Evidence for Cardiomyocyte Repopulation by Extracardiac Progenitors in Transplanted Human Hearts

Michael A. Laflamme, David Myerson, Jeffrey E. Saffitz, Charles E. Murry

Abstract—Human myocardium has long been considered to have essentially no intrinsic regenerative capacity. Recent studies in rodent models, however, have suggested the presence of an extracardiac stem cell population, perhaps in bone marrow, that is capable of some reconstitution of cardiomyocytes after injury. To determine whether similar mechanisms exist in the human heart, we evaluated human female allograft hearts transplanted into male patients. The presence of Y chromosomes in cardiomyocytes would indicate these cells arose from the recipient, rather than the donor heart. We identified 5 male patients who had retained a female heart at least 9 months before death and necropsy. Remarkably, in each case, the transplanted heart contained a minute but readily detectable fraction of Y chromosome–positive cardiomyocytes. The mean percentage of cardiomyocytes arising from the host was estimated to be 0.04% with a median of 0.016%. Most Y-positive cardiomyocytes were associated with regions of acute rejection, suggesting such chimerism involves an injury event. Furthermore, the sole patient whose immediate cause of death was allograft rejection showed a much higher percentage of host-derived cardiomyocytes, up to 29% in local, 1-mm² “hot spots.” Thus, adult humans have extracardiac progenitor cells capable of migrating to and repopulating damaged myocardium, but this process occurs at very low levels. (Circ Res. 2002;90:1111–1118.)

Key Words: human cardiac allografts ■ stem cells ■ transdifferentiation ■ Y chromosome in situ hybridization ■ regeneration

Because irreversible injury to myocardium, such as by infarction, typically results in scar formation,1 the human heart has long been considered to lack any appreciable regenerative ability. The replacement of muscle by scar tissue leads to loss of contractile function, and, consequently, many such patients develop progressive heart failure. Indeed, although there is debate about whether adult cardiomyocytes possess any intrinsic proliferative capacity at all,2,3 such activity, if it exists, must be physiologically inadequate in most circumstances. Despite this seemingly gloomy situation, some hope was provided by recent experiments in mouse models suggesting the presence of extracardiac progenitors capable of generating new cardiomyocytes.4,5 Using genetically tagged cells to follow lineage, two groups of investigators showed apparent incorporation of bone marrow–derived cells within infarcted myocardium, whether by bone marrow transplantation4 or direct injection into the infarct zone.5 Although perhaps not surprising given the obviously different experimental designs, the two studies reported widely disparate fractions of apparently transdifferentiated cardiomyocytes possessing the transgenic tag.

These findings in mouse models beg the question whether such mechanisms exist in the human heart. To address whether extracardiac progenitors give rise to cardiomyocytes, we chose to study human female-to-male cardiac transplants, because the presence of the Y chromosome in such hearts serves as an unambiguous extracardiac lineage marker. Moreover, experience in rodent models suggested that recruitment of bone marrow–derived precursors to the heart requires an injury event.4,5 This requirement should be met by transplant hearts, given the frequent occurrence of acute cellular rejection, chronic graft vascular disease (and resulting ischemic injury), and mechanical injury from routine surveillance biopsies to exclude rejection. Finally, based on animal evidence that such cells were likely rare,4 we chose to examine the large tissue blocks obtained at autopsy rather than the small tissue fragments obtained via surveillance biopsies. We report here the presence of a minute but readily detectable fraction of Y chromosome–positive cardiomyocytes (mean Y-positive percentage of 0.02% and median of 0.008%), thereby demonstrating very rare but definite replacement of the myocardium by cells of recipient origin. Correcting for the inherent false-positive rate of this technique as determined on male control hearts, we estimated a mean of ≈0.04% of cardiomyocytes to be of recipient origin.

While this manuscript was in the final stages of preparation, Quaini et al6 reported the discovery of Y-positive cardiomyocytes using confocal fluorescent microscopy on a...
similar series of human female-to-male cardiac transplant patients. In contrast to our own findings, these authors described a very remarkable degree of chimerism, estimating that a mean of 18% of the cardiomyocytes in their transplant hearts were of recipient origin. Although our own data confirm that the phenomenon of chimerism does occur, the much lower rate of incorporation has obviously different implications with regard to its physiological significance.

Materials and Methods
A review of the Washington University School of Medicine autopsy files identified 5 men who had received a female cardiac allograft and survived for >9 months thereafter. Similarly processed autopsy hearts from 5 female and 4 male subjects were used as negative and positive controls, respectively. Approval for the study of archived human tissue was granted by the institutional review committee of the Washington University School of Medicine. Five-micron sections were prepared from formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization and quenching of endogenous peroxidase activity (15 minutes in 100% methanol with 2% H2O2), sections were submitted to heat-induced epitope retrieval by boiling for 20 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0), blocked with 1.5% normal horse serum (Vector, Burlingame, Calif), incubated overnight at 4°C with either mouse anti-human CD45 (1:50, clones PD7/26 and 2B11; Dako, Carpinteria, Calif) or MF-20 hybridoma supernatant to detect sarcomeric myosin heavy chain (1:2, Developmental Studies Hybridoma Bank, University of Iowa). Antibodies were visualized via biotinylated, rat-absorbed horse anti-mouse secondary antibody, avidin-biotin complex alkaline phosphatase, and detection was by peroxidase-conjugated sheep anti-digoxigenin (DIG high-prime DNA labeling kit; Roche Diagnostics). Detection was by peroxidase-conjugated sheep anti-digoxigenin antibody Fab fragments (1:100, Roche Diagnostics) and the chromagen Vector Red (all from Vector).

The Y chromosome was detected by subsequent in situ hybridization with pY3,4,6,13 generously provided by Dr Y. F. Lai (University of California, San Francisco). The DNA probe was prepared from EcoRI-linearized plasmid, random prime-labeled with digoxigenin (DIG high-prime DNA labeling kit; Roche Diagnostics). Sections underwent repeated heat-induced epitope retrieval (sodium citrate buffer adjusted to pH 4.0 to 4.5), proteinase K digestion (0.01 µg/mL at 37°C for 20 minutes in 200 mMol/L Tris-HCl, 300 mMol/L sodium acetate, 50 mmol/L EDTA, 1% SDS; pH 9.0), three brief rinses in 2× SSC (0.30 mol/L sodium chloride and 0.03 mol/L sodium citrate), and prehybridization (15 minutes at 40°C in 2× SSC with 50% deionized formamide and 0.1% Tween-20). Denaturation was performed at 98°C for 15 minutes with probe (600 pg/mL, concentration, estimated by dot blot), in 2× SSC with 50% deionized formamide, 10% dextran sulfate, 0.1% Tween-20, and 400 µg/mL salmon sperm DNA. Overnight hybridization at 40°C was followed by a stringent wash (2× SSC with 50% formamide), at 40°C. Detection was by peroxidase-conjugated sheep anti-digoxigenin antibody Fab fragments (1:100, Roche Diagnostics) and the chromagen diaminobenzidine. Nuclei were counterstained with hematoxylin with Scott’s blue, and slides were coverslipped with Permount (Fisher).

Because the cardiac allografts typically contained large numbers of host-derived infiltrating leukocytes (containing Y chromosomes), rigorous criteria were used to determine whether a nucleus belonged to a cardiomyocyte. Nuclei had to be surrounded by red-colored, MF-20-stained cytoplasm and in the same focal plane as the MF-20-positive cytoplasm. Furthermore, cardiomyocytes had to be in a normal morphological relation to surrounding cardiomyocytes. Staining for the Y chromosome was regarded as positive if a punctate, dark brown signal was present within a given blue-stained nucleus and in the same focal plane. Y chromosome signals frequently showed an eccentric localization within the nucleus, both within cardiomyocytes and other cell types. The total count of Y-positive cardiomyocytes per section was determined by systematically raster-scanning the entire slide at ×1000 magnification with an oil-immersion objective. The density of cardiomyocyte nuclei per section was determined by enumerating 30 square fields of 102 square microns each using a gridded ocular reticule at ×1000. Fields were divided equally among subendocardial, midmyocardial, and subepicardial locations. The area of the section was quantitated by digital image analysis (Scion Image). The total number of cardiomyocyte nuclei per section was obtained by multiplying nuclear density times section area. Microscopic fields of interest were acquired via digital photography (Spot Diagnostic Instruments).

Results
To assess whether sections of allograft heart contained cardiomyocytes possessing a Y chromosome, we used dual labeling with a Y chromosome in situ hybridization probe and immunostaining with the monoclonal antibody MF-20. The latter antibody recognizes sarcomeric myosin heavy chain and is therefore a very specific marker for striated muscle. Suitable tissue was obtained from 5 male patients, each of whom had received a female heart before death and necropsy. The time interval between cardiac transplantation and death in these individuals ranged from 9 months to 4 years, and the respective cause of death in each case was different (see Table 1 for additional clinical details). In addition, similarly prepared sections of myocardium from autopsy cases of 5 female and 4 male individuals who had never undergone organ transplantation were used as negative and positive controls, respectively. Representative examples of the dual-labeled female and male myocardium are depicted in Figures 1A and 1B, respectively. Note that, despite a careful search, absolutely no Y-positive cardiomyocyte nuclei were identified in any sections of female heart, indicating that background labeling was not a problem with this technique. Further, 500 cardiomyocyte nuclei were quantitated on each section of male heart, and, in so doing, we found a mean of 53.3% of these nuclei to exhibit a Y chromosome signal (standard deviation of 8.5%), a value in good agreement with Quaini et al. This serves as an estimate of the inherent false-negative rate of the technique and is an expected result, given that the target sequence in the Y chromosome will not always be intercepted within a given plane of section.

Interestingly, many cardiomyocyte nuclei in male control hearts contained two Y chromosome signals, presumably indicating polyploid nuclei. An additional positive control intrinsic to the allograft tissue sections was the presence of infiltrating leukocytes. Indeed, as would be expected, the combination of in situ hybridization for the Y chromosome and immunostaining with the pan-leukocyte antigen CD45 demonstrated the presence of a Y chromosome body within the vast majority of inflammatory cells present within each allograft tissue section (data not shown).

Remarkably, all sections examined except one (septal myocardium from patient 3) contained unequivocal cardiomyocyte nuclei with a Y chromosome signal (see Figures 2 and 3 for representative examples). Overall, results for the 5 allograft hearts are summarized in Table 2. It should be noted that, although definite Y-positive cardiomyocytes could be found in virtually every specimen, these were extremely rare. The mean percentage of Y-positive cardiomyocytes was 0.02%, with a range of 0.005% to 0.07% and a median of 0.008%. Correcting for the false-negative rate as determined on the male control sections (47%), we estimated a mean of 0.04% of cardiomyocytes to be of recipient origin. In all instances, Y-positive cardiomyocytes appeared normally in-
integrated and were morphologically indistinguishable from their Y-negative neighbors. No binucleated Y-positive cardiomyocytes were observed. One additional finding of interest was that, in contrast to the pattern of in situ hybridization noted in male control myocardium, the Y-positive cardiomyocytes in transplant hearts uniformly demonstrated a single punctate signal. (Compare inset of Figure 1B with insets of Figures 2 and 3).

In general, Y-positive cardiomyocytes were quite widely distributed, with one notable exception. Myocardium from patient 5 (see Figure 3) showed an appreciably higher fraction of Y-positive cardiomyocytes (0.07%), and, in this case, they quite obviously coincided with foci of inflammation, signifying acute cellular rejection. Associated with rejection within the patient’s right ventricular myocardium were two “hot spots” with substantially higher densities of Y-positive cardiomyocytes, 14.5% and 10.8% (22 of 152 and 21 of 194 cardiomyocyte nuclei within a 1-mm-square area in each case). Because of the comparative rarity of Y-positive cardiomyocytes in the remaining subjects, no attempt was made to quantitate the “local” density of cardiomyocytes within these individuals. That said, where they occurred, Y-positive cardiomyocytes generally were in proximity to foci of acute rejection, as defined by the presence of inflammatory cell infiltrates. The converse was not true, however: in many areas, inflammation was widespread and the tissue was devoid of Y-positive cardiomyocytes (including portions of the myocardium in patient 4, who also demonstrated significant acute rejection; see Table 2).

Finally, in all sections, numerous additional Y-positive cells were observed that would not appear to be either cardiomyocytes or leukocytes. Further studies to determine what cell types these represent are being undertaken.

### Discussion

The results reported in the present study provide evidence that the transplanted human heart can be repopulated by extracardiac progenitors, albeit at an extremely low level. This study could not address the source of these progenitors, and, admittedly, they could have even arisen from within the small amount of host atrial myocardium typically retained after cardiac transplantation, should cardiac stem cells reside there. Furthermore, a small number of studies in mouse models have indicated that such progenitors might reside within bone marrow. Jackson et al transplanted hematopoietic “side population” stem cells from ROSA 26 (constitutive β-galactosidase–expressing) mice into lethally irradiated hosts and, 2 to 4 weeks after infarction, they found expression of β-galactosidase in a small number of cardiomyocytes in the peri-infarct region. Interestingly, they estimated that 0.02% of cardiomyocytes in these hearts were derived from the donor marrow, a number that agrees quite well with the fraction of Y chromosome–positive cardiomyocytes in the present study. Bittner et al performed a similar study in which female mdx mice (a model of Duchenne muscular dystrophy) received bone marrow transplants from congenic males. They found Y chromosome signals in rare

### TABLE 1. Clinical Data for 5 Male Transplant Recipients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Death, y</th>
<th>Transplant–Death Interval, mo</th>
<th>Reason for Transplant</th>
<th>Cause(s) of Death</th>
<th>No. of Antemortem Biopsies With at Least Multifocal Moderate (3A) Acute Rejection/Total No. of Biopsies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>50</td>
<td>IHD</td>
<td>Metastatic pancreatic cancer</td>
<td>2/16</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>34</td>
<td>IHD</td>
<td>Congestive heart failure, cause undetermined</td>
<td>2/22</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>19</td>
<td>IHD</td>
<td>Immediate: bronchopneumonia, ARDS, pulmonary hemorrhage</td>
<td>4/19</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>10</td>
<td>DCM</td>
<td>Sudden death; cause undetermined</td>
<td>1/15</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>9</td>
<td>DCM</td>
<td>Immediate: acute and chronic cardiac allograft rejection</td>
<td>2/14</td>
</tr>
</tbody>
</table>

IHD indicates ischemic heart disease; DCM, dilated cardiomyopathy; and ARDS, acute respiratory distress syndrome.

*Grades of acute rejection had been assigned on surveillance biopsies during the posttransplant period using International Society for Heart and Lung Transplantation standardized grading system. For every recipient except patient 5, all such episodes of rejection were classified as multifocal moderate (3A). Patient 5 was the sole individual to be diagnosed with an even higher grade of rejection: moderate diffuse (3B) and moderate diffuse-to-severe (3B/4) rejection, the latter shortly preceding death. Note that these data do not include evidence of rejection noted in autopsy tissue.
cardiomyocytes, although they did not attempt quantitation. Finally, Orlic et al.\(^5\) reported a remarkable degree of myocardial regeneration when bone marrow cells (lineage marker-negative, c-kit-positive) were injected directly into myocardial infarcts. We have been unable to reproduce these findings using similar direct injection methods,\(^15\) so their relation to the present study is not clear.

In addition to those found in bone marrow, other cell types have been reported to give rise to cardiomyocytes. For example, Malouf et al.\(^16\) reported that a cell line from rat liver transdifferentiated into cardiomyocytes after intramyocardial implantation. Clarke et al.\(^17\) reported that neural stem cells transdifferentiated into cardiomyocytes, among other cell types, after implantation into blastocysts. Some investigators have proposed that skeletal myoblasts can transdifferentiate into cardiomyocytes after intramyocardial grafting, but detailed studies from our laboratory showed no transdifferentiation of either neonatal\(^18\) or adult skeletal muscle cells.\(^19\) Finally, Condorelli et al.\(^20\) reported that neural stem cells and certain types of endothelial cells transdifferentiated into cardiomyocytes when co-cultured with neonatal cardiomyocytes. Indeed, adult stem cells in general seem to have a much greater degree of pluripotency than originally established.\(^21-24\)

As mentioned above, Quaini et al.\(^6\) very recently reported that 18% of cardiomyocytes in transplanted hearts were derived from the host. Although our data confirm that this phenomenon of chimerism does occur, we found vastly lower fractions of host-derived cells (0.02% Y-positive myocytes, corresponding to \(\approx 0.04\%\) myocytes of recipient origin, a three orders of magnitude difference from the aforementioned study). In general terms, this enormous discrepancy could result from clinical differences in the patients under study or from technical differences in the ways the two estimates were derived. The only clinical parameter apparently different is the shorter interval between transplantation and death in their series versus our own. (We deliberately avoided cases with short transplant intervals, because we were concerned about the greater inflammatory infiltrate in these hearts leading to false-positive results; see below). The patients studied by

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**Figure 1.** Dual labeling with MF-20 (Vector Red chromagen) antibody to detect sarcomeric myosin heavy chain and Y chromosome in situ hybridization (brown diaminobenzidine nuclear signal) on typical female (A) and male (B) control heart sections. Occasional cardiomyocytes in both sections show granular, yellow-brown lipofuscin pigment in a perinuclear distribution, but this can be readily distinguished from the punctate, dark brown Y chromosome signal. The female myocardium is devoid of Y chromosome in situ signal. In contrast, the majority of cardiomyocytes in male myocardium demonstrate one or more discrete Y chromosome signals, as is demonstrated by the inset in panel B (magnified an additional 2-fold). This multiplicity of Y chromosome signals in cardiomyocytes, not seen in other cell types, which only show a single nuclear dot, may be reflective of their reportedly frequent polyploid nature. In sections from 4 male control hearts, a mean of 53.3% of cardiomyocytes was found to be Y-positive. Nuclear counterstain was Scott’s blue/hematoxylin. Scale bar=20 \(\mu\)m.
Quaini et al. had transplant-to-death intervals ranging from 4 to 552 days, versus the range of 9 to 50 months in our own series. Counterintuitively, they reported the highest rates of chimerism in patients who had their hearts the shortest time: 30% Y-positive cardiomyocytes in three patients who died 4 to 28 days after transplantation (corresponding to ~100 grams of new myocardium), versus 8% for their two patients at 396 and 552 days. Furthermore, they reported that the new cardiomyocytes were morphologically indistinguishable from host cells by posttransplant day 4. This would require that blast-to-adult myocyte differentiation exceeded normal development rates by several orders of magnitude. In contrast, previous studies from this laboratory have shown that neonatal rat cardiomyocytes hypertrophied at their normal developmental rate after grafting into the injured heart, taking >2 months to reach adult size.\textsuperscript{25} Finally, it is conceivable that immunosuppressive therapy could alter the rate of accumulation of chimerism. Because all the patients in the present series were on standard regimens, however, we would not expect this parameter to be substantially different between the patient population used by Quaini et al.\textsuperscript{6} and our own. Further, a review of the clinical history of our own patient population did not reveal any additional pharmacological agents that would intuitively be expected to alter stem cell mobilization or differentiation (although this possibility is obviously impossible to totally exclude in human patients, given our current level of understanding). None of our transplant patients underwent cytotoxic chemotherapy, including patient 1 who expired from metastatic pancreatic cancer.

Although our data cannot definitively exclude a role for clinical variables, it seems more likely to us that technical differences underlie the vast difference in the results. Human
cardiac allografts are infiltrated with a population of host-derived leukocytes within hours of engraftment, particularly macrophages and T lymphocytes, and cellular rejection is typically worst in the first year after transplantation. In female-to-male transplants, these leukocytes, of course, will contain Y chromosomes. To obtain a true count of cardiomyocyte chimerism, it is imperative that leukocyte nuclei not be mistaken for cardiomyocyte nuclei. To avoid this pitfall, we established very stringent criteria for identifying Y-positive cardiomyocytes (see Materials and Methods). Furthermore, our use of two chromagens detected at visible wavelengths permitted interpretation of the signals in a more familiar morphological context. This contrasts with the use of fluorescence microscopy by Quaini et al. Conventional microscopy offers much better detection of cell borders than does fluorescence microscopy, and identification of cell borders is critical in assigning a given nucleus to a cell type. We found a great many cases where leukocytes were sandwiched between cardiomyocytes and could have been misinterpreted as cardiomyocyte nuclei under fluorescent microscopy. Thus, it is possible that many of the chimeric cardiomyocytes reported by Quaini et al were actually leukocytes, misidentified as cardiomyocytes because of technical limitations. Finally, we note that in a much earlier attempt to use Y chromosome in situ hybridization to assess for transplant chimerism, Hruban et al were not able to detect any cardiomyocytes of recipient origin within two transplant hearts subsequently removed for graft failure. Given the

Figure 3. Multiple Y chromosome signals present within cardiomyocytes of female heart transplanted into patient 5. Patient 5 was the only patient in this series whose immediate cause of death was acute and chronic cardiac allograft rejection, and his heart showed a substantially higher fraction of Y-positive cardiomyocytes, locally numbering up to 14.5% within “hot spots” found in the right ventricular myocardium. Note occasional brown Y chromosome bodies evident within nuclei of red-colored, MF-20–immunostained cardiomyocytes, as illustrated by 2-fold magnified insets in panels A and B. Both images also show scattered Y chromosome–positive interstitial cells (some likely representing host inflammatory cells). Panel B contains at least two Y chromosome–positive cardiomyocytes (see inset and arrowhead). For both, nuclear counterstain was Scott’s blue/hematoxylin. Scale bar=10 μm.
The existence of extracardiac stem cells capable of renewing myocardium represents a new paradigm for how the human heart, formerly viewed as essentially static in this regard, may respond to injury. In addition to determining the time course for such a phenomenon, obvious remaining questions of considerable interest include determination of the anatomic location of this stem cell population, whether such repopulation occurs in humans in other pathophysiologic contexts (such as myocardial infarction), elucidation of the sequence of migration and transdifferentiation events, and whether this pathway is amenable to therapeutic intervention.

Acknowledgments

These studies were supported in part by NIH Grants RO1 HL61553, PO1 HL03174, and R24HL64387 (to C.E.M.) and CA-15074 and CA-18029 (to D.M.). The authors wish to thank Veronica Poppa and Carol Bevan for their expert technical assistance.

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Circ Res. published online March 7, 2002;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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