Physiological Coupling of Donor and Host Cardiomyocytes After Cellular Transplantation

Michael Rubart, Kishore B.S. Pasumarthi, Hidehiro Nakajima, Mark H. Soonpaa, Hisako O. Nakajima, Loren J. Field

Abstract—Cellular transplantation has emerged as a potential approach to treat diseased hearts. Although cell transplantation can affect global heart function, it is not known if this results directly via functional integration of donor myocytes or indirectly via enhanced revascularization and/or altered postinjury remodeling. To determine the degree to which donor cardiomyocytes are able to functionally integrate with the host myocardium, fetal transgenic cardiomyocytes expressing enhanced green fluorescent protein were transplanted into the hearts of nontransgenic adult mice. Two-photon molecular excitation laser scanning microscopy was then used to simultaneously image cellular calcium transients in donor and host cells within the intact recipient hearts. Calcium transients in the donor cardiomyocytes were synchronous with and had kinetics indistinguishable from those of neighboring host cardiomyocytes. These results strongly suggest that donor cardiomyocytes functionally couple with host cardiomyocytes and support the notion that transplanted cardiomyocytes can form a functional syncytium with the host myocardium. (Circ Res. 2003;92:1217-1224.)

Key Words: cellular transplantation • heart regeneration

Many forms of cardiac disease are precipitated by cardiomyocyte death. Although it is accepted that there is some capacity for cardiomyocyte DNA synthesis in the adult heart, the degree to which this occurs and the cellular consequences (eg, polyploidization, karyokinesis, and/or cytokinesis) have been the subject of considerable debate.1,2 It has also been suggested that cardiomyogenic adult stem cells may contribute to myocardial reconstitution, but again the degree to which this occurs is controversial.3–6 The potential contribution of cell fusion events2,8 to apparent stem cell-mediated cardiomyogenesis also needs to be resolved. Despite data supporting the presence of at least some degree of myocardial renewal via either proliferation or stem cell recruitment, the propensity for cumulative decreases in cardiomyocyte number during cardiovascular disease indicates that the rate at which renewal occurs naturally is insufficient to restore myocyte loss. Interventions aimed at augmenting cardiomyocyte number in diseased hearts therefore could be of considerable therapeutic value. Current strategies to accomplish this include cellular transplantation,9 adult stem cell mobilization,10 and cardiomyocyte cell cycle activation.11 Cellular transplantation approaches have to date used fetal cardiomyocytes, embryonic stem cell–derived cardiomyocytes, skeletal myoblasts, endothelial stem cells, and adult stem cells with myogenic potential as donor cells.

Although a large body of data supports the notion that cardiomyocyte transplantation can have a positive effect on global cardiac function,12–15 it is not clear if this results from direct functional integration of the donor cells with the host myocardium.16 A review of the literature indicates that cardiomyocyte transplantation can augment and/or enhance myocardial angiogenesis and revascularization.9 Although such indirect effects can clearly be of therapeutic benefit, replacement of lost systolic function remains the major goal of myocardial regeneration strategies. The ability to monitor donor cell functional activity at the cellular level is therefore of critical importance. Although ultrastructural analyses suggest physical coupling between donor and host cardiomyocytes,17,18 these analyses do not address functional activity, nor are they amenable to the analysis of a large number of donor cells.

Traditional confocal imaging approaches permit the imaging of intracellular calcium transients in Langendorff-perfused hearts at high spatial and temporal resolution.19,20 Two-photon molecular excitation (TPME) laser scanning microscopy (also known as 2-photon laser scanning fluorescent microscopy or TPLSM21) permits imaging within intact tissue at depths in excess of 100 μm.21,22 This system has recently been used to monitor spontaneous and stimulation-evoked calcium transients in individual cardiomyocytes.
with intact hearts at depths superior to those obtainable with traditional single-photon confocal imaging approaches.\textsuperscript{23} Given the ability to image at greater tissue depths, TPME laser scanning microscopy could be used to monitor physiological coupling between transplanted cardiomyocytes and the host myocardium, provided that the donor and host cells could be distinguished.

In this report, enhanced green fluorescent protein (EGFP)-expressing donor cardiomyocytes were transplanted into the hearts of adult nontransgenic recipients. TPME laser scanning microscopy was used to simultaneously monitor calcium-dependent changes of indicator dye fluorescence and EGFP status in the intact recipient heart. Spontaneous and stimulation-evoked calcium transients were observed to occur simultaneously in donor and host cardiomyocytes, strongly suggesting that the donor cells were functionally coupled with the host myocardium. These data support the concept that transplanted cardiomyocytes can contribute to myocardial function in vivo.

**Materials and Methods**

**Generation of the MHC-EGFP Transgene Mice**

The MHC-EGFP transgene used the mouse \(\alpha\)-cardiac myosin heavy chain (MHC) promoter\textsuperscript{24} and sequences encoding an EGFP reporter (Figure 1A). The SV40 early region transcription terminator/polyadenylation site\textsuperscript{25} was inserted downstream from the EGFP sequence. Transgenic mice were generated and screened as described.\textsuperscript{28} Two patterns of transgene expression were observed in the resulting lineages. Several of the lines exhibited very high penetrance of transgene expression, with virtually all of the cardiomyocytes exhibiting EGFP positivity (Figure 1B). To date, no deleterious consequences of myocardial EGFP expression have been noted in these lines. Other lines exhibited a mosaic pattern of transgene expression, with anywhere from 1\% to \(\sim\)75\% of the cardiomyocytes exhibiting EGFP fluorescence of varying strength in the adult heart (Figure 1C).

**TPME Imaging System**

Images were recorded with a Bio-Rad MRC 1024 Laser Scanning microscope modified for TPME (described in detail in the online Materials and Methods). Illumination for 2-photon excitation was provided by a mode-locked Ti:Sapphire laser (Spectraphysics, Mountain View, Calif); the excitation wavelength was 810 nm. Hearts were imaged with a Nikon \(\times 60\) 1.2 numerical aperture water-immersion lens with a working distance of 200 \(\mu\)m. Using 2-photon molecular excitation with a high numerical aperture objective, Denk\textsuperscript{29} previously demonstrated that the majority of all excitations are confined to less than femtoliter volumes around the focal points, with \(\sim\)1-\(\mu\)m resolution along the laser propagation axis. Measurements of the axial resolution were in excellent agreement with these values.\textsuperscript{31} Emitted light was collected by 2 photomultiplier tubes fitted with narrow bandwidth filters for 560 to 650 nm and 500 to 550 nm, respectively. Images were collected at a resolution of 0.43 \(\mu\)m/pixel along the \(x\)-\(y\)-axis. For full-frame mode analyses (512\(\times\)512 pixels), hearts were scanned at 1.46 and 0.73 frames per second on horizontal (\(x, y\)) planes and the resulting images digitized at 8-bit resolution and stored directly on the hard disk. For line-scan mode analyses, hearts were scanned repetitively along a line spanning at least 2 juxtaposed cardiomyocytes (scan speed was 6.88 \(\mu\)m/ms, corresponding to a pixel dwell time of 62.5 \(\mu\)s). Line-scan images were then constructed by stacking all lines vertically. Postacquisition analysis was performed using MetaMorph software version 4.6r (Universal Imaging Incorporation, Downington, Pa). For determination of the time course of [Ca\textsuperscript{2+}]\textsuperscript{c}, decay, rhod-2 fluorescence intensity was normalized to the difference between peak and baseline intensity, and intervals from 90\% to 50\% (\(t_{90\%\text{--}50\%}\)) and 50\% to 10\% (\(t_{50\%\text{--}10\%}\)) decay were calculated.\textsuperscript{30}

**Fetal Cardiomyocyte Transplantation**

Single-cell preparations of embryonic day 15 transgenic fetal donor cardiomyocytes were prepared and injected into the left ventricular wall of syngeneic mice as described previously.\textsuperscript{17,20} One hundred thousand cells were injected directly into the ventricular myocardium of host animals. Immune localization of connexin43 was performed as described (see online Materials and Methods).

**Results**

**Generation and Analysis of MHC-EGFP Mice**

A fluorescence-based reporter transgene was generated to facilitate the identification of donor cardiomyocytes after transplantation into nontransgenic recipient hearts. The reporter transgene (designated MHC-EGFP) used the \(\alpha\)-cardiac MHC promoter to target expression of EGFP to cardiomyocytes (Figure 1A). Nine transgenic lineages were generated. Two patterns of transgene expression were observed in the resulting lineages. Several of the lines exhibited very high penetrance of transgene expression, with virtually all of the cardiomyocytes exhibiting EGFP positivity (Figure 1B). To date, no deleterious consequences of myocardial EGFP expression have been noted in these lines. Other lines exhibited a mosaic pattern of transgene expression, with anywhere from 1\% to \(\sim\)75\% of the cardiomyocytes exhibiting EGFP fluorescence of varying strength in the adult heart (Figure 1C).
Interestingly, the level of mosaic transgene expression appeared to be influenced by the genetic background of the mice. The transgenic lineages were generated in C3HeB/FeJ inbred mice; after crossing the mosaic-expressing mice into a DBA/2J background, 100% of the cardiomyocytes expressed EGFP (1000 cells counted in a dispersed cell preparation). We have observed a similar genetic background effect with a number of other MHC-promoted transgenes (L. Field, unpublished observation).

Simultaneous Imaging of \([\text{Ca}^{2+}]_i\) Transients and EGFP Status in Intact Hearts

A TPME imaging system was configured to permit simultaneous monitoring of fluorescence from a calcium-sensitive dye and EGFP. Either fura-2 or rhod-2 can be used to monitor intracellular calcium \((\left[\text{Ca}^{2+}\right]_i)\) transients in individual myocytes within the intact heart using this imaging system. Based on the maximal emission wavelengths of 579 and 509 nm for calcium-bound rhod-2 and EGFP, respectively, we reasoned that this combination of fluorophores would provide a good separation of fluorescence signals. This prediction was confirmed by comparative analysis of rhod-2–loaded nontransgenic mouse hearts with nonloaded MHC-EGFP transgenic hearts (see online Materials and Methods for validation of signal separation). MHC-EGFP transgenic mice with mosaic EGFP reporter gene expression were used to demonstrate the ability of the TPME system to simultaneously image \([\text{Ca}^{2+}]_i\), transients and EGFP status in intact hearts. During imaging, hearts were perfused with Tyrode’s solution containing cytochalasin D to uncouple contraction from excitation (and thereby eliminate motion artifacts during the imaging protocol).

The hearts were then point-stimulated (1 to 4 Hz) at a site remote from the epicardial surface being imaged. Under these conditions, the occurrence of electrically evoked calcium transients across the imaged areas was dependent on cell-to-cell action potential propagation (as opposed to field stimulation). Although cytochalasin D can influence action potential duration and calcium transient amplitude, these properties do not interfere with the ability of the TPME imaging system to monitor synchronous calcium transients. Images of the full-frame mode emission (Figure 2A) revealed that red rhod-2 fluorescence was uniformly distributed throughout the cardiomyocyte cytoplasm and was also prominent in endothelial cell nuclei (in agreement with our previous observations). A typical myocardial cytoarchitecture was apparent, with neighboring cardiomyocytes well aligned with one another. The spatially restricted, increased rhod-2 fluorescence apparent across the middle of the image (as well as in all subsequent rhod-2 full-frame mode images) reflects the relatively slower rate for data acquisition for a full-frame mode image compared with the kinetics of the \([\text{Ca}^{2+}]_i\) transients. It was apparent in the full-frame mode image that EGFP and rhod-2 were excited simultaneously with an input wavelength of 810 nm (Figure 2A). The EGFP-expressing cardiomyocytes (which appeared yellow as
a result of the overlay of green EGFP and red rhod-2 fluorescence) were well aligned with and morphologically indistinguishable from cardiomyocytes that did not express EGFP. Fluorescence signals were also recorded in the line-scan mode to quantify temporal changes in $[\text{Ca}^{2+}]$. The scan line (Figure 2A, white bar) traversed 3 juxtaposed cardiomyocytes at a speed of 6.88 µm/ms. The stacked line-scan images (Figure 2B) showed that the EGFP-expressing cardiomyocyte (cell 2) and the nonexpressing neighbors (cells 1 and 3) simultaneously exhibited transient increases in rhod-2 fluorescence, corresponding to stimulation-evoked increases in $[\text{Ca}^{2+}]$. Simultaneous transients between the EGFP-expressing and nonexpressing cells persisted when the stimulation rate was increased from 1 to 2 Hz (Figure 2B).

Averaged traces of the red and green fluorescence from cells 1, 2, and 3 were generated from the line-scan data (Figure 2C). The transient changes in rhod-2 fluorescence (red traces) appeared qualitatively very similar to $[\text{Ca}^{2+}]$, transients recorded from isolated cells under similar experimental conditions. Appreciable EGFP signal (green traces) was only apparent in cell 2. Importantly, there was no change in EGFP fluorescence associated with the electrical stimulus; thus, all changes in the fluorescence in the red range resulted solely from changes in rhod-2 fluorescence. The kinetics of stimulation-evoked changes in $[\text{Ca}^{2+}]$, were essentially superimposable in the EGFP-expressing and nonexpressing cells (Figure 2D). For each cell, the relative changes in fluorescence were normalized such that 0 represents the prestimulus value. Peak increases in $[\text{Ca}^{2+}]$ occurred simultaneously in both cell types (within the temporal resolution of our imaging system) and decayed with $t_{50}$ values of 78±5.8 and 137±10.3 ms, respectively, in the EGFP-expressing cells and 81±4.3 and 146±9.9 ms in the nonexpressing cells (1 Hz; 14 EGFP-expressing cardiomyocytes and their neighboring nonexpressing cardiomyocytes were analyzed, distributed between 3 of the MHC-EGFP mosaic hearts; $P>0.05$; t test). Doubling the stimulation rate similarly shortened $t_{50}$ values in EGFP-positive (64±4.3 and 98±5.7 ms) and EGFP-negative myocytes (65±3.7 and 115±7.9 ms; $P>0.05$). These data indicated that the mechanisms underlying depolarization-induced increase in cytosolic intracellular calcium as well as subsequent removal of calcium ions from the cytosol were unaltered in EGFP-expressing cardiomyocytes. The TPME system was thus suitable for the simultaneous imaging of $[\text{Ca}^{2+}]$, transients and EGFP status in the intact heart.

**Imaging of $[\text{Ca}^{2+}]$, Transients and EGFP**

Fluorescence of Donor and Host Cardiomyocytes After Transplantation Into the Heart of a Syngeneic Adult Nontransgenic Mouse

To determine if transplanted cardiomyocytes were able to functionally couple with the host myocardium, single-cell suspensions of embryonic day 15 ventricular cardiomyocytes from MHC-EGFP mice were injected directly into the left ventricle and septum of nontransgenic adult mice. A transgenic lineage showing high levels of transgene expression (100% EGFP-positive cardiomyocytes) was used. Hearts were harvested from 8 to 37 days after cellular transplantation and subjected to TPME analysis. Donor cardiomyocytes (which appeared yellow as a result of the overlay of green EGFP and red rhod-2 fluorescence) were well aligned with and morphologically indistinguishable from the host cardiomyocytes when examined in full-frame mode (Figure 3A). The imaged cells were located ~40 µm from the epicardial surface. Line-scan imaging was also performed (the scan line traversed 7 juxtaposed cardiomyocytes at a speed of 6.88 µm/ms, Figure 3A). Stacked line-scan images (Figure 3B) revealed that spontaneous rhod-2 fluorescence transients occurred simultaneously in EGFP-expressing cardiomyocytes (cells 2, 3, 5, and 6) and host cardiomyocytes (cells 1, 4, and 7). This 1:1 association of the rhod-2 transients in host and donor cardiomyocytes was maintained during remote point stimulation pacing at either 2 or 4 Hz, as well as after the resumption of spontaneous activity (Figure 3B).

Averaged traces for the red and green fluorescence present in cells 1 and 2 were generated from the line-scan data (Figure 3C). Appreciable EGFP signal (green traces) was only apparent in the donor cardiomyocyte (cell 2). Moreover, there was no change in EGFP fluorescence in the donor cardiomyocyte during spontaneous or evoked depolarizations. Collectively, these data confirmed that transplanted donor cardiomyocytes were able to functionally couple with the host myocardium.

Superimposition of normalized $[\text{Ca}^{2+}]$, transients obtained from a second group of cells imaged at a myocardial depth of ~50 µm demonstrated that the peak changes in $[\text{Ca}^{2+}]$, occurred simultaneously in donor and host myocytes and that the kinetics of $[\text{Ca}^{2+}]$, decay were quite similar (Figure 3D). Values for $t_{50}$ in donor (60±4.6 and 112±4.9 ms) and host cardiomyocytes and their neighboring host cardiomyocytes were analyzed, distributed between 6 recipient hearts; $P>0.05$). Doubling the stimulation rate similarly shortened $t_{50}$ values in donor (60±4.6 and 112±4.9 ms) and host cells (71±3.4 and 110±4.8 ms; $P>0.05$). Importantly, the kinetics of the $[\text{Ca}^{2+}]$, transients in the donor cardiomyocytes and their neighboring host cells were identical to remotely localized host cardiomyocytes (data not shown) as well as to cardiomyocytes in hearts that did not receive cell transplants (also compare Figures 2D and 3D). These data provide compelling albeit indirect proof that these cells are electrically coupled. To further explore this point, immune histological analyses were performed to monitor connexin43 localization (Figure 4). As anticipated, connexin immune reactivity (red signal) was readily detected between donor (green) and host (blue) cardiomyocytes.

**Transplanted Atrial Cardiomyocytes Retain Discrete Functional Characteristics**

Over the course of these studies, it became apparent that in intact hearts, $[\text{Ca}^{2+}]$, transient duration in atrial cardiomyocytes was shorter than in ventricular cardiomyocytes under the imaging conditions used (Figure 5A versus 5B, red traces). When paced at 2 Hz, values for $t_{50}$ averaged 33±1.9 and 81±6.2 ms, respectively, for atrial cardiomyocytes and 62±3.0 and 94±4.6 ms for ventricular cardiomyocytes.
cardiomyocytes (20 atrial cardiomyocytes and 19 ventricular cardiomyocytes were analyzed, distributed between 3 different hearts; \( P < 0.05 \), atrial versus ventricular). To determine if atrial cardiomyocytes retained this characteristic after transplantation, embryonic day 15 MHC-EGFP hearts were harvested and single-cell suspensions prepared from the atria were injected directly into the left ventricle and septum of nontransgenic adult mice. Hearts were harvested 28 to 40 days later and subjected to TPME imaging. Examination of averaged traces from the transplanted atrial cardiomyocytes and the bordering host ventricular cardiomyocytes revealed that the atrial cells retained the short [Ca\(^{2+}\)] transient duration phenotype (Figure 5A versus 5B, green traces). Values for \( t_{50-50\%} \) and \( t_{50-10\%} \) averaged 30\(\pm\)1.5 and 63\(\pm\)3.7 ms, respectively, for the transplanted atrial cardiomyocytes and 56\(\pm\)2.3 and 106\(\pm\)4.5 ms for the bordering host ventricular cardiomyocytes (44 donor atrial cardiomyocytes and 38 host ventricular cardiomyocytes were analyzed, distributed between 4 different transplanted hearts; \( P < 0.05 \), atrial versus ventricular). The retention of a discrete atrial phenotype after transplantation suggests that the milieu of the normal ventricular myocardium is insufficient to markedly modulate the donor cells and furthermore argues against the occurrence of donor cell/host cell fusion events in this system.

**Discussion**

The studies described in the present study demonstrated that transplanted EGFP-expressing fetal cardiomyocytes couple with the host myocardium, using spontaneous and evoked calcium transients as a surrogate assay for action potential propagation. The presence of simultaneous [Ca\(^{2+}\)] transients in the imaged donor and host cardiomyocytes, as well as the similarities in [Ca\(^{2+}\)] transient kinetics, strongly support the notion that the transplanted cells form a functional syncytium with the host myocardium. Thus far, rhod-2 fluorescence has been imaged in a total of 177 EGFP-expressing donor cardiomyocytes that were transplanted within the host myocardium (from 7 independent animals with cellular transplants). All of these donor cardiomyocytes exhibited transients that occurred in synchrony with those in the neighboring host cardiomyocytes during normal sinus rhythm, as well as in response to remote electrical stimula-
Twenty additional donor cardiomyocytes were observed within the pericardial space and were thus separated from host cardiomyocytes by the epicardial endothelium. Image analyses indicated that these cells did not exhibit stimulation-evoked \([\text{Ca}^{2+}]\) transients (data not shown). This observation indicates that the pacing protocol used in this study does not result in field stimulation of the heart and thus further supports the notion of direct donor to host cardiomyocyte coupling. Moreover, these data also suggest that the cell-to-cell coupling observed between myocytes and nonmyocytes in vitro is insufficient to support coupling of the transplanted donor cardiomyocytes in the pericardial space. Collectively, these observations strongly suggest that direct cell contact with concomitant gap junction formation is required for action potential propagation between donor and host cardiomyocytes.

Recent studies have shown that, in vitro, cell fusion events may account for several instances of apparent cardiomyogenic induction.\(^7,8\) Previous time-course studies revealed that donor cardiomyocytes undergo progressive differentiation and hypertrophic growth after transplantation, suggesting that cell fusion events are not likely to occur at significant levels in cardiomyocyte transplantation studies. The retention of discrete functional characteristics after transplantation of atrial cardiomyocytes further supports the absence of donor to host cell fusion after cardiomyocyte transplantation.

The high rate of donor to host cardiomyocyte coupling reported here and the observation that transplanted cardiomyocytes can successfully seed the infarct border zone raises the possibility that cardiomyocyte transplantation may be able to impart functional improvement in diseased hearts. However, the ability of donor cardiomyocytes to seed either normal or injured hearts is currently somewhat limited. Use of a nuclear-localized transgenic reporter system revealed that only 1.3±0.40% of embryonic day 15 cardiomyocytes survive the initial transplantation procedure (M.H. Soonpaa, unpublished observation, 2002); this value is in good agreement with results from other laboratories.\(^12,37\) In contrast, once successfully transplanted, long-term donor cardiomyocyte viability is well established.\(^17,18\) Therapeutic utility of cardiomyocyte transplantation will likely also require interventions aimed at enhancing donor cell viability during the transplantation process and/or enhancing donor cell proliferation after transplantation.

It remains to be determined to what degree other cell types integrate after cellular transplantation. For example, recent clinical studies suggest that skeletal myoblast transplantation may generate a transient arrhythmogenic substrate.\(^39\) This could result from cellular heterogeneity at the myocardial/skeletal muscle interface (ie, by mimicking the heterogeneity seen at infarct border zones) from partial coupling between the nascent skeletal myocytes and the host myocardium at the border zone or from the formation of heterokaryons with aberrant electrical properties. In other studies, adult stem cells with apparent cardiomyogenic potential have been identified. These include hematopoietic stem cells, neuronal stem cells, hepatic stem cells, mesenchymal stem cells, and endothelial cell precursors.\(^40\) Many of these later studies used only a limited number of markers to characterize the degree

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**Figure 4.** Connexin43 immune reactivity between donor and host cardiomyocytes. A thin section prepared from a heart transplanted with MHC-EGFP fetal ventricular cardiomyocytes was reacted with an anti-connexin43 antibody, followed by a rhodamine-conjugated secondary antibody. EGFP fluorescence was captured at 505 to 540 nm (excitation at 488 nm), and rhodamine fluorescence (red) was captured in the 580- to 620-nm range (excitation at 568 nm). Transmitted light was recorded during laser illumination at 647 nm.

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**Figure 5.** Atrial cardiomyocytes retain discrete functional properties after transplantation into the ventricular myocardium. A. Tracings of electrically evoked changes in rhod-2 fluorescence as a function of time from normal atrial cardiomyocytes (red trace) and transplanted atrial cardiomyocytes (blue trace) paced at a rate of 2 Hz. For each cell, the relative changes in fluorescence were normalized such that 0 represents the prestimulus fluorescence intensity and 1 represents the peak fluorescence intensity. B. Tracings of electrically evoked changes as a function of time in a normal ventricular cardiomyocyte (red trace) and a ventricular cardiomyocyte neighboring transplanted atrial cardiomyocyte (blue panel). Conditions were as described in panel A.
of cardiomyogenic differentiation, and there was no characterization of cellular function in situ. Cellular TPMF imaging in intact hearts could provide insight into the functional consequences of transplantation with these cell types, provided that appropriate fluorescent reporters can be incorporated into the donor cells.

In summary, donor fetal cardiomyocytes can couple with the host myocardium after transplantation. The [Ca$^{2+}$] transient profiles in the donor cardiomyocytes strongly suggest that these cells form a functional syncytium with the host myocardium. These observations bode well for the notion that cell transplantation approaches can directly augment systolic function, in addition to the established effects on remodeling.

Acknowledgments

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References


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ONLINE MATERIALS AND METHODS

Generation of the MHC-EGFP transgene mice
The MHC-EGFP transgene used the mouse alpha-cardiac myosin heavy chain (MHC) promoter and sequences encoding an EGFP reporter (Clontech, Palo Alto, CA). The SV40 early region transcription terminator/poly-adenylation site (nucleotide residues #2586-2452) was inserted downstream from the EGFP sequences. Transgene insert was purified and microinjected into inbred C3HeB/FeJ (Jackson Laboratories, Bar Harbor MA) zygotes as described. The resulting pups were screened using diagnostic PCR amplification.

TPME imaging system
Images were recorded with a Bio-Rad MRC 1024 Laser Scanning microscope modified for TPME (see Figure). Illumination for 2-photon excitation was provided by a mode-locked Ti:Sapphire laser (Spectraphysics, Mountain View, CA), which generated a train of 100-fs pulses at a repetition rate of 82 MHz. The excitation wavelength was 810 nm. Hearts were imaged through a Nikon 60x 1.2 numerical aperture water-immersion lens with a working distance of 200 microns. Using two-photon molecular excitation with a high numerical aperture objective, Denk previously demonstrated that the majority of all excitations are confined to less than femtoliter volumes around the focal points, with ~1 micron resolution along the laser propagation axis. Measurements of the axial resolution of our imaging system are in excellent agreement with these values, thus the rhod-2 and EGFP fluorescence reported here originated...
from within individual cardiomyocytes. Emitted light was split by 2 dichroic mirrors in series and collected simultaneously by photo-multiplier tubes fitted with narrow bandwidth filters for 560 to 650 nm and 500 to 550 nm, respectively. Images were collected at a resolution of 0.43 microns/pixel along the xy-axis. For full-frame mode analyses (512 x 512 pixels), hearts were scanned at 1.46 and 0.73 frames per second on horizontal (X, Y) planes and the resulting images digitized at 8-bit resolution and stored directly on the hard disc. For line-scan mode analyses, hearts were scanned repetitively along a line spanning at least 2 juxtaposed cardiomyocytes. The scan speed was 6.88 microns/ms, corresponding to a pixel dwell time of 62.5 microseconds. Line-scan images were then constructed by stacking all lines vertically. Post-acquisition analysis was performed using MetaMorph software version 4.6r (Universal Imaging Incorporation, Downingtown, PA). The fluorescent profiles of [Ca\textsuperscript{2+}], transients were obtained by averaging the line-plot data of sequential line-scans. For determination of the time course of [Ca\textsuperscript{2+}], decay, rhod-2 fluorescence intensity was normalized to the difference between peak and baseline intensity, and intervals from 90 to 50% (t\textsubscript{90-50%}) and 50 to 10% (t\textsubscript{50-10%}) decay were calculated\textsuperscript{7}.

**Heart preparation for TPME imaging**

Hearts were heparinized (125 international units per kg bodyweight), cannulated via the ascending aorta and perfused in Langendorff mode at a constant mean perfusion pressure of 60 cm H\textsubscript{2}O at 21 “C with oxygenated Tyrode’s solution containing (in mmol/L) 134 NaCl, 4 KCl, 1.2 MgSO\textsubscript{4}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 10 Hepes, 11 D-glucose, and 2 CaCl\textsubscript{2} (pH = 7.35 adjusted with 1 M NaOH). During dye loading and wash out, the solution also contained 50 mmol/L 2,3-butanedione monoxime. During [Ca\textsuperscript{2+}]\textsubscript{i} imaging, cytochalasin D (50 micromol/L; stock solution: 3.9 mmol/L in DMSO) and acetylcholine (10 micromol/L; stock solution: 10 mmol/L in deionized water) were added to the Tyrode’s solution to eliminate contraction-induced movement of the heart\textsuperscript{8} and to lower the intrinsic heart rate, respectively. After an initial period of ~30 minutes, the perfusion was switched to a Tyrode’s solution containing the acetoxyxmethylenelester (AM) of the calcium fluorophore, rhod-2 (10 micromol/L; Molecular Probes, Eugene, OR) as described\textsuperscript{5}. After a 15-min loading period, the perfusion was reverted to dye-free Tyrode’s solution to wash out rhod-2 and to allow for a 20-min incubation period, ensuring intracellular de-esterification of rhod-2/AM. Hearts were stimulated via 2-ms square wave pulses with 1.5 times threshold current amplitude. The stimuli were delivered by a constant current isolator (Krannert Engineering, Indianapolis, IN) driven by a programmable stimulator (SD9, Grass Instruments).

**Fetal Cardiomyocyte transplantation**

Single cell preparations of embryonic day 15 transgenic fetal donor cardiomyocytes were prepared and injected into the left ventricular wall of syngeneic [C3HeB/FeJ x DBA/2J]F\textsubscript{1} mice essentially as described previously\textsuperscript{9,10}. One hundred thousand cells were injected directly into the ventricular myocardium of host animals. C3Heb/FeJ and DBA/2J progenitor mice were obtained from the Jackson Laboratory (Bar Harbor, MA).

**Validation of rhod2 and EGFP signal separation**

Based on the maximal emission wavelengths of 579 nm and 509 nm for calcium bound rhod-2\textsuperscript{11} and EGFP\textsuperscript{12}, respectively, we reasoned that this combination of fluorophores would provide a good separation of fluorescence signals. This prediction was confirmed by comparative
analysis of rhod-2 loaded non-transgenic mouse hearts with non-loaded MHC-EGFP transgenic hearts (see Figure). Images of the full-frame mode emission of a rhod-2 loaded non-transgenic heart revealed robust emission in the red range, but virtually no fluorescence in the green range (panel a, upper and lower images, respectively; the corresponding color-specific intensity histograms are also shown). Red rhod-2 fluorescence was uniformly distributed throughout the cardiomyocyte cytoplasm, and was also prominent in endothelial cell nuclei (in agreement with our previous observations). A typical myocardial cyto-architecture was apparent, with neighboring cardiomyocytes well aligned with one another. The spatially restricted increased rhod-2 fluorescence apparent across the middle of the image (as well as in all subsequent rhod-2 full-frame mode images) reflects the relatively slower rate for data acquisition for a full-frame mode image as compared to the kinetics of the [Ca\(^{2+}\)]\(_i\) transients. The imaged cells were located approximately 30 microns from the epicardial surface.

Validation of Signal Separation:

A similar optical analysis was performed with a non-loaded, mosaic-expressing, MHC-EGFP transgenic heart (Figure 3b). Images of the full-frame mode emission revealed robust emission in the green range, as well as low but measurable emission in the red range (the gains of the two photo-multiplier tubes were set to identical values). This result was expected, as the published emission spectrum of EGFP overlaps slightly with the passed wavelength range of the band-pass filter used. The imaged cells were located approximately 30 microns from the epicardial surface. As expected a typical myocardial cyto-architecture was apparent, with the EGFP fluorescence homogeneously distributed throughout the cytosol of the expressing cardiomyocytes. Although there was a slight contribution from EGFP in the red range, these data nonetheless indicated that calcium dependent changes in rhod-2 fluorescence and EGFP fluorescence could be imaged simultaneously provided that the intensity of EGFP fluorescence was not affected by cellular depolarization.

Simultaneous imaging of EGFP and rhod-2 fluorescence in intact hearts

To further document the imaging system, an MHC-EGFP heart with mosaic transgene expression (series A) and a non-transgenic adult heart transplanted with embryonic day 15 fetal ventricular cardiomyocytes (series B) were subjected to TPME imaging as described above. The
Figure shows full frame images. In each case, the left panel shows the EGFP fluorescence (pseudo-colored with green); the center panel shows rhod-2 fluorescence (pseudo-colored with red), and the right panel shows the merged image. Examination of the non-merged images clearly reveals the presence of \([\text{Ca}^{2+}]_i\) transients in the EGFP positive cells. Scale bars, 20 \(\mu m\) (upper panel) and 10 \(\mu m\).

**Anti-Connexin43 immune histology**

For anti-connexin 43 immune histology, hearts were isolated, the ascending aorta was cannulated with an 18G needle, and the hearts were retrogradely perfused with 4% paraformaldehyde in PBS at 60 cm H\(_2\)O for 20 min. Perfusion fixed hearts were incubated in 30% sucrose for ~12 hours for cryoprotection, embedded in Tissue-Tek Oct compound, and frozen at –80 °C. Cryosections (10 microns) were equilibrated to room temperature and subsequently incubated with 0.2% Triton X-100 (Sigma) in PBS for 1 hour, followed by 30 minutes of blocking with 2% bovine serum albumin (BSA). Sections were incubated for 12 hours with primary antibody in PBS supplemented with 2% BSA and 10% normal goat serum. A commercially available monoclonal anti-connexin43 antibody (raised against residues 252 to 270 of rat connexin43; MAB 3068, Chemicon, Temecula, CA) was used at 1:250 dilution. Sections were subsequently preincubated with 2% BSA in PBS. Immunolabeling was carried out with a rhodamin-conjugated rabbit anti-mouse IgG secondary antibody (AP160R, Chemicon) at 1:50 dilution. All
incubation steps were performed at room temperature, and between all incubation steps the slides were thoroughly washed with PBS three times for 5 min each. After application of the cover slip, sections were examined with a BioRad 1024 three-channel laser scanning confocal microscope equipped with an argon/krypton laser. For each section, serial highly confocal optical images at 0.5 micron intervals were recorded. EGFP fluorescence was measured in the 505 to 540 nm range during excitation at 488 nm, whereas rhodamin fluorescence was captured in the 580 to 620 nm range during illumination at 568 nm. Transmitted light was recorded during laser illumination at 647 nm. Five confocal images were stacked to produce the final image.

References