Published in final edited form as:

Mol Imaging Biol. 2016 August ; 18(4): 510–518. doi:10.1007/s11307-016-0929-x.

Noninvasive Monitoring of the Mitochondrial Function in Mesenchymal Stromal Cells

Federico Franchi¹, Karen M. Peterson¹, Ramasamy Paulmurugan³, Clifford Folmes¹, Ian R. Lanza², Amir Lerman¹, and Martin Rodriguez-Porcel¹

¹Division of Cardiovascular Diseases, Department of Internal Medicine, Mayo Clinic, 200 First St. SW, Rochester, MN, 55905, USA

²Division of Endocrinology and Metabolism, Department of Internal Medicine, Mayo Clinic, Rochester, MN, USA

³Department of Radiology and Molecular Imaging Program at Stanford (MIPS), Stanford University School of Medicine, Stanford, CA, USA

Abstract

Purpose—Mitochondria are a gatekeeper of cell survival and mitochondrial function can be used to monitor cell stress. Here we validate a pathway-specific reporter gene to noninvasively image the mitochondrial function of stem cells.

Procedures—We constructed a mitochondrial sensor with the firefly luciferase (Fluc) reporter gene driven by the NQO1 enzyme promoter. The sensor was introduced in stem cells and validated *in vitro* and *in vivo*, in a mouse model of myocardial ischemia/reperfusion (IR).

Results—The sensor activity showed an inverse relationship with mitochondrial function ($R^2 = -0.975$, $p = 0.025$) and showed specificity and sensitivity for mitochondrial dysfunction. *In vivo*, NQO1-Fluc activity was significantly higher in IR animals vs. controls, indicative of mitochondrial dysfunction, and was corroborated by ex vivo luminometry.

Conclusions—Reporter gene imaging allows assessment of the biology of transplanted mesenchymal stromal cells (MSCs), providing important information that can be used to improve the phenotype and survival of transplanted stem cells.

Keywords

Bioluminescence; Mesenchymal stem cells; Mitochondrial function; Reporter gene

Correspondence to: Martin Rodriguez-Porcel; rodriguez.m@mayo.edu.
Federico Franchi and Karen M. Peterson contributed equally to this work.

Compliance with Ethical Standards.

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical Approval. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Introduction

There is significant interest in the use of stem cells (SCs) as an alternative therapy for cardiac repair after myocardial infarction (MI) [1, 2]. However, while some clinical studies have shown benefit from cell therapy, others have been neutral or shown a transient improvement in left ventricular (LV) function [3–7]. This variable response may, in part, be due to poor survival and functionality of transplanted SCs within the post-ischemic myocardium, which has been shown to be pro-inflammatory, hypoxic, pro-fibrotic, and apoptotic [2, 8, 9]. Thus, identifying the conditions when SCs are most vulnerable to the hostile post-ischemic myocardial microenvironment [10–12] will provide critical insight that can be used to optimize regenerative therapies.

The survival and functionality of SCs, as with other cell types, is largely dependent on their capacity to generate chemical energy (e.g., adenosine triphosphate [ATP]), the majority of which is produced within the mitochondria [13]. These organelles are postulated as the “gatekeepers” of cell survival [14–19]. Furthermore, stem cell therapy is being tested in the ischemic myocardium, a condition that is characterized by hypoxia, inflammation, and oxidative stress [20–24], all of which are known inducers of mitochondrial dysfunction, as well as deleterious to the phenotype/survival of transplanted SCs. Thus, it becomes of critical importance to expand our understanding of the interaction of transplanted SCs with the host ischemic myocardium, providing information that can be used to improved SC functionality and survival.

Current approaches to assess the fate and biology of transplanted stem cells involve traditional ex vivo assays (histology) and molecular techniques (gene expression by real-time polymerase chain reaction (PCR) and western blotting), which are both invasive and restricted in their capacity to monitor temporal changes in a given subject [25].

Developments in molecular imaging techniques, such as reporter gene technology, have enabled the noninvasive surveillance of cell fate in the living subject [26–31]. In fact, bioluminescence imaging (BLI) has been used to study the kinetics of the viability of myocardial-implanted cells in living animals [27]. Furthermore, we recently reported that reporter gene imaging can also be used to monitor specific biological processes using conditional transgenes whose expression is regulated by pathway-specific promoters [32].

In this manuscript, we describe a novel, noninvasive imaging strategy to monitor the mitochondrial function of mesenchymal stromal cells (MSCs) transplanted into the ischemic myocardium.

Materials and Methods

In Vitro Studies

MSCs were isolated from mice (friend virus B-type (FVB) background) and characterized as previously described [33, 34]. MSCs were, then, challenged with increasing doses of diethylmaleate (DEM, 50–150 μ M), a known mitochondrial stressor [35–37]. Mitochondrial function was assessed by measuring oxygen consumption rate (OCR), membrane

polarization (JC-1), cellular ATP content, and the rate of apoptosis/necrosis (annexin V/propidium iodide—PI—with FACS).

Cellular Respiration (Oxygen Consumption Rate)

The OCR was analyzed on a Seahorse extracellular flux analyzer (XF24, Seahorse Bioscience, North Billerica, MA) as previously described [38]. Briefly, MSCs were seeded at a density of 7.5×10^4 cells/well in a Seahorse XF24 plate ($n = 5$). The seeding density was determined with an initial assay that optimized OCR and minimized variability. After an overnight stabilization period to ensure cell attachment, cells were washed once with phosphate-buffered saline (PBS) and treated with DEM (0, 50, 75, and 150 μ M) for 6 h at 37 °C. Immediately prior to the assay, cultured media was replaced with minimal assay media (Seahorse Biosciences) supplemented with 25 mM glucose, 1 mM sodium pyruvate, 2 mM glutaMAX, 1 $\times$ nonessential amino acids, and 1 % FBS and equilibrated for 45 min in a non-CO₂ incubator. The OCR was measured over a period of 20 min. Data are presented as the mean of three measurements normalized to protein content (pmol/min/ μ g prot).

Membrane Potential Assay

Mitochondrial membrane potential ($n = 6$) was determined by JC-1 mitochondrial membrane potential kit (Abcam, Cambridge, MA) following manufacturer instructions, and as previously described [39–41]. Briefly, MSCs were plated in clear-bottom black 96-well plates at a density of 1×10^4 cells/well. Cells were then incubated with JC-1 (20 μ M) dissolved in complete Dulbecco's minimal essential medium (DMEM) without phenol red for 10 min at 37 °C. JC-1 was then removed and cells treated with medium containing DEM (75 μ M for 6 h) and returned to the incubator. At the end of the experiment, the red (aggregated) and green (monomeric) forms of JC-1 were quantified in a Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA) with excitation at 475 nm and emission at 590 and 525 nm, respectively. The fluorescence values of unstained cells were used to determine background fluorescence and were subtracted from the raw data. The ratio of aggregate to monomeric fluorescence was expressed as percent of normal control value.

Measurement of ATP

Cellular ATP ($n = 6$) was measured by StayBrite ATP assay kit (Biovision Inc., Milpitas, CA) according to manufacturer's instructions. Briefly, experiments were performed on cells seeded in 12-well plates, lysed with provided buffer, centrifuged, and then transferred to sterile tubes. Samples and ATP standards (10 μ l each) were assayed with 80 μ l of reaction mix, and luminescence was measured on a Turner Biosystems 20/20n luminometer.

Measurement of Apoptosis and Necrosis

Apoptosis and necrosis of MSCs ($n = 4$) was determined by FACS analysis (FACSCalibur flow cytometer, BD Biosciences, San Jose, CA) after staining with the Annexin V-FITC/PI Detection Kit (Abcam, Cambridge, MA) according to kit instructions. Briefly, MSCs were grown in 10-cm culture dishes for 24 h. Then, the cells were collected by gentle trypsinization and resuspended in the provided buffer (500 μ l) with 5 μ l of annexin V-FITC and 5 μ l of PI. FACS data were analyzed by BD CellQuest Pro software.

Real-Time Reverse Transcription PCR

RNA was isolated using a RNeasy Mini extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions followed by treatment with DNase I, amplification grade (Invitrogen). One microgram of RNA was reverse transcribed to synthesize complementary DNA (cDNA) using M-MLV Reverse Transcriptase Kit and random primers (Life Technologies, Grand Island, NY). The cDNA underwent 40 rounds of amplification (LightCycler® 480, Roche Diagnostics GmbH, Mannheim, Germany) with the following reaction conditions: 40 cycles of a two-step polymerase chain reaction (PCR; 95 °C for 15 s, 60 °C for 1 min) after initial denaturation (95 °C for 10 min) with LightCycler® 480 Probes Master mix (Roche Diagnostics GmbH, Mannheim Germany), 500 nM of each primer and 250 nM of probe in a 12-µl final volume reaction. Gene expression levels were normalized using β-actin as housekeeping gene. Data were presented in terms of relative expression using the comparative cycle threshold (Ct) method (2^{-Ct}) [42].

PrimeTime® qPCR Assay primer and probe sequences for the genes studied are provided in Table 1 (Integrated DNA Technologies, IDT). Due to the diverse functions of the mitochondria, we focused on the expression of markers involved in a wide variety of mitochondrial functions. Real-time reverse transcription PCR (RT-PCR) was performed for the following candidates: STAT-3 (apoptosis), Tfam (regulator of mitochondrial DNA), ATP5a1 (ATP synthase), CyC1 (complex III of mitochondrial respiration), and NAD(P)H dehydrogenase quinone 1 (NQO1, mitochondrial-related oxidative stress).

Western Blotting

Protein expression of the most promising candidate was assessed by immunoblot analysis (NQO1, Abcam #ab34173, Cambridge, CA; dilution 1:1000).

Luminometer Measurements

NQO1-Fluc reporter enzyme activity was determined by luminometry ($n = 9$) as previously reported [27, 32]. Briefly, cells were lysed with passive lysis buffer for 10 min at 4 °C, and lysates were centrifuged for 15 min at 15,000 rpm at 4 °C. Aliquots of supernatant (20 µl) were mixed with 100 µl of luciferase assay reagent II (LARII, substrate for Fluc, Promega, Madison, WI) or coelenterazine (1 µg, substrate for Rluc in 100 µl PBS, Nanolight Technology, Pinetop, AZ) and analyzed using a luminometer (Turner Designs 20/20n, Sunnyvale, CA) to measure total light emission. This enabled quantification of both Fluc and Rluc activity in relative light units (RLU). Data were corrected for background signal, using lysates from untransfected cells, and normalized to protein content.

Specificity and Sensitivity of the Sensor

To test the specificity of the sensor, we exposed MSCs to a non-mitochondrial challenge (dimethyl sulfoxide, DMSO, 1 %), and measured its effect on cell toxicity (LDH release, $n = 8$) [34], OCR ($n = 5$) and the mitochondrial sensor (NQO1-Fluc) activity. To further examine if this sensor is sensitive to modulations of mitochondrial function, MSCs exposed to DEM (75 µM for 6 h) were treated with a mitochondrial-specific ROS scavenger (mito-Tempo, 100 µM, $n = 6$). To test whether the sensor was specific to mitochondrial activity and would

not react to a non-mitochondrial antioxidant, a separate group of MSCs was treated (in conjunction with DEM-75 μM) with a cytoplasmic antioxidant (Tempol, 2.5 mM, $n = 6$).

Moreover, we sought to determine the response of the NQO1-Fluc sensor after exposure of MSCs to challenges used to study the effect of reactive oxygen species (ROS), highly produced in the pro-inflammatory processes that occur after an ischemia/reperfusion injury, on cellular function. Cells were treated with either hydrogen peroxide (H_2O_2 , 100 μM during 6 h, $n = 4$) or hypoxia (1 % O_2 during 72 h, $n = 4$). Cellular ATP content and the mitochondrial sensor activity were analyzed. MSCs exposed to hypoxic conditions (30,000 cells/well) were transfected with the NQO1-Fluc vector after 48 h of culture, and in vivo BLI of whole intact cells was performed 24 h post-transfection. Cells were imaged for 10 min using 1-minute high-sensitivity acquisition scans, and data were expressed as total radiance (photons/s/cm²/sr) of each well.

Animal Studies

All animal experiments complied with the standards stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and were approved by the Mayo Clinic College of Medicine Institutional Animal Care and Use Committee (IACUC).

Studies were performed on female FVB/NJ inbred mice (Jackson Laboratory, Bar Harbour, ME, USA) of 11 weeks of age, weighing 20–25 g. Mice underwent a surgical procedure for the induction of ischemia/reperfusion (IR) injury: following endotracheal intubation, animals were mechanically ventilated. Isoflurane (1.8 %) was used to maintain general anesthesia. After visualization of the left anterior descending coronary artery (LAD), a 9–0 Ethilon suture was inserted into the myocardium enclosing the LAD just 1.5 mm distal to the left auricle. Then, the LAD was tightly compressed by a thin piece of plastic tubing secured by the suture. Ischemia was confirmed by the appearance of pallor over the apical LV myocardium, along with hypokinesis/akinesis. After 35 min of ischemia, the suture was cut and the tubing removed to allow reperfusion. Allogeneic MSCs (4×10^5 in 30 μl of saline), co-transfected with CMV-Rluc and NQO1-Fluc, were administered transeptically into the peri-ischemic territory of animals ($n = 8$), 10 min after reperfusion or sham procedure ($n = 9$).

BLI was performed 6 and 24 h after MSC delivery. After intraperitoneal injection of the reporter substrate D-luciferin (50 mg/kg body weight), animals were imaged for 20 min using 5-minute high-sensitivity acquisition scans, and data were expressed as total radiance (photons/s/cm²/sr) over the area of the heart. Data are expressed as ratio of Fluc to Rluc activity.

Statistical Analysis

Statistical comparisons between groups were performed using nonparametric Mann–Whitney *U* test. Results are expressed as mean $\pm$ standard error of the mean (s.e.m.). Statistical significance was established at two-tailed $p < 0.05$. All analyses were conducted using GraphPad Prism 6.0 software.

Results

Diethylmaleate as a Mitochondrial Challenge

We observed that exposure of MSCs to increasing doses of DEM resulted in a dose-dependent decrease in OCR (Fig. 1a), as well as a membrane depolarization (Fig. 1b), leading to increased apoptosis (Fig. 1a). Despite significant mitochondrial dysfunction, there was minimal yet significant necrosis/cell death at 50 μ M DEM (Fig. 1a). Furthermore, when MSCs were simultaneously treated with DEM (75 μ M) and mito-Tempo (100 μ M for 24 h), both ATP content and cell viability were improved (Fig. 1c, d).

Identification of Markers of Mitochondrial Dysfunction

Figure 2a shows that DEM (75 μ M, 6 h) triggered the upregulation of many mitochondria-related genes. In these conditions, the antioxidant NQO1 displayed the highest increase both in gene and protein expression (Fig. 2b), when compared to untreated cells. Moreover, when we challenged MSCs with another mitochondrial stressor (Oligomycin A, 10 μ g/ml, 90 min) [43], we again observed a 1.3-fold increase in NQO1 gene expression compared to control.

Construction of Reporter Gene Vectors

Therefore, we constructed a mitochondrial reporter gene sensor whose activity is driven by the NQO1 promoter. A plasmid vector (pNQO1-ARE-FLuc2) expressing firefly luciferase (Fluc) under a mitochondrial-specific antioxidant responsive promoter sequence derived from human NQO1 gene upstream of exon 1 from position 1347–1387 (*acgcgtaaatgcagtcacagtcagtcagcagaatct-gagcctagggagatct*) was cloned into *Mlu*I and *Bgl*II restriction enzyme sites of pGL4.12-Luc2 plasmid. Due to the relatively weak signal obtained when using pathway-specific “single step” reporter genes, and in preparation for *in vivo* use, a two-step transcriptional amplification (TSTA) system-based mitochondrial-specific antioxidant responsive vector was constructed. The above hNQO1-ARE sequence was cloned upstream of minimal-TK-promoter driving the expression of the GAL4-(VP16)₂ fusion protein in *Bsu*361/*Mlu*I restriction enzyme site by replacing the NFkB promoter sequence in pNFkB-TK-minimal-TSTA-FLuc2 vector from our library (Fig. 3). The vector possesses a FLuc2 gene with five repeats of GAL4-binding DNA sequence (G5-DBD) followed by E4-TATA box. When the mitochondrial stress-specific GAL4-VP16 protein expression increases, it binds to a G5-DBD inducing Fluc protein expression.

Reporter Gene Detection of Cellular Mitochondrial Function In Vitro

MSCs were co-transfected with the CMV-Rluc and NQO1-Fluc plasmids (10 and 500 ng per well, respectively) as previously described [27, 32] and exposed to different concentrations of DEM. The NQO1-Fluc signal was inversely proportional to the amount of OCR in the cells (Fig. 4a). Importantly, MSCs exposed to a non-mitochondrial challenge (DMSO) had elevated LDH release, compared to controls (5.04 ± 0.63 vs. 3.56 ± 0.13 %, $p = 0.02$), but no change in mitochondrial function (OCR, 24.40 ± 1.74 vs. 25.33 ± 2.30 pmol/min/ μ g protein, $p = 0.7143$), and no increased signal from the mitochondrial Fluc sensor (0.28 ± 0.02 vs. 0.27 ± 0.01 RLU-Fluc/RLU-Rluc/ μ g protein, $p = 0.5$), showing that the mitochondrial sensor does not react to non-mitochondrial cellular stress, but is specific to the mitochondria.

Furthermore, when MSCs were treated simultaneously with DEM and mito-Tempo, they exhibited lower NQO1-Fluc signal (Fig. 4b). On the other hand, the Fluc signal from MSCs treated with Tempol (0.05–5 mM, concentrations shown to normalize cytoplasmic oxidative stress [34]) was similar to that from cells treated with DEM alone (2.5 mM Tempol, Fig. 4c). Moreover, exposure to H₂O₂ induced a significant reduction in ATP content (Fig. 5a), which was partially improved by mito-Tempo, as well as an increased activity of the mitochondrial sensor, which was significantly reduced by mito-Tempo (Fig. 5b). Finally, MSCs cultured in hypoxic conditions (1 % O₂) displayed a significant decrease in cellular ATP content (Fig. 5c) associated with a significant increase in Fluc activity as assessed by luminometry (Fig. 5d) and *in vivo* BLI (Fig. 5e, f).

Monitoring of Mitochondrial Function in MSCs

Finally, we validated the mitochondrial oxidative stress sensor in *in vivo* studies. Both at 6 and 24 h, the Fluc signal was highest in MSCs transplanted to IR animals compared to sham, suggesting that MSCs in the former group are undergoing a higher degree of mitochondrial dysfunction (Fig. 6a, b at 6 h and Fig. 6c, d at 24 h). In the current conditions, and likely due to its absorption/emission properties, the Rluc signal could not be reliably detected by *in vivo* BLI. Thus, ex vivo luminometry of heart homogenates was used to enable correction of mitochondrial function activity (Fluc) for the number of viable MSC (Rluc) [32]. The ex vivo luminometry showed a similar behavior (Fig. 6e). Moreover, MSCs transfected with a Null-Fluc vector displayed very low levels of Fluc activity (Fig. 6e), whereas MSCs treated ex vivo with mito-Tempo (100 μM) showed Fluc signals that were similar to those in shams (Fig. 6e, n = 5).

Discussion

In this study, we validated a reporter gene sensor to detect mitochondrial dysfunction in stem cells transplanted into the ischemic myocardium. Moreover, this study provides evidence that this novel mitochondrial sensor may be used to monitor the effect of adjuvant interventions targeted to improve the biology of transplanted progenitor cells.

Cell therapy studies, regardless of the cell type used, have consistently encountered a significant rate of cell death after transplantation [28, 44, 45]. As mitochondrial function is a major determinant of cell survival, there is interest in understanding the mitochondrial status in transplanted cells. The mitochondria have many functions, including cellular respiration/ATP synthesis, apoptosis regulation, calcium handling, and redox signaling, among others. For the purpose of the development of the mitochondrial sensor, we chose to screen several candidates that reflect many (but not all) of the functions associated with the mitochondria. We chose some of the markers/functions deemed more critical to cell survival and, therefore, more likely to result in cell death. Notwithstanding, it is possible that other mitochondrial functions/markers not tested could have also been affected in these conditions.

In these studies, we chose DEM as the mitochondrial challenge. DEM is a compound that leads to increased oxidative stress, thereby destabilizing mitochondria, leading to mitochondrial dysfunction, as shown in previous studies [35, 46]. Our results show that

DEM affected cellular respiration and membrane polarization leading to cell death, validating the mitochondrial dependence of this challenge. In these settings, NQO1 was the most promising candidate among the genes evaluated. NQO1 is an enzyme that is associated with mitochondrial metabolism and stress, which has been closely linked to cellular respiration, and ultimately, cell survival. The intention of the use of DEM is not to reproduce the noxious environment that MSCs face after transplantation, but rather to induce mitochondrial dysfunction in a controlled, predictable way to allow proof-of-principle experiments to validate the candidate marker probe. We have previously shown that the post-ischemic myocardium is a noxious environment characterized by increased inflammation, fibrosis, and oxidative stress, all in a state of relative hypoxia [8, 9]. Due to the complexity of the stimuli involved, and the dynamic nature of the changes, we believe that the post-ischemic microenvironment cannot be accurately reproduced *in vitro*, hence the critical importance of our *in vivo* studies to provide conclusive evidence of the activation of the mitochondrial sensor in that setting. Nevertheless, we studied *in vitro* the effect of ROS in MSCs (after exposure to H₂O₂), as it would occur in a post-IR environment, to confirm the utility of the NQO1-Fluc sensor for our studies. The small, but statistically significant, improvement in the amount of ATP and sensor activity observed in MSCs treated with the mitochondrial-specific mito-Tempo may be due to the fact that the majority of the ROS are in fact produced in the cytoplasm.

From the imaging perspective, we elected to use the Fluc reporter gene system, which has been a cornerstone of BLI to evaluate cell fate and pathway-specific processes [32, 47] in small animal studies, in large part due to the high wavelength (500–700 nm) of the Fluc signal [27, 29, 31]. In this manuscript, we extend our previous work and show the versatility of this imaging approach in the understanding and monitoring of cell biology directly in the living subject. Furthermore, these systems can be used not only for monitoring purposes but also as an endogenous trigger for therapy, in a “Theranostics” approach. One could conceive the use of endogenous mitochondrial signals, to trigger the activation of different endogenous or exogenous therapeutic genes, with the ultimate goal of improving cell function, and ultimately, cell survival. Because Fluc uses ATP to catabolize luciferin, it is possible that, under mitochondrial stress conditions, the *in vivo* Fluc signal may have been “minimized” by the dependence of Fluc activity on the availability of ATP in mitochondrially stressed cells, and that having used a non-ATP-dependent reporter gene, results might have been even more impressive. It should be mentioned that ATP is not needed for the *in vitro* luminometer testing and that *in vivo* and *in vitro* studies had quite comparable results. In future studies, reporter genes that do not depend on ATP for its activity can be considered. Available options may not be sensitive enough or not in the desired light spectrum to be applicable to animal studies. Positron emission tomography reporter genes like thymidine kinase may be an option but are not ideal for high-throughput small animal studies [25].

Furthermore, the absolute level of signal produced by the NQO1 reporter vector, when compared to the Fluc signal expressed under a CMV promoter, is several folds lower and is below the threshold of luciferase protein required to optically image myocardial implants in murine model. Hence, we constructed a two-step transcriptional amplification strategy, which we previously developed and applied for imaging various endogenous promoters

(prostate-specific antigen-PSA, hypoxia inducible factor one alpha-HIF 1 α , vascular endothelial growth factor-VEGF) in living animals [48–50].

Conclusions

In conclusion, this study demonstrates the feasibility of using a conditional reporter gene approach to monitor the mitochondrial function of transplanted progenitor cells, providing a novel platform for optimizing the use of cell therapies.

Acknowledgments

This study was supported in part by the National Institutes of Health awards R56 HL113371 (MR-P) and RO1CA161091 (RP). We acknowledge the Todd and Karen Wanek Family Program for Hypoplastic Left Heart Syndrome for the assistance with the metabolic analysis of stem cells (Seahorse experiments). The luminometer used was obtained through a grant from Turner Biosystems, Sunnyvale, CA.

References

1. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001; 410:701–705. [PubMed: 11287958]
2. Mazo M, Araña M, Pelacho B, Prósper F. Mesenchymal stem cells and cardiovascular disease: a bench to bedside roadmap. *Stem Cells Int*. 2012; 2012:1–11.
3. Schächinger V, Erbs S, Elsässer A, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med*. 2006; 355:1210–1221. [PubMed: 16990384]
4. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002; 106:1913–1918. [PubMed: 12370212]
5. Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet*. 2004; 364:141–148. [PubMed: 15246726]
6. Assmus B, Dimmeler S, Zeiher AM. Cardiac cell therapy: lost in meta-analyses. *Circ Res*. 2015; 116:1291–1292. [PubMed: 25858060]
7. Gyöngyösi M, Wojakowski W, Lemarchand P, et al. Meta-Analysis of Cell-based CaRdiac stUdiEs (ACCRUE) in patients with acute myocardial infarction based on individual patient data. *Circ Res*. 2015; 116:1346–1360. [PubMed: 25700037]
8. Lee SH, Wolf PL, Escudero R, et al. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. *N Engl J Med*. 2000; 342:626–633. [PubMed: 10699162]
9. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. *Circulation*. 2000; 101:2981–2988. [PubMed: 10869273]
10. Forrester JS, Makkar RR, Marban E. Long-term outcome of stem cell therapy for acute myocardial infarction. *J Am Coll Cardiol*. 2009; 53:2270–2272. [PubMed: 19520250]
11. Mohsin S, Siddiqi S, Collins B, Sussman MA. Empowering adult stem cells for myocardial regeneration. *Circ Res*. 2011; 109:1415–1428. [PubMed: 22158649]
12. Don CW, Murry CE. Improving survival and efficacy of pluripotent stem cell-derived cardiac grafts. *J Cell Mol Med*. 2013; 17:1355–1362. [PubMed: 24118766]
13. Boengler K, Heusch G, Schulz R. Mitochondria in postconditioning. *Antioxid Redox Signal*. 2011; 14:863–880. [PubMed: 20578961]
14. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. 2007; 87:99–163. [PubMed: 17237344]
15. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. *Circ Res*. 2012; 111:1208–1221. [PubMed: 23065344]
16. Orrenius S. Reactive oxygen species in mitochondria-mediated cell death. *Drug Metab Rev*. 2007; 39:443–455. [PubMed: 17786631]

17. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. Apoptosis. 2007; 12:913–922. [PubMed: 17453160]
18. Bouchier-Hayes L, Lartigue L, Newmeyer DD. Mitochondria: pharmacological manipulation of cell death. J Clin Invest. 2005; 115:2640–2647. [PubMed: 16200197]
19. Lam E, Kato N, Lawton M. Programmed cell death, mitochondria and the plant hypersensitive response. Nature. 2001; 411:848–853. [PubMed: 11459068]
20. Biniecka M, Fox E, Gao W, et al. Hypoxia induces mitochondrial mutagenesis and dysfunction in inflammatory arthritis. Arthritis Rheum. 2011; 63:2172–2182. [PubMed: 21484771]
21. Garedew A, Moncada S. Mitochondrial dysfunction and HIF1alpha stabilization in inflammation. J Cell Sci. 2008; 121:3468–3475. [PubMed: 18827009]
22. Milano G, Bianciardi P, Corno AF, et al. Myocardial impairment in chronic hypoxia is abolished by short aeration episodes: involvement of K⁺ATP channels. Exp Biol Med (Maywood). 2004; 229:1196–1205. [PubMed: 15564447]
23. Zhu W, Chen J, Cong X, et al. Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. Stem Cells. 2006; 24:416–425. [PubMed: 16253984]
24. Ong SB, Gustafsson AB. New roles for mitochondria in cell death in the reperfused myocardium. Cardiovasc Res. 2012; 94:190–196. [PubMed: 22108916]
25. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. Genes Dev. 2003; 17:545–580. [PubMed: 12629038]
26. Sinusas AJ, Bengel F, Nahrendorf M, et al. Multimodality cardiovascular molecular imaging, part I. Circ Cardiovasc Imaging. 2008; 1:244–256. [PubMed: 19808549]
27. Rodriguez-Porcel M. In vivo imaging and monitoring of transplanted stem cells: clinical applications. Curr Cardiol Rep. 2010; 12:51–58. [PubMed: 20425184]
28. Wu JC. Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography. Circulation. 2003; 108:1302–1305. [PubMed: 12963637]
29. Wu JC, Tseng JR, Gambhir SS. Molecular imaging of cardiovascular gene products. J Nucl Cardiol. 2004; 11:491–505. [PubMed: 15295418]
30. Chen IY, Greve JM, Gheysens O, et al. Comparison of optical bioluminescence reporter gene and superparamagnetic iron oxide MR contrast agent as cell markers for noninvasive imaging of cardiac cell transplantation. Mol Imaging Biol. 2009; 11:178–187. [PubMed: 19034584]
31. Nguyen PK, Riegler J, Wu JC. Stem cell imaging: from bench to bedside. Cell Stem Cell. 2014; 14:431–444. [PubMed: 24702995]
32. Psaltis PJ, Peterson KM, Xu R, et al. Noninvasive monitoring of oxidative stress in transplanted mesenchymal stromal cells. JACC Cardiovasc Imaging. 2013; 6:795–802. [PubMed: 23643284]
33. Franchi F, Ezenekwe A, Wellkamp L, et al. Renin inhibition improves the survival of mesenchymal stromal cells in a mouse model of myocardial infarction. J Cardiovasc Transl Res. 2014; 7:560–569. [PubMed: 25030734]
34. Peterson KM, Aly A, Lerman A, et al. Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress. Life Sci. 2011; 88:65–73. [PubMed: 21062632]
35. Garcia-Ruiz C, Colell A, Morales A, et al. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-kappa B: studies with isolated mitochondria and rat hepatocytes. Mol Pharmacol. 1995; 48:825–834. [PubMed: 7476912]
36. Fujii Y, Johnson ME, Gores GJ. Mitochondrial dysfunction during anoxia/reoxygenation injury of liver sinusoidal endothelial cells. Hepatology. 1994; 20:177–185. [PubMed: 7517381]
37. Goossens V, Grooten J, De Vos K, Fiers W. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci U S A. 1995; 92:8115–8119. [PubMed: 7667254]
38. Folmes CD, Martinez-Fernandez A, Perales-Clemente E, et al. Disease-causing mitochondrial heteroplasmy segregated within induced pluripotent stem cell clones derived from a patient with MELAS. Stem Cells. 2013; 31:1298–1308. [PubMed: 23553816]

39. Chen TL, Zhu GL, Wang JA, et al. Apoptosis of bone marrow mesenchymal stem cells caused by hypoxia/reoxygenation via multiple pathways. *Int J Clin Exp Med*. 2014; 7:4686–4697. [PubMed: 25663966]
40. Nie Y, Han BM, Liu XB, et al. Identification of microRNAs involved in hypoxia- and serum deprivation-induced apoptosis in mesenchymal stem cells. *Int J Biol Sci*. 2011; 7:762–768. [PubMed: 21698002]
41. Sun X, Fang B, Zhao X, et al. Preconditioning of mesenchymal stem cells by sevoflurane to improve their therapeutic potential. *PLoS ONE*. 2014; 9(3):e90667.doi: 10.1371/journal.pone.0090667 [PubMed: 24599264]
42. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008; 3:1101–1108. [PubMed: 18546601]
43. Liu Y, Schubert DR. The specificity of neuroprotection by antioxidants. *J Biomed Sci*. 2009; 16:98–14. [PubMed: 19891782]
44. Rodriguez-Porcel M, Gheysens O, Chen IY, et al. Image-guided cardiac cell delivery using high-resolution small-animal ultrasound. *Mol Ther*. 2005; 12:1142–1147. [PubMed: 16111921]
45. Hou D, Youssef EA, Brinton TJ, et al. Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials. *Circulation*. 2005; 112:1150–6. [PubMed: 16159808]
46. Tong V, Teng XW, Chang TK, Abbott FS. Valproic acid II: effects on oxidative stress, mitochondrial membrane potential, and cytotoxicity in glutathione-depleted rat hepatocytes. *Toxicol Sci*. 2005; 86:436–443. [PubMed: 15858222]
47. Terrovitis J, Kwok KF, Lautamaki R, et al. Ectopic expression of the sodium-iodide symporter enables imaging of transplanted cardiac stem cells in vivo by single-photon emission computed tomography or positron emission tomography. *J Am Coll Cardiol*. 2008; 52:1652–1660. [PubMed: 18992656]
48. Chen IY, Gheysens O, Li Z, et al. Noninvasive imaging of hypoxia-inducible factor-1alpha gene therapy for myocardial ischemia. *Hum Gene Ther Methods*. 2013; 24:279–288. [PubMed: 23937265]
49. Iyer M, Wu L, Carey M, et al. Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc Natl Acad Sci U S A*. 2001; 98:14595–14600. [PubMed: 11734653]
50. Wang Y, Iyer M, Annala A, et al. Noninvasive indirect imaging of vascular endothelial growth factor gene expression using bioluminescence imaging in living transgenic mice. *Physiol Genomics*. 2006; 24:173–180. [PubMed: 16410544]

Fig. 1.

Dose-dependent effect of DEM on MSC mitochondrial function. **a** Increasing doses of DEM lowered OCR (*open triangles*, $n = 5$) and increased apoptosis (*closed squares*), with minimal cell death (*open circles*) ($n = 4$). **b** DEM lowered polarization of the cell ($n = 6$). The use of a mitochondrial-specific reactive oxygen species scavenger (mito-Tempo) improved **c** cellular ATP content ($n = 6$) as well as **d** apoptosis ($n = 4$). PI and Annexin: all DEM doses were statistically different from baseline. DEM diethylmaleate, MSCs mesenchymal stromal cells, OCR oxygen consumption rate, ATP adenosine triphosphate. * $p < 0.05$ vs. control (untreated cells), # $p < 0.05$ vs. DEM only.

Fig. 2.

Candidate marker screening. **a** Several candidate markers were screened by real-time RT-PCR in MSCs exposed to DEM (75 μ M) for 6 h, with NQO1 showing the highest change expression. **b** NQO1 protein expression was quantified using immunoblotting. *NQO1* NAD(P)H dehydrogenase, quinone 1. * $p < 0.0001$ compared to control.

Fig. 3.

Reporter vector constructs. **a** Single step vector in which the NQO1-ARE promoter drives the expression of the firefly luciferase (Fluc) reporter gene. **b** TSTA vector expresses GAL4-VP16 fusion protein under the NQO1-ARE promoter. The GAL4-VP16 protein binds to five time repeats of GAL4-binding DNA sequence located upstream of a minimal TK promoter which drives the expression of firefly luciferase. The VP16 peptide of GAL4-VP16 fusion protein transcriptionally activates minimal TK promoter and increases the expression of Fluc protein by recruiting co-activators. The *arrows* represent the direction of the transcription. The reporter gene vector is bi-directional, providing a significant increase in firefly luciferase expression.

Fig. 4.

Validation of the mitochondrial sensor *in vitro*. **a** Increasing doses of DEM impaired MSC mitochondrial function (OCR, *open triangles*) and increased the NQO1-Fluc signal (*closed circles*). The Fluc signal showed strong inverse correlation with OCR. Furthermore, the use of mito-Tempo **b** decreased the NQO1-Fluc signal (control and DEM $n = 9$, DEM + Mito-Tempo $n = 6$), while the use of Tempol **c** did not change the Fluc signal ($n = 6$). * $p < 0.05$ vs. control (untreated cells).

Fig. 5.

Specificity of the mitochondrial sensor. Exposure to H₂O₂ or hypoxia (1 % O₂) reduces cellular ATP (**a** and **c**, respectively) and increases the activity of the NQO1-Fluc sensor (**b** and **d** respectively). **a, b** Both the ATP content and the mitochondrial sensor showed partial improvement when MSCs exposed to H₂O₂ were simultaneously treated with mito-Tempo. **e** Representative *in vivo* bioluminescence imaging (BLI) of living intact MSCs treated with hypoxia, showing increased Fluc signal. **f** Quantification of the NQO1 expression via *in vivo* BLI of cultured MSCs. *N* = 4 for all groups. **p* < 0.05 vs. control (untreated cells), #*p* < 0.05 vs. DEM only.

Fig. 6. Validation of the mitochondrial sensor in animal studies. Representative bioluminescence images for Fluc signal at **a** 6 h and **c** 24 h after MSC transplantation in the different experimental groups. Quantitation of Fluc activity was measured *in vivo* at **b** 6 h and **d** 24 h and confirmed using **e** ex vivo luminometry, corrected for Rluc activity. Sham ($n = 9$), IR ($n = 8$), IR mito-Tempo ($n = 5$), IR null vector ($n = 1$). * $p < 0.05$ vs. sham animals, # $p < 0.05$ vs. I/R animals.

Table 1

Primers and probe sequences for the genes studied

Gene	Forward sequence	Reverse sequence	Probe sequence
<i>Actb</i>	AGGTCTTTACGGATGTCAACG	ATTGGCAACGAGCGGT	ATTCCATACCCAAGAAGGAAGGCTGG
<i>Stat3</i>	CAAGCATTTGGCATCTGACAG	ACCTGATCACCTTCGAGACT	ACCTAGAGACCCACTCCTTGCCA
<i>Cyc1</i>	GCGACATCCTTAGCTACTTGT	CCTCTATTTC AACCTTACTTTCCC	TGATGATGGCACCCCAGTACC
<i>Atp5a1</i>	GGAGCCAAGTACTGAAGCG	AAGCTGTACTGCATCTACGTC	CTGGTGAAGAGACTGACGGATGCG
<i>Tfam</i>	ATCCTTTGCCTCCTGGAAG	CTGATGGGTATGGAGAAGGAG	ATGCTGAACGAGGTCTTTTGGTTTTCC
<i>NQO1</i>	AGATGACTCGGAAGGATACTGA	GTACTCGAATCTGACCTCTATGC	ACATCACAGGTGAGCTGAAGGACTC