MBq) were injected intravenously via the tail vein immediately after intra-myocardial cell transplantation, to determine stem cell retention and myocardial perfusion. The isoflurane was turned off and the animal was monitored for spontaneous breathing and allowed to fully recover prior to imaging.

*In vivo* dual isotope SPECT imaging was performed 1hr after injection of \(^{99m}\)Tc-pertechnetate and \(^{201}\)TlCl. CT imaging was performed prior to SPECT imaging. Both scans were performed on a small animal SPECT/CT system (X-SPECT-CT from Gamma Medica Inc., Northridge, CA) using inhalational isoflurane as the anesthetic agent, administered via a nose cone. Animals were allowed to recover in their cages after completion of imaging on day 0. After 24hrs, the same rats were re-injected with \(^{99m}\)Tc-pertechnetate (555-740 MBq) and \(^{201}\)TlCl (37-74 MBq) via the tail vein and *in vivo* dual isotope SPECT-CT imaging was performed. The rats were euthanized after completing the 24hr imaging protocol.

In a second study, rats were serially imaged at 1d, 3-4d and 7d following intra-myocardial transplantation of NIS\(^{+}\) or fluc\(^{+}\) rCDCs.

**SPECT/CT image acquisition and processing:** The SPECT module X-SPECT-CT system is composed of two gamma camera heads each consisting of pixelated NaI(Tl) with a total area of 125 mm × 125 mm, divided into 80 × 80 number of pixels with 1.56 mm pitch. Low-energy knife-edge pinhole collimators were used with a pinhole aperture of 1 mm diameter and a focal length of 9cm; a radius-of-rotation of 5.42 cm was used. Each camera head acquired 128 projections over a 180-degree range, with an acquisition time of 30s for each projection for all scans except for the ex vivo heart scans which was changed to 40s per projection.

In the dual isotope SPECT imaging, data were acquired in listmode and were subsequently re-binned into two energy windows (“75 keV +10%/-10%” and “140 keV +10%/-10%”) to obtain separate sets of \(^{201}\)Tl and \(^{99m}\)Tc projections. The \(^{99m}\)Tc and \(^{201}\)Tl projection datasets were reconstructed using a 3D pinhole ordered-subset expectation-maximization (OS-EM) imaging reconstruction algorithm with 8 and 4 updates, respectively with an isotropic reconstructed image voxel size of 0.7 mm.

X-ray computed tomography (CT) was performed on the microCT module with an X-ray tube voltage of 75 kVp. A total of 512 projections were acquired over a 360-degree range. The projections with 1,184 × 1,120 isotropic pixels (100 µm) were reconstructed into a CT volume of 512\(^{3}\) isotropic voxels with 170µm pixel size. The SPECT and CT were then registered using rigid body transform, with pre-set parameters specific to the system.

**SPECT image quantification:** For absolute quantification, a calibration factor (CF) was calculated from an experimental study by inserting a small hollow sphere filled with a known amount of radioactivity of \(^{99m}\)Tc or \(^{201}\)Tl in water in an average rat-size water-filled cylindrical phantom to simulate a rat scan. Dose-response plot for \(^{99m}\)Tc-pertechnetate was obtained by dual isotope SPECT imaging of varying doses of \(^{99m}\)Tc-pertechnetate and \(^{201}\)Tl and then used to calculate the calibration factor. SPECT data for the phantom were acquired using exactly the same acquisition settings as those used in the animal experiments. In this case, CF (MBq/i.i.) was defined as the quotient of the known activity concentration (MBq/ml) within the radioactive sphere in the phantom divided by the measured mean image intensity (i.i/cm\(^{3}\)) within a ROI drawn over the small sphere in the SPECT image of the phantom.
To quantify the tracer uptake in vivo, regions-of-interest (ROI) were manually defined on a region of increased focal tracer uptake and on a contra-lateral normal region of a mid-myocardial section. In the case of no observable increased focal myocardial tracer accumulation, an ROI was placed on the distal anterior wall. The total radioactivity at the region of interest was calculated by the image intensity within the ROI multiplied by the CF. The radioactivity concentration (MBq/mL) within the ROI was calculated by the total activity divided by the volume of the ROI. The background activity was calculated by placing an ROI on the baso-lateral wall of the heart.

Myocardial perfusion polar maps were generated by combining the short-axis image slices through the left ventricular myocardium of the $^{201}$TI SPECT dataset. A perfusion defect was extracted from a threshold of 60% of the maximum intensity of the myocardium.

**Ex-Vivo SPECT imaging**

The same procedure was adopted as for the in vivo imaging group, except that only $^{99m}$Tc-pertechnetate was injected. For the 1hr ex-vivo group, the rats were injected with $^{99m}$Tc-pertechnetate (555-740 MBq) immediately after injection of the NIS+ rCDCs and imaged 1hr later. Immediately after the SPECT scan, the rat was sacrificed and the heart was rinsed with PBS and washed thoroughly to remove any remaining blood before ex vivo scanning. Imaging parameters were identical to the ones used in the in vivo acquisitions, with the exception of the time per projection which was decreased to 40s per projection. For the second group of rats, the same procedure was performed as the 1hr ex vivo rats with the exception that the rats were injected with $^{99m}$Tc-pertechnetate (555-740 MBq), imaged and sacrificed 24hrs following cell transplantation.

**Quantification of engraftment by ex vivo luciferase assay:** We performed the ex vivo luciferase assay in a separate set of animals, to quantify engraftment of fluc+ rCDCs in the first 24hrs following transplantation. This assay has high sensitivity and is not affected by conditions such as ischemia/hypoxia, cellular metabolism and diffusion of luciferin to the cell transplantation site, which can all affect the in vivo signal, thus permitting reliable quantification of engraftment. For this purpose, we performed intra-myocardial injection of 1million fluc+ rCDCs in 12 animals (6 animals without myocardial infarction and 6 animals with infarction). Three animals from each group were sacrificed at 1hr and 24hrs after cell transplantation. A standard curve was constructed to calculate cell number.

Hearts were harvested and cut into 200mg pieces, from apex to base (4-5 pieces/heart). The tissue samples were mixed with 1ml of lysis buffer (Promega) and 0.5ml of 10% BSA (as a non-specific protease inhibitor). Tissue samples were homogenized by manually grinding (Duall 24 Glass homogenizer, Kontes, Vineland, NJ, USA), the homogenates were collected in 50ml conical tubes (VWR, West Chester, PA, USA) and centrifuged at 25,000 g for 45min, at 4° C. The supernatants were collected and a luciferase assay was performed using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, USA). Twenty microliters of each sample was mixed with 100µl of luciferase assay reagent (Promega) in 75mm glass tubes (VWR) and placed in the instrument (2s measurements). Results were reported as relative light units (RLUs) and converted to cell numbers using the regression equation of the corresponding standard curve.

**Standard curve preparation:** Luciferase over-expressing rCDCs (from the same isolates that were used for the in vivo experiments) were also used for the standard curve preparation. Different numbers of rCDCs (starting from 500 up to 100,000 in duplicates) were pelleted. Hearts from normal male WKY rats were harvested and cut into small pieces. Cell pellets were lysed (Luciferase lysis buffer, Promega) and 200mg of rat heart tissue was added to the cell lysate. Cell lysates and tissue samples were processed as described above and values
obtained by the luciferase assay were used to construct the standard curve. Luciferase activity demonstrated excellent linear correlation to cell numbers.

**Quantification of engraftment by quantitative polymerase chain reaction (qPCR):**
Genomic DNA was isolated from aliquots of the homogenate corresponding to 12.5mg of myocardial tissue, according to the manufacturer's instructions (Qiagen). Real time PCR was performed using the TaqMan® chemistry (Applied Biosystems), with the rat SRY gene as target (forward primer: 5’-GGA GAG AGG CAC AAG TTG GC-3’, reverse primer: 5’-TCC CAG CTG CTT GCT GAT C-3’, TaqMan probe: 6FAM CAA CAG AAT CCC AGC ATG CAG AAT TCA G TAMRA). For absolute quantification of gene copy number, a standard curve was constructed with samples derived from multiple log dilutions of genomic DNA isolated from male rat CDCs. All samples were spiked with 50ng of female genomic DNA to control for any effects this may have on reaction efficiency in the actual samples. The copy number of the SRY gene at each point of the standard curve is calculated based on the amount of DNA in each sample and total mass of the rat genome per diploid cell. (http:www.cbs.dtu.dk/databases/DOGS/index.html). All samples were tested in triplicate. The qPCR assay was repeated twice with DNA samples isolated on 2 occasions, from each heart. For each reaction, 50ng of template DNA was used. Real-time PCR was performed in an ABI PRISM 7700 instrument. The result from each reaction, i.e. copies of the SRY gene in 50ng of genomic DNA, was expressed as the number of engrafted cells/heart, by first calculating the copy number of the SRY gene in the total amount of DNA corresponding to 12.5mg of myocardium and then extrapolating to the total weight of each heart.

**Supplemental Figures and Figure Legends**

A

![Graph A](image1)

B

![Graph B](image2)

Supplemental Figure 1: Longitudinal in vivo SPECT/CT imaging of NIS⁺ rCDCs (A) and BLI of fluc⁺ rCDCs (B) over 7days revealed progressive cell loss from day1 to 7 post-transplantation (n=3).
Supplemental Figure 2: In vitro studies reveal that cellular metabolism is stable after adhesion for 24hrs.
Supplemental Figure 3: In vitro studies: Ouabain (100 µM), a blocker of Na⁺-K⁺-ATPase reduced ⁹⁹mTc-pertechnetate uptake by ~17% in adherent CDCs (p<0.01) and in CDCs suspended in glucose-containing media (p=0.01), but had no effect when CDCs were suspended in PBS (p=0.76), confirming a link between cellular Na⁺-K⁺-ATPase activity and NIS-mediated ⁹⁹mTc-pertechnetate uptake.

**Supplemental References**
