Adult progenitor cell transplantation influences contractile performance and calcium handling of recipient cardiomyocytes


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Submitted 26 August 2008; accepted in final form 28 January 2009

Lee J, Stagg MA, Fukushima S, Soppa GK, Siedlecka U, Youssef SJ, Suzuki K, Yacoub MH, Terraciano CM. Adult progenitor cell transplantation influences contractile performance and calcium handling of recipient cardiomyocytes. Am J Physiol Heart Circ Physiol 296: H927–H936, 2009. First published January 30, 2009; doi:10.1152/ajpheart.00931.2008.—Adult progenitor cell transplantation has been proposed for the treatment of heart failure, but the mechanisms effecting functional improvements remain unknown. The aim of this study was to test the hypothesis that, in failing hearts treated with cell transplantation, the mechanical properties and excitation-contraction coupling of recipient cardiomyocytes are altered. Adult rats underwent coronary artery ligation, leading to myocardial infarction and chronic heart failure. After 3 wk, they received intramyocardial injections of either 107 green fluorescence protein (GFP)-positive bone marrow mononuclear cells or 5 × 106 GFP-positive skeletal myoblasts. Four weeks after injection, both cell types increased ejection fraction and reduced cardiomyocyte size. The contractility of isolated GFP-negative cardiomyocytes was monitored by sarcomere shortening assessment, Ca2+ handling by indo-1 and fluo-4 fluorescence, and electrophysiology by patch-clamping techniques. Injection of either bone marrow cells or skeletal myoblasts normalized the impaired contractile performance and the prolonged time to peak of the Ca2+ transient observed in failing cardiomyocytes. The smaller and slower L-type Ca2+ current observed in heart failure normalized after skeletal myoblast, but not bone marrow cell, transplantation. Measurement of Ca2+ sparks suggested a normalization of sarcoplasmic reticulum Ca2+ leak after skeletal myoblast transplantation. The increased Ca2+ wave frequency observed in failing myocytes was reduced by either bone marrow cells or skeletal myoblasts. In conclusion, the morphology, contractile performance, and excitation-contraction coupling of individual recipient cardiomyocytes are altered in failing hearts treated with adult progenitor cell transplantation.

Heart failure; cell therapy; excitation-contraction coupling; cell electrophysiology; paracrine mechanisms

HEART FAILURE (HF) represents a major disease burden in Western countries. The disease is progressive with limited therapeutic options. Cell transplantation has been tested in a number of preclinical (37) and clinical (1, 24) studies with small but encouraging improvements in heart function.

To understand the basis for cell therapy, and possibly increase its efficacy, it is essential to define the physiological mechanisms of improvement. The original concept that the transplanted cells would survive and transdifferentiate into cardiomyocytes in significant numbers to provide extra contractile force is still a matter of considerable debate (24, 33). A number of alternative hypotheses have been put forward to account for the observed effects of cell transplantation (24). One widely held hypothesis is for cell transplantation to affect the surviving myocardium through paracrine secretions (17). The targets of the putative paracrine mediators during cell transplantation may include the surviving cardiomyocytes, the extracellular matrix, or the microcirculation, all of which undergo pathological changes in HF. While there is evidence that the extracellular matrix and microcirculation can be affected by cell transplantation (12, 28), the effects on cardiomyocyte function are still poorly defined. Cardiomyocyte structure and function are profoundly affected in HF. The impairment of cardiomyocyte contractile performance, Ca2+ handling, and electrophysiological parameters has been extensively demonstrated in failing hearts (5, 18, 39, 41). It has also been shown that targeting cardiomyocyte excitation-contraction (E-C) coupling defects can lead to an improvement in whole heart function (21).

The important question of whether cell transplantation affects the functional properties of recipient cardiomyocytes in failing hearts has not been previously addressed. Here, we show, for the first time, that the morphology, contractile performance, and E-C coupling of individual recipient cardiomyocytes are altered in failing hearts treated with adult progenitor cell transplantation.

METHODS

Induction of HF. All procedures conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). All animal procedures were approved by the Harefield Heart Science ethical review process and authorized by the UK Home Office according to the Animals (Scientific Procedures) UK Act 1985. Adult female Sprague-Dawley rats were anaesthetised with 1–2% isoflurane and ventilated using an 18-gauge vascular cannula connected to a volume-controlled ventilator (model 683 Rodent Ventilator, Harvard Apparatus). The tidal volume was adjusted to ~8 ml/kg, and the ventilatory rate was adjusted to ~60 breaths/min.

Animals underwent a left thoracotomy at the fourth intercostal space followed by a pericardectomy. The left anterior descending coronary artery was ligated permanently at the level of the left atrial appendage using a 6-0 suture to cause myocardial infarction. Sham-operated animals were also subjected to a thoracotomy but underwent no coronary artery ligation.

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Preparation of bone marrow mononuclear cells. To determine the origin of the myocytes studied, green fluorescent protein-positive (GFP+) male Sprague-Dawley rats (Rat Resource and Research Centre) were used as donors. Although there was a concern that the use of Sprague-Dawley rats for the donors/recipient of cell transplantation might cause an immune response, previous results using athymic nude rats as recipients (data not shown) were the same as described in this study. More detail on this point are described in other publications where identical animals/procedures were used (14).

GFP+ rats were killed by cervical dislocation. Both tibia were removed, and their marrow cavities were flushed using 10% FBS in HBSS to obtain whole marrow aspirate. This was then spun through a Percoll (Amersham) gradient at 1,600 g for 25 min to yield the bone marrow mononuclear cell (BM) fraction, which was washed and stored in HBSS containing 10% FBS. Cells were washed using PBS and transplanted within 6 h.

Preparation of skeletal myoblasts. Skeletal myoblasts (SK) were cultured using the single fiber method as previously described (32). Extensor digitorum longus muscles were removed from GFP+ male Sprague-Dawley rats and shaken for 60 min at 35°C in DMEM containing 2 mg/ml collagenase (type I, Sigma). Partially digested muscle tissue was triturated, and individual myofibers were plated in Matrigel (Becton Dickinson) on culture dishes and bathed in DMEM with 10% added horse serum, 0.5% chick embryo extract, and 1% penicillin-streptomycin. Fibers were removed 24–48 h after myofibers had been plated, and 20% FBS and 5 ng/ml basic fibroblast growth factor were added to the bathing medium. Thereafter, SK were passaged every 2 days and used before the fifth passage. At the time of injection, myoblasts were washed with PBS.

Cell injection. Three weeks after coronary artery ligation, a thoracotomy via the fifth intercostal space was performed. Either 107 BM or 5 × 106 SK were suspended in 150 μl PBS. These numbers of cells were chosen following a previous study performed within our research group (14) as well as another study (30). To date, there has been no report establishing the optimal injection numbers in any animal model or in patients. Using a 25-gauge needle, half of this volume was injected with the viable peri-infarct myocardium at the left ventricular (LV) apex above the scar and the other half into the LV lateral wall. Control HF animals and in sham-operated animals, PBS only was used.

Efficiency of cell grafting. The grafting properties could not be directly assessed in the hearts used in the experiments reported in the present study because of the enzymatic digestion for cardiomyocyte isolation. However, we performed the assessment of grafting of BM and SK in identical experiments for another study (14), and the results have already been published. Briefly, we did not find significant differences in the numbers of surviving cells between the groups. However, after 4 wk, more CD45-positive cells, possibly of inflammatory origin, were apparent after SK injections.

Echocardiography. Animals were anesthetized by the administration of 1–1.5% isoflurane in 100% oxygen via a nose cone and allowed to self-ventilate while in a supine position. Attempts were made to keep the level of anesthesia minimal and constant throughout image acquisition to avoid the hemodynamic artifacts induced by the myocardial depression and vasodilatory effects of isoflurane. Animals were shaved over the anterior chest wall, and ultrasound gel was applied. Using a 15-MHz probe (model 15L8) on an Acuson Sequoia 256 system (Siemens Medical Systems), transthoracic echocardiography was performed to obtain parasternal short-axis two-dimensional views at the level of the midpapillary muscle.

Experiments were performed immediately before cell injection and also 4 wk after cell injection. Heart rate (HR) measured during echocardiography was similar in all groups before injection [HF: 373 ± 20 beats/min (n = 10), SK: 374 ± 9 beats/min (n = 10), and BM: 357 ± 7 beats/min (n = 9)]. After 4 wk of treatment, HR was significantly higher in the groups treated with saline only [HF: 406 ± 12 beats/min (n = 10), SK: 354 ± 9 beats/min (n = 10), and BM: 350 ± 10 beats/min (n = 9), P < 0.05], possibly indicating further deterioration of heart function over the 4 wk after injection in this group.

Cell isolation and cell area planimetry. Cells were killed 4 wk after cell injection. Cardiomyocytes were isolated by standard enzymatic digestion. The infarct scar was discarded, and cardiomyocytes were isolated only from the remaining LV tissue. Overall cardiomyocyte viability was 70–80%. Cardiomyocytes were used within 6 h, and those for study were selected at random and only excluded if they did not have a rod-shaped appearance. Given the limited amount of viable ventricular tissue remaining after infarction, no further regional subdivision was performed. This would be a desirable strategy for future studies. Isolated cells were examined using an Olympus IX-71 inverted epifluorescence microscope with ×60 objective. Photographs were taken, and the projected 2-dimensional area for each cell was measured using ImageJ software (http://rsb.info.nih.gov/ij). GFP fluorescence was assessed by exciting the cells at 488 nm.

Assessment of sarcomere shortening. Isolated cardiomyocytes were field stimulated at 1–5 Hz while being superfused at 37°C with Tyrode solution containing (in mM) 140 NaCl, 6 KCl, 10 glucose, 1 MgCl2, 1 CaCl2, and 10 HEPES (pH 7.4). Images were acquired at 240 Hz through a ×60 microscope objective using a variable field rate camera (IonOptix, Milton, MA). Using an IonOptix interface system, real-time Fourier analyses of images of cardiomyocytes were performed to measure their sarcomere lengths and contraction profiles. For offline analysis, 10–20 contraction traces at steady state were averaged with respect to the field stimulation signal. The baseline sarcomere length and contraction amplitude of the resulting trace were measured using this field stimulation signal as a reference point. The average diastolic sarcomere lengths were 1.732 μm (normal), 1.702 μm (HF), 1.73 μm (BM), and 1.726 μm (SK), and there were no statistically significant differences between groups. Peak contraction and relaxation velocities were determined by identifying the steepest tangents during contraction and relaxation, respectively. Only rod-shaped, clearly striated cardiomyocytes that were Ca2+ tolerant were used in all the experiments.

Indo-1 fluorescence. Isolated cardiomyocytes were loaded with indo-1 AM (Molecular Probes) at a concentration of 10 μM for 20 min and allowed to deesterify for at least 1 h. They were field stimulated at 1, 2, or 3 Hz while being superfused with normal Tyrode solution as above. The excitation wavelength was 385 nm, and emissions at 405 and 485 nm were acquired during steady-state contraction. After background levels had been subtracted, the indo-1 fluorescence ratio was calculated (405-to-485-nm fluorescence ratio). For analysis, 10–20 transients were averaged with reference to the field stimulation signal. The transient peak amplitude and time to peak (Tpeak) were calculated from the field stimulation signal baseline, and decay times (time to 50% and 90% decay) were calculated from Tpeak.

To elicit caffeine-induced indo-1 transients, field stimulation was stopped, and caffeine was rapidly applied using a solenoid switcher device. Immediately before the application of caffeine, the superfusate was switched to a Na+-free/Ca2+-free solution containing (in mM) 140 LiCl, 10 glucose, 10 HEPES, 0.75 EGTA, 1 MgCl2, and 6 KOH (pH 7.40) for 100 ms to prevent Ca2+ extrusion by the Na+/Ca2+ exchanger (NCX). This was followed by a 1-s application of 20 mM caffeine dissolved in the same Na+-free/Ca2+-free solution and then 20 mM caffeine in normal Tyrode solution for 5 s. The amplitude of the caffeine-induced transient was taken as a measure of total sarcoplasmic reticulum (SR) Ca2+ content.

Measurement of intracellular pH and Na+/H+ exchanger activity. Cardiomyocytes were loaded with 10 μM 5 (and 6)-carboxy-SNARF-1 AM (Molecular Probes) for 8 min and allowed to deesterify for 1 h. They were then superfused with Tyrode solution as above. Fluorescence excitation was at 480 nm, and the ratio of emissions at 580 and 640 nm (580-to-640-nm fluorescence ratio) was calculated. NH4Cl (15 mM) was applied for 5 min and then removed when acid pH was decreased to ∼6.5.
Extrusion was measured. Calibration of SNARF in situ was performed as previously described (35).

**Electrophysiological parameters.** Cells were studied using an Axon 2B amplifier (Axon Instruments) in discontinuous (switch clamp) mode. The pipette resistance was ~30 MΩ, and the pipette filling solution contained (in mM) 2,000 KCl, 5 HEPES, and 0.1 EGTA (pH 7.2). Action potentials (APs) were measured in current-clamp mode after stimulation at 1, 3, and 5 Hz using a 1-ms, 1.2- to 1.4-nA pulse. Times to 20%, 50%, and 90% repolarization were measured from the stimulation pulse. L-type Ca^2+ current (I_CaL) was measured in voltage-clamp mode. Current-voltage relationships for I_CaL were built using 450-ms depolarization steps from a holding potential of −40 mV (range: −45 to +50 mV). The measured current was nifedipine sensitive and 4-aminopyridine insensitive and, thus, attributable to I_CaL. The voltage-dependent steady-state activation profile was derived from the same protocol. Steady-state inactivation of I_CaL was studied using a double-pulse protocol. Inactivating prepulses of 200 ms were applied from a holding potential of −50 mV (range: −55 to +50 mV in 5-mV increments), after which the membrane potential was held at −40 mV for 5 ms and the test pulse was applied at +5 mV for 200 ms.

For the measurement of NCX current density, cells were superfused with K+-free Tyrode solution containing (in mM) 140 NaCl, 10 HEPES, 10 glucose, 1 MgCl₂, 1 CaCl₂, 6 CsCl, 0.01 prostaglandin, and 0.01 nifedipine (pH 7.4). Continuous voltage-clamp mode on a Multiclamp 700A amplifier (Axon Instruments) was used. Pipette resistances were 2.3–3.5 MΩ. The pipette filling solution contained (in mM) 45 CsCl, 20 HEPES, 11 MgCl₂, 10 Na₂ATP, 100 CsOH, 50 CaCO₃ (pH 7.2). The intracellular Ca^2+ concentration ([Ca^2+]i) was calculated to be ~200 nM, as previously described (31). The access resistance was between 2 and 8 MΩ, and the calculated junction potential at the pipette tip was −12 mV. From a holding potential of −40 mV, a 3-s descending ramp was applied from +80 to −120 mV at 0.1 Hz until a steady state was reached. NCX current (I_NCX) was taken as the 5 mM Ni^2+-sensitive component of the active current.

**Ca^2+ imaging of Ca^2+ sparks and waves.** The Ca^2+-sensitive, single-excitation, single-emission fluorescent dye fluo-4 AM was used to monitor localized changes in Ca^2+ concentration. Aliquots of cells were incubated with fluo-4 AM (10 μM) for 20 min, and cells were allowed to deesterify for at least 30 min before being used. The experimental chamber was mounted on the stage of a Zeiss Axiovert microscope with the LSM 510 confocal attachment, and myocytes were incubated with fluo-4 AM (10 μM) for at least 30 min before being used. The intracellular Ca^2+ concentration ([Ca^2+]i) was measured in voltage-clamp mode. Current-voltage relationships for I_CaL were built using 450-ms depolarization steps from a holding potential of −40 mV (range: −45 to +50 mV). The measured current was nifedipine sensitive and 4-aminopyridine insensitive and, thus, attributable to I_CaL. The voltage-dependent steady-state activation profile was derived from the same protocol. Steady-state inactivation of I_CaL was studied using a double-pulse protocol. Inactivating prepulses of 200 ms were applied from a holding potential of −50 mV (range: −55 to +50 mV in 5-mV increments), after which the membrane potential was held at −40 mV for 5 ms and the test pulse was applied at +5 mV for 200 ms.

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**RESULTS**

HF was induced in adult rats by left anterior descending coronary artery ligation. Three weeks after surgery, the ejection fraction (EF) measured by echocardiography was 35 ± 0.8% (n = 57) for failing groups, whereas sham-operated hearts had an EF of 76 ± 1% (n = 17). Injection of 10⁷ BM or 5 × 10⁶ SK from transgenic rats constitutively expressing GFP or PBS only was then performed. After 4 wk, echocardiography demonstrated an increased EF in cell transplantation groups, whereas no change was seen after the injection of PBS (Fig. 1A). Ventricular end-diastolic areas were significantly smaller in animals treated with either SK or BM compared with animals treated with PBS only [PBS: 98.41 ± 1.6 mm² (n = 17), BK: 66.4 ± 5.4 mm² (n = 7), and SK: 74.1 ± 2.6 mm² (n = 9), P < 0.001]. These results confirm previous observations by others (23, 30) and us (7, 14). To characterize the cellular mechanisms involved, LV cardiomyocytes were isolated. Cardiomyocytes were studied for GFP fluorescence to determine their origin. The levels of green fluorescence in the isolated cardiomyocytes were low, similar to the level of background autofluorescence emitted by cardiomyocytes isolated from wild-type rat hearts. The lack of GFP^+ myocytes in the studied cell population does not exclude the presence of GFP^+ myocytes in the heart but suggests that, if present, their number was small. This indicated that the cardiomyocytes under study were of recipient origin. Cell area measurements using planimetry revealed that cardiomyocytes isolated from failing hearts were larger than those from normal hearts (Fig. 1D), as seen in ventricular remodeling. This hypertrophy was reduced after transplantation of either BM or SK, suggesting regression (Fig. 1B). Similar results were seen when whole cell capacitance, obtained from the electrophysiological experiments, was used to measure the cell surface area (Fig. 1C).

**Signaloremyocyte shortening profiles revealed that isolated failing cardiomyocytes contracted less and slower compared with normal** (Fig. 2, A–C). Injection of either SK or BM restored the contraction amplitude (Fig. 2B) as well as the peak contraction (Fig. 2C) and relaxation (Fig. 2D) velocities.

The dynamics of intracellular Ca^2+ are a major determinant of E-C coupling, and altered Ca^2+ homeostasis is central to the pathophysiology of HF (4, 20). To investigate the cellular mechanisms of functional improvement, we monitored changes in [Ca^2+]i using indo-1. Failing cardiomyocytes showed indo-1 transients with prolonged T_peak and decay time courses (Fig. 3A). There were no changes in diastolic baseline levels or in transient peak amplitudes. Transplantation of either cell type normalized the prolonged T_peak (Fig. 3B). However, the time to 50% (Fig. 3C) and 90% decay (not shown), measures of Ca^2+ removal from the cytoplasm during diastole, were not significantly affected by cell transplantation. Similar results were observed at 2- and 3-Hz field stimulation (data not shown). SR Ca^2+ content measured during the rapid application of 20 mM caffeine was similar in all groups (Fig. 3D).

Prolongation of the AP is a hallmark of HF, regardless of etiology. APs were prolonged in failing cardiomyocytes by up to 40%. The prolonged AP duration (APD) at 90% repolarization remained unaffected after cell transplantation. Similar changes were seen when APD at 50% repolarization was analyzed. At APD at 20% repolarization, there were no differences between groups (Fig. 4 and Table 1).
Ca,L, the main trigger for Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), was studied (Fig. 5). Peak \(I_{\text{Ca,L}}\) density was reduced in failing cardiomyocytes (Fig. 5A). This reduction was normalized by transplantation of SK. In contrast, transplantation of BM had no effect on the reduced \(I_{\text{Ca,L}}\). To study time-dependent inactivation, a two-exponential decay of \(I_{\text{Ca,L}}\) elicited by a step from -40 to 0 mV was calculated, and fast and slow components of inactivation were identified (fast component, Fig. 5B; and slow component, Fig. 5C). Both time constants were prolonged in HF compared with normal cardiomyocytes. The delay was normalized by transplantation of SK but not BM. Voltage-dependent activation (\(G_{\text{max}}\); Fig. 5D) and inactivation (\(F_{\text{max}}\); Fig. 5E) profiles of \(I_{\text{Ca,L}}\) were obtained by fitting Boltzmann curves to the measured currents as previously described (22). There were no difference between the groups in \(G_{\text{max}}\). HF resulted in a leftward shift in \(F_{\text{max}}\), indicating further limitation of \(I_{\text{Ca,L}}\). This was corrected by transplantation of SK but not BM. Overall, our data showed that the impaired \(I_{\text{Ca,L}}\) is normalized by SK transplantation. Changes in this current determine the rate of Ca\(^{2+}\) release from the sarcoplasmic reticulum.

**Fig. 1.** A: ejection fraction was increased by cell transplantation in failing hearts. Numbers of animals and groups were as follows: 18 animals in the sham-operated group (sham group), 18 animals in the heart failure group (HF group), 18 animals in the bone marrow mononuclear cell (BM)-transplanted group (BM group), and 21 animals in the skeletal myoblast (SK)-transplanted group (SK group). B: planimetry data representing cell area. HF cardiomyocytes were larger than normal (\(p < 0.001\)), but cell size was reduced by transplantation of either BM (\(p < 0.001\) vs. HF) or SK (\(p < 0.001\) vs. HF). Numbers of animals/cells were as follows: normal, 6 animals/220 cells; HF, 5 animals/225 cells; BM, 5 animals/259 cells; and SK, 5 animals/328 cells. C: cell capacitance data from whole cell patch-clamp experiments. As with planimetry, failing cardiomyocytes were larger than normal (\(p < 0.001\)). Transplantation of either BM (\(p < 0.05\) vs. HF) or SK (\(p < 0.05\) vs. HF) reduced this hypertrophy. ANOVA was used for statistical comparison. Numbers of animals/cells were as follows: normal, 6 animals/45 cells; HF, 5 animals/57 cells; BM, 5 animals/53 cells; and SK, 5 animals/45 cells. D: example photographs of representative cardiomyocytes isolated from the four groups of hearts. Scale bars = 10 \(\mu\)m.

**Fig. 2.** A: representative traces of sarcomere shortening profiles elicited at 1 Hz. Contraction amplitude (B) and maximum contraction velocity (C) were reduced in the HF group (***\(p < 0.001\) vs. normal) but were restored to normal after transplantation of either cell type. D: transplantation of SK (\(p < 0.001\) vs. HF) and BM (\(p < 0.001\) vs. HF) also restored the maximum speed of relaxation compared with the HF group. Numbers of animals/cells were as follows: normal, 6 animals/66 cells; HF, 6 animals/60 cells; BM, 5 animals/53 cells; and SK, 6 animals/57 cells.
the SR and could contribute to the faster indo-1 transient development and improved contractile function observed.

It has been previously suggested that changes in $I_{\text{NCX}}$ may be relevant to the abnormal Ca$^{2+}$/H$^+$ homeostasis in HF. In our study, no differences were seen between groups in terms of average $I_{\text{NCX}}$ density or reversal potentials (data not shown). It has also been suggested that disturbances in intracellular Na$^+$/H$^+$ concentration and intracellular pH (pH$_i$) via altered Na$^+$/H$^+$ exchange occur during HF, leading to dysfunctional intracellular Ca$^{2+}$/H$^+$ homeostasis (2). The pH-sensitive indicator SNARF was used to study pH$_i$ and acid extrusion ability. Resting pH$_i$ was unchanged in the HF group (not shown). Acid extrusion after NH$_4$Cl prepulse was measured between pH$_i$ 6.8 and 7.3 and also showed no significant differences between groups [at pH$_i$ 6.9; normal: 3.16 ± 0.39 meq·l$^{-1}$·min$^{-1}$ ($n = 7$), HF: 3.33 ± 0.89 meq·l$^{-1}$·min$^{-1}$ ($n = 117$), SK: 2.5 ± 0.33 meq·l$^{-1}$·min$^{-1}$ ($n = 19$), and BM: 2.55 ± 0.4 meq·l$^{-1}$·min$^{-1}$ ($n = 18$)].

Given the observation that the rate of [Ca$^{2+}$]$_i$ increase is faster after cell transplantation (Fig. 3B), we tested the hypothesis that unitary SR Ca$^{2+}$ release events, Ca$^{2+}$ sparks, were affected. Ca$^{2+}$ spark frequency was increased in failing cardiomyocytes but was not changed after cell transplantation (Table 2). There were no differences in spark amplitude between groups. The width of Ca$^{2+}$ sparks, expressed as FWHM, was greater in failing cardiomyocytes compared with normal, suggesting wider diffusion of Ca$^{2+}$. This increase was reduced after transplantation of either cell type. There were no differences seen in the decay rate of the sparks, measured as FDHM, between normal and failing cardiomyocytes. Transplantation of SK reduced FDHM compared with both normal and failing cells. From these parameters, SR Ca$^{2+}$ leak was calculated as previously reported (27):

\[
\text{SR Ca}^{2+} \text{ leak} = \frac{\text{Ca}^{2+} \text{ spark frequency} \times \text{FWHM} \times \text{FDHM}}{	ext{Peak}}
\]

SR Ca$^{2+}$ leak increased in response to HF and was normalized by SK transplantation (Fig. 6A). Furthermore, failing cardiomyocytes had a higher frequency of Ca$^{2+}$ waves, suggesting increased aberrant leakage of Ca$^{2+}$ from the SR and dysfunctional local control of E-C coupling. This increase was significantly reduced after transplantation of either BM or SK (Fig. 6B). These observations suggest that adult progenitor cell transplantation affects Ca$^{2+}$ regulation at the level of SR Ca$^{2+}$ release and the local control of E-C coupling.
DISCUSSION

Changes in recipient cardiomyocyte morphology after cell transplantation. This study identified, for the first time, individual recipient cardiomyocytes in failing hearts as one of the targets of the effects of transplanted BM and SK. After cell transplantation, the cardiomyocyte hypertrophy resulting from HF was reduced. Cardiac hypertrophy is recognized as a maladaptive process associated with an unfavorable outcome, and its reduction is thought to improve prognosis (13).

Effects of cell transplantation on E-C coupling of recipient cardiomyocytes. Our data show that the contractile performance of individual cardiomyocytes, which is impaired in HF, was restored after cell transplantation. It is likely that this improvement of cardiomyocyte contractile performance would contribute to the improved whole heart function. We also show that changes in Ca\(^{2+}\) regulation, however, cannot entirely explain the reduced contractility as the amplitude of the Ca\(^{2+}\) transient was maintained, implying that other Ca\(^{2+}\)-independent mechanisms are also important.

It is interesting to note that several established and experimental strategies for the treatment of HF affect cardiomyocyte E-C coupling. Studies using different approaches, such as \(\beta\)-adrenergic receptor blockers or HR-reducing agents (26), mechanical unloading with LV assist devices (11, 38), and cardiac resynchronization therapy (29), have all been associated with changes in E-C coupling. Direct intervention on E-C coupling mechanisms using overexpression of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (8) or reduction of SR Ca\(^{2+}\) leak (21) also improves function of improvement in HF. This suggests a crucial importance for E-C coupling in the pathophysiology of HF.

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<th>Table 1. APDs, measured as APD(<em>{20}), APD(</em>{50}), and APD(_{90}) at 1, 3, and 5 Hz of stimulation</th>
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Data are means ± SE (in ms). APD, action potential duration; APD\(_{20}\), APD at 20% repolarization; APD\(_{50}\), APD at 50% repolarization; APD\(_{90}\), APD at 90% repolarization. Groups were as follows: normal group, heart failure (HF), bone marrow mononuclear cell transplantation (BM), and skeletal myoblast transplantation (SK). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the normal group; *P < 0.01 and **P < 0.001 vs. the HF group.
HF and a possible role of the effects on E-C coupling observed after cell transplantation.

Several E-C coupling mechanisms are involved in the pathophysiology of HF with varying relative contributions that depend on the model, species, and severity (3). The two main aspects of Ca\(^{2+}\) regulation, Ca\(^{2+}\) entry and Ca\(^{2+}\) extrusion, have both been implicated. Altered Ca\(^{2+}\) entry, disrupting the complex interplay between sarcolemmal and SR Ca\(^{2+}\) release, affects the development of contraction and is thought to play an important role in HF. In our study, the \(T_{peak}\) values of indo-1 transients were prolonged in HF, implying impaired Ca\(^{2+}\) release, but normalized after transplantation of either cell type. In addition, failing cardiomyocytes also showed reduced \(I_{Ca,L}\) density and delayed inactivation. Reduced Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels during the AP decreases Ca\(^{2+}\) release from the SR, leading to slower and less effective contraction. Transplantation of SK, but not BM, normalized \(I_{Ca,L}\) density and inactivation properties, and this could explain, at least in part, the faster development of Ca\(^{2+}\) transients in this group. However, cell contractility and Ca\(^{2+}\) transients of SK and BM groups were similar, indicating that the changes in \(I_{Ca,L}\) are unlikely to be the sole factor accounting for the cellular improvement. Ca\(^{2+}\)-independent mechanisms, such as myofilament function or subcellular morphological changes, could be responsible for this discrepancy, and more experiments are required on this aspect.

Another surprising finding was that the \(T_{peak}\) of the Ca\(^{2+}\) transient was equally affected in BM and SK groups, whereas \(I_{Ca,L}\) was only affected in the SK group. Other mechanisms, such as a different ability of the SR to release Ca\(^{2+}\), could be responsible. A previous study (41) has shown that in HF altered Ca\(^{2+}\) release can be due to a modification in ryanodine receptor (RyR) function. The faster development of Ca\(^{2+}\) transients in the absence of any improvement of \(I_{Ca,L}\) content after BM transplantation points toward improvements in CICR at the RyR as a likely mechanism. The functional state of the RyR and the local control of E-C coupling were assessed by studying Ca\(^{2+}\) sparks. The calculated SR leak product (27), substantially increased in failing myocytes, was significantly reduced in the SK group. In addition, the increased frequency of Ca\(^{2+}\) waves in failing myocytes, as the result of dysfunctional control of local E-C coupling, was normalized after transplantation of both BM and SK. Since alterations of the phosphorylation status of the failing myocardium are likely to occur (34), and L-type Ca\(^{2+}\) channels and RyRs are closely regulated by levels of phosphorylation (41), transplanted cells may be acting via these targets. However, the functional role of these and other post-translational modifications of the channels remains controversial (15).

It is also possible that a disrupted relationship between Ca\(^{2+}\) current and the consequent SR Ca\(^{2+}\) release exists. We show here that some parameters of Ca\(^{2+}\) sparks, related to SR Ca\(^{2+}\) release function, were altered in this model of HF (Fig. 6) and were partially restored after cell transplantation. The spatial relationship between dihydropyridine receptors and RyRs could also be altered in hypertrophy, resulting in altered CICR (18).

Ca\(^{2+}\) extrusion is affected by changes in SR Ca\(^{2+}\) uptake and NCX function, resulting in slower Ca\(^{2+}\) decay and reduced SR Ca\(^{2+}\) content (20). However, in our study, no changes were detected in these parameters after cell transplantation. In contrast, sarcomere relaxation returned to normal values, suggesting a contribution of other factors, such as myofilament regulation. Whether myofilament sensitivity to Ca\(^{2+}\) is affected in our particular model of HF and a target for cell transplantation requires further testing.

Factors mediating the effects of cell transplantation in recipient cardiomyocytes. Our observations were made 4 wk after cell transplantation, when only few of the transplanted
cells had survived (7, 14). In a recent study (36), we have shown that at day 28 after BM or SK injection, GFP\(^+\)/H11001 grafts were largely decreased in size, GFP\(^+\)/H11001 cells appeared to be isolated from the native myocardium, and GFP\(^+\)/H11001 cardiomyocyte-like cells could not be observed (36). This suggests that transplanted cells had enhanced heart function by mechanisms other than provision of additional contractile elements. One possibility is the secretion of paracrine factors affecting neighboring cardiomyocytes, as previously proposed by others (10, 16, 17). The “paracrine hypothesis” could offer a likely

Table 2. Morphometric analysis of Ca\(^{2+}\) sparks

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Ca(^{2+}) Spark Frequency, no. of events per 100 (\mu)m/s</th>
<th>Ca(^{2+}) Spark Peak Intensity, peak fluorescence/background fluorescence</th>
<th>Full Width at Half Maximum, (\mu)m</th>
<th>Full Duration at Half Maximum, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>60</td>
<td>0.952±0.13</td>
<td>1.82±0.017</td>
<td>2.88±0.04</td>
<td>20.4±0.83</td>
</tr>
<tr>
<td>HF</td>
<td>53</td>
<td>1.953±0.27(\dagger)</td>
<td>1.88±0.019</td>
<td>3.13±0.03(\ddagger)</td>
<td>20.4±0.44</td>
</tr>
<tr>
<td>BM</td>
<td>46</td>
<td>1.423±0.23</td>
<td>1.89±0.019</td>
<td>3.03±0.03(\dagger)</td>
<td>18.7±0.40</td>
</tr>
<tr>
<td>SK</td>
<td>44</td>
<td>1.28±0.18</td>
<td>1.89±0.017</td>
<td>2.99±0.03(\ast)</td>
<td>18.1±0.4(\ddagger)</td>
</tr>
</tbody>
</table>

Data are means ± SE; \(n\), no. of observations. \(\ast P < 0.05\) vs. the HF group; \(\dagger P < 0.05\), \(\ddagger P < 0.01\), and \(\ast P < 0.001\) vs. the normal group.

Fig. 6. A: Ca\(^{2+}\) spark parameters were determined in quiescent cells after a 1-min stimulation protocol at 1 Hz (left). SR Ca\(^{2+}\) leak was calculated as the product of Ca\(^{2+}\) spark frequency, peak amplitude, size (full width at half maximum), and duration (full duration at half maximum). There was an increase in SR Ca\(^{2+}\) leak in the HF group, which was restored in the SK group (right). B: spontaneous Ca\(^{2+}\) waves were recorded over 30,000 line scans (left). HF increased the incidence of Ca\(^{2+}\) waves compared with the sham group, whereas failing hearts injected with either BM or SK had significantly fewer Ca\(^{2+}\) waves compared with the HF group (right). \(\ast P < 0.05\); \(\ast\ast P < 0.01\). Numbers of animals/measurements were as follows: normal, 6 animals/60 measurements; HF, 5 animals/53 measurements; BM, 5 animals/46 measurements; and SK, 5 animals/44 measurements.
explanation to our observations, although the actual mediators remain unidentified. Gnecci et al. (16) have shown that mesenchymal stem cells overexpressing Akt1 secrete factors that limit myocyte death in myocardial infarction. Uemura et al. (40) have also described prevention of ventricular remodeling after myocardial infarction by injection of bone marrow cells. We (25) have recently reported that coculture of BM or SK and cardiac myocytes results in improved contractility of the myocytes by secreted mediators and that skeletal myoblasts secrete a number of factors, including IGF-1, VEGF, tissue inhibitor of metalloproteinase (TIMP)-1, monocyte chemotactant protein-1, and possibly many others. Whether these secreted substances affected cardiomyocyte function directly in vivo or through other components of the myocardium such as the extracellular matrix or microcirculation remains to be established. The inflammatory response that follows cell injection may also play a part in determining the effects on the recipient cardiomyocytes reported in this study. We (7) have previously shown that more CD45-positive cells were apparent after SK injections compared with BM injections. This cell-specific inflammatory response may contribute other paracrine factors in addition to those secreted by the injected cells, with a role that remains to be established.

Regarding BM cells, we used the whole mononuclear cell fraction, as this was employed in the largest clinical trial performed to date (9). A preclinical study (19) has suggested that selected mesenchymal stromal or lineage-negative cells may be more effective in cell therapy strategies. The use of these selected cell populations was not adopted here, and further studies are required to determine whether the effects on recipient cardiomyocytes can be ascribed to specific cell types. Testing the causal relationship between paracrine secretions and functional changes requires a different model than that used in the present study. The direct application of the candidate paracrine substance(s) in vivo may not be sufficient to recapitulate the effects on cardiomyocytes observed in the present study since a continuous, chronic, and local production is a likely requirement.

Conclusions. We have demonstrated, for the first time, that in hearts treated with cell transplantation, contractile performance and E-C coupling of individual recipient cardiomyocytes are altered and that these effects vary with the type of cell used. This improvement of contractile performance may contribute to the improved whole heart function observed after cell transplantation. Our findings confirm the hypothesis that indirect mechanisms, including possible paracrine effects, alter recipient cardiomyocytes after cell transplantation.

GRANTS
This work was supported by the Wellcome Trust and the British Heart Foundation.

REFERENCES


