Extracardiac Progenitor Cells Repopulate Most Major Cell Types in the Transplanted Human Heart

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Background—Extracardiac progenitor cells are capable of repopulating cardiomyocytes at very low levels in the human heart after injury. Here, we explored the extent of endothelial, smooth muscle, and Schwann cell chimerism in patients with sex-mismatched (female-to-male) heart transplants.

Methods and Results—Autopsy specimens from 5 patients and endomyocardial biopsies from 7 patients were used for this study. Endothelial, vascular smooth muscle, and Schwann cells were stained with antibodies against CD31 or Ulex europaeus lectin, smooth muscle α-actin, and S-100, respectively, and the Y chromosome was identified with in situ hybridization. Biopsy specimens from 1, 4, 6, and 12 months and 5 and 10 years after heart transplantation were evaluated. Y-positive cells were counted by conventional bright-field microscopy and confirmed by confocal microscopy. Endothelial cells showed the highest degree of chimerism, averaging 24.3±8.2% from extracardiac sources. Schwann cells showed the next highest chimerism, averaging 11.2±2.1%; vascular smooth muscle cells averaged 3.4±1.8%. All 3 cell types showed substantially higher chimerism than we previously observed for cardiomyocytes (0.04±0.05%). Endothelial chimerism was much higher in the microcirculation than in larger vessels. Analysis of serial endomyocardial biopsies revealed that high levels of endothelial chimerism occurred as early as 1 month after transplantation (22.6±6.6%) with no significant increases even up to 10 years after cardiac transplantation.

Conclusions—Extracardiac progenitor cells are capable of repopulating most major cell types in the heart, but they do so with varying frequency. The signals for endothelial progenitor recruitment occur early and could relate to injury during allograft harvest or transplantation. The high degree of endothelial chimerism may have immune implications such as for myocardial rejection or graft vasculopathy. (Circulation. 2005;112:2951-2958.)

Key Words: endothelium • pathology • stem cells • transplantation • Y chromosome

The heart has marginal ability to regenerate. As a consequence, it heals after infarction by scar formation and wall thinning in the infarct region, compensatory hypertrophy in the viable myocardial regions, and eventual dilation.1–5 In more severe cases, this process translates into clinical heart failure and marked functional decline. Recently, there is growing evidence that extracardiac progenitor cells may contribute to heart repair after injury. Although first described in animal models,6–12 several groups, including our own, have shown similar derivation of different cell types from progenitor cells in humans.13–17

One way to trace the lineage of these progenitor cells in humans is to study sex-mismatched heart transplants.18 When a female heart is transplanted into a male patient, in situ hybridization with a Y-chromosome probe allows identification of extracardiac cells in the female allograft. Using this approach, we have recently shown that 0.04% of cardiomyocytes in the transplant heart are extracardiac derived and are recipient in origin.13

The goal of this study was to determine whether extracardiac progenitor cells are capable of repopulating multiple other cell types in the human transplanted heart. We also sought to determine the time course of endothelial cell chimerism to shed more light on its mechanism and its influence on clinical outcome.

Methods

Specimen Selection
This study was approved by the Institutional Review boards of the University of Washington in Seattle and Washington University School of Medicine in St Louis. Five autopsy cases were used to investigate the extent of chimerism in the transplanted heart. These specimens were identified as having sex-mismatched transplants.
after review of the Washington University School of Medicine pathology files. For the endothelial chimerism time course study, we identified 7 male patients with female cardiac allograft transplants who are still alive and who had serial endomyocardial biopsies from 1 month to 10 years after transplantation. For comparison of autopsy and biopsy patients in the time course evaluation, 3 of the autopsy patients (patients 1, 2, and 3) were included because they lived from a range of 19 to 50 months after transplantation. Because patients 4 and 5 did not survive past 12 months, their specimens were not included in the time course study. Five female and 4 male subjects who did not undergo heart transplantation were used as negative and positive controls, respectively.

**Immunohistochemistry and In Situ Hybridization**

We prepared 5-μm sections from formalin-fixed, paraffin-embedded tissue blocks from both autopsy and biopsy specimens and subjected them to immunohistochemical staining and in situ hybridization techniques as previously described. In brief, these sections were deparaffinized and quenched for endogenous peroxidase activity (15 minutes in 100% methanol and 3% hydrogen peroxide). These sections were then blocked with 1.5% normal horse serum (Vector) and incubated overnight with primary antibodies to smooth α-actin (smooth muscle cells, mouse monoclonal, DAKO) and S-100 (Schwann cells/peripheral nerve, rabbit polyclonal, DAKO). For endothelial cells, biotinylated Ulex europeus lectin (Vector) was used for the biopsy specimens, and CD31 was used for the autopsy specimens. Pilot studies showed that the autopsy fixation protocol was not consistently compatible with Ulex staining, and many of the biopsy specimens, in which the endothelium stained well with Ulex, did not stain uniformly with CD31. Because 2 different endothelial markers were used in this study as a result of fixation obstacles, we confirmed that the percentage of endothelial chimerism did not vary between Ulex and CD31 staining in the biopsy specimens that stained for both (data not shown). As shown in Figure I of the online-only Data Supplement (found at http://circ.ahajournals.org/cgi/content/full/112/19/2951/DC1), Ulex staining was specific to endothelial cells and did not label leukocytes. Microscopic fields of interest were acquired via digital photography (Spot Diagnostic instruments). Primary antibodies were visualized by avidin-biotin complex alkaline phosphatase after incubation with appropriate secondary antibodies. Chromagen Vector Red (Vector) was used to visualize the specific antibody or lectin.

After immunostaining was complete, Y-chromosome in situ hybridization was performed on the same tissue sections. A digoxigenin-labeled probe was prepared by random priming and hybridized to a highly repetitive target sequence in the human Y chromosome as previously described. After overnight hybridization and stringent washes, the probe was visualized with a peroxidase-conjugated anti-digoxigenin antibody (Roche) and diaminobenzidine as substrate. Slides were counterstained with hematoxylin with Scott’s blue.

To confirm the in situ hybridization and immunohistochemical data, 3 of the autopsy patients were studied by confocal fluorescent microscopy. The sections were deparaffinized and treated with 2× standard sodium citrate at 80°C for 10 minutes, followed by antigen retrieval (pH 4.5) for 20 minutes. The sections were then subjected to proteinase K treatment at 37°C for 15 minutes and placed in denaturation solution (2× standard saline citrate, 70% formamide) at 73°C for 5 minutes. The sections were then incubated with the probe (CEP Y SG Sat III DNA probe, Vyssis) for 10 minutes at 75°C and overnight at 37°C. Slides were then washed with 2× standard saline citrate. Primary antibodies to endothelial, smooth muscle, and Schwann cells were applied in a manner similar to that of the standard immunohistochemical technique mentioned above, incubated overnight, and labeled with Alexa Red 598 secondary antibody (Molecular Probes) to identify the specific cell type. The nucleus was counterstained with DAPI II (Vyssis).

**Cell Counting**

For conventional bright-field microscopy, rigorous criteria were used to determine whether a Y-chromosome signal was indeed within the nucleus of the cell and whether that nucleus resided within a labeled cell population. For all cell types counted, the nuclei had to be completely surrounded by the cell type–specific immunostaining product. In addition, the cells had to be in a normal morphological context within the tissue. Staining for 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. The density of nuclei per section was determined by enumerating 30 square fields of 100 μm² each with a gridded ocular reticle. Y-positive cells were manually counted for all cell types and all nuclei under each gridded ocular reticle by raster scanning the entire slide at ×100 objective. The percent of each cell type that was Y positive was calculated by dividing the total number of Y-positive cells counted over the total number of nuclei counted for each specimen and for each cell type. All Y chromosome–positive counts were corrected for false negatives determined by hybridization efficiency, which was defined as Y chromosome–positive nuclei divided by total nuclei counted in male control subjects. The hybridization efficiency was 47.7±2.7% for endothelial cells, 45.4±1.3% for smooth muscle cells, and 29.5±8.4% for Schwann cells. The corrected mean±SD percentage incorporates this false-negative correction.

For cell counting with confocal microscopy, 30 to 40 visual fields from each section were obtained by use of the Leica model TCS-SP1-MP confocal microscope under a ×40 oil objective. The range of wavelength detected for each fluorochrome is as follows: FITC channel, between 498 and 600 nm; Alexa Red channel, between 580 and 742 nm; and DAPI channel, between 395 and 560 nm. The number of nuclei counted per section ranged from 95 to 1197. Cells were considered positive if the Y signal was contained in the nucleus and completely surrounded by cytoplasm for each cell type.

The hybridization efficiency for fluorescent in situ hybridization (FISH) using male control subjects was as follows: 26.9±2.6% for endothelial cells, 24.4±0.57% for smooth muscle cells, and 27.2±3.11% for Schwann cells. Possible explanations for the lower hybridization efficiency of FISH protocol include the lower time of denaturation at high temperature during the hybridization step, the thinner optical section used for confocal analysis compared with the 5-μm section for conventional microscopy, and a lower sensitivity of the fluorescent Y probe.

For quantifying endothelial chimerism in the autopsy specimens, the specimens were divided into epicardial and intramyocardial compartments and endocardium. In the epicardial compartment, separate counts were obtained for small (defined in this case as ≤250 μm in diameter) and large (>250 μm) vessels; the latter category was further subdivided histologically into arteries and veins. In the intramyocardial compartment, vessels were subdivided as large vessels (again defined as >250 μm), intramyocardial arterioles (defined as small, 30- to 100-μm arteries with definite circumscribed smooth muscle), and all other small vessels (defined as <30 μm in diameter). Of note, the last category of small vessels was composed predominantly of capillaries (defined as 7 to 8 μm in diameter). Smooth muscle cell chimerism was quantified in the autopsy specimens with the same categories of vessel localization and size.

For the time course study, biopsy specimens from 7 patients were obtained from time points of 1, 4, 6, and 12 months and 5 and 10 years after cardiac transplantation. For the 3 autopsy patients whose biopsy specimens were used for the time course study, we looked at specimens from 1, 4, 6, and 12 months and their last biopsies after cardiac transplantation.

**Statistical Analysis**

All data are presented as the mean±SD percentage. Calculations were made with the InStat statistical program. Comparisons of chimerism in vessels by location and size were made by ANOVA. Values of P<0.05 were considered significant.
Results

For the first phase of our study, male autopsy patients who underwent a female allograft heart transplantation were investigated so that we could identify recipient-derived cells using Y-chromosome in situ hybridization. The age at autopsy ranged from 23 to 62 years, and the transplantation–death interval ranged from 9 to 50 months (Table 1). Patients 1 through 3 were included in the study for time course measurements of endothelial chimerism because they survived at least 19 months to as long as 50 months after transplantation. The biopsy specimens used for the time course analysis were obtained from 7 male post–cardiac transplantation patients with female allografts who continue to remain alive as of this writing (Table 2). The most common reason for transplantation was ischemic heart disease, and mild coronary atherosclerosis was present in all patients on their most current angiogram except for patients 6 and 7, who demonstrated moderate narrowing.

Extent of Chimerism in Autopsy Specimens

In contrast to our earlier study, which detected 0.04% cardiomyocytes repopulated from extracardiac cells, there was much greater chimerism in other cell types of the heart, namely endothelial, vascular smooth muscle, and Schwann cells. The vascular samples available for study did not have significant transplant arteriopathy. For endothelial cells, an overall mean of 24.3%/H11006 8.2% was derived from extracardiac progenitors in the autopsy cases (Table 3); this measurement correlated well with confocal microscopy (31.2%/H11006 12.8%). Representative examples are shown in Figures 1A, 1B, 2A, and 2B. These figures show multiple Y-positive endothelial cells in a single vascular cross section, a common anatomic finding. The endothelial cells, however, were not distributed uniformly throughout the vasculature. Large coronary arteries and veins (>250-μm diameter) had fewer extracardiaderived endothelial cells, ranging from 4.4% to 11.6% (Table 3). In contrast, coronary microvessels (≤100-μm diameter) had endothelial chimerism that ranged from 23.1% to 36.3%. No systematic differences in chimerism were noted between arteries and veins regardless of size. The endocardial endothelium averaged 22.8% extracardiac derived.

Next, we studied chimerism in vascular smooth muscle cells from the autopsy specimens. Smooth muscle chimerism was ∼7-fold less common than that of endothelial cells, with an overall corrected mean of 3.4%/H11006 1.8% of extracardiac origin (Table 4 and Figure 1C and 1D). We searched for differences in smooth muscle cell chimerism in microvessels versus large vessels, arteries versus veins, and location on the epicardial surface versus within the myocardium. No significant difference in smooth muscle cell chimerism was noted (Table 4). Using confocal microscopy, we counted modestly higher levels of smooth muscle cell chimerism (5.9%/H11006 1.2%), although we think these values fall within the error margin of the 2 approaches.

Our final cell population of interest was the peripheral nerve Schwann cell. Using S-100 to identify these cells, we observed a corrected mean of 11.2%/H11006 2.1% Schwann cells derived from outside the heart (Figure 1E and 1F). This corrected mean was comparable to confocal microscopy (14.1%/H11006 2.9%; Figure 2E and 2F). The Y-positive Schwann cells were typically isolated (Figures 1E and 2F) or occasionally clustered in small groups (Figure 1F). We did not observe

**TABLE 1. Clinical Data for 5 Male Autopsy Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Death, y</th>
<th>Transplantation-Death Interval, mo</th>
<th>Reason for Transplantation</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>50</td>
<td>IHD</td>
<td>Pancreatic cancer</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>34</td>
<td>IHD</td>
<td>Undetermined</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>19</td>
<td>IHD</td>
<td>Bronchopneumonia, ARDS, pulmonary hemorrhage</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>10</td>
<td>DCM</td>
<td>Sudden death, undetermined</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>9</td>
<td>DCM</td>
<td>Acute/chronic cardiac allograft rejection</td>
</tr>
</tbody>
</table>

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

**TABLE 2. Clinical Data of 7 Patients in the Biopsy Study**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Posttransplantation Interval, mo</th>
<th>Reason for Transplantation</th>
<th>Current Angiogram Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>144</td>
<td>DCM</td>
<td>Mild narrowing of coronaries</td>
</tr>
<tr>
<td>2</td>
<td>136</td>
<td>IHD</td>
<td>Mild narrowing of proximal and mid portion of RCA</td>
</tr>
<tr>
<td>3</td>
<td>142</td>
<td>IHD</td>
<td>Mild diffuse narrowing of epicardial, mid caliber, and small caliber vessels</td>
</tr>
<tr>
<td>4</td>
<td>135</td>
<td>IHD</td>
<td>Mild narrowing of LAD, circumflex, and RCA</td>
</tr>
<tr>
<td>5</td>
<td>135</td>
<td>IHD</td>
<td>Mild narrowing of circumflex and RCA</td>
</tr>
<tr>
<td>6</td>
<td>191</td>
<td>DCM</td>
<td>Moderately severe circumflex, moderate RCA narrowing</td>
</tr>
<tr>
<td>7</td>
<td>182</td>
<td>IHD</td>
<td>Moderate diffuse narrowing</td>
</tr>
</tbody>
</table>

DCM indicates dilated cardiomyopathy; IHD, ischemic heart disease; RCA, right coronary artery; and LAD, left anterior descending artery.

*All patients in this group are currently living.*
nerve segments with coherent labeling comparable to male control hearts.

**Time Course of Endothelial Chimerism**

Endomyocardial biopsies are performed frequently during the first year after transplantation to monitor for rejection and at least annually thereafter. This allows an opportunity to study the temporal development of endothelial chimerism. We hypothesized that chimerization would evolve gradually, possibly taking months or even years to reach its maximal extent. To our surprise, biopsies taken as early as 1 month showed high levels of endothelial chimerism (21.7% ± 6.6%; Table 5 and Figure 3). Although there was some variation in the extent of chimerism within a given patient from biopsy to Table 3. Endothelial Cell Chimerism by Location in Autopsy Specimens

<table>
<thead>
<tr>
<th>Patient</th>
<th>Large Epicardial Arteries, %</th>
<th>Large Epicardial Veins, %</th>
<th>Small Intramyocardial Vessels, %</th>
<th>Large Intramyocardial Arterioles, %</th>
<th>Other Intramyocardial Vessels, %</th>
<th>Endocardium, %</th>
<th>Total Average, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7</td>
<td>2.5</td>
<td>40</td>
<td>8.0</td>
<td>24.8</td>
<td>16.8</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>4</td>
<td>64.9</td>
<td>11.1</td>
<td>33.8</td>
<td>24.8</td>
<td>23.3</td>
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<td>3</td>
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<td>NA</td>
<td>30</td>
<td>21.4</td>
<td>38.6</td>
<td>26</td>
<td>41.3</td>
</tr>
<tr>
<td>4</td>
<td>7.6</td>
<td>8.6</td>
<td>21.6</td>
<td>7.8</td>
<td>25.8</td>
<td>24.4</td>
<td>23.9</td>
</tr>
<tr>
<td>5</td>
<td>11.8</td>
<td>2.3</td>
<td>24.8</td>
<td>9.7</td>
<td>18.5</td>
<td>23.3</td>
<td>18.5</td>
</tr>
<tr>
<td>Corrected mean*</td>
<td>5.3</td>
<td>4.4</td>
<td>36.3†</td>
<td>11.6</td>
<td>28.3‡</td>
<td>23.1</td>
<td>22.8</td>
</tr>
<tr>
<td>SD</td>
<td>5.4</td>
<td>2.9</td>
<td>17.4</td>
<td>5.6</td>
<td>7.9</td>
<td>3.6</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Each of the autopsy specimens was divided into regions of the epicardium, intramyocardium, and endocardium during counting. Large epicardial arteries and veins were defined as vessels >250 μm in diameter. Vessels <250 μm in the epicardial region were classified as small epicardial veins. Large intramyocardial vessels were defined as vessels >250 μm in diameter. Intramyocardial arterioles are vessels ranging from 30 to 100 μm in diameter. Other intramyocardial vessels were <30 μm, which predominantly represented capillaries. Endocardium represents the cells lining the chamber of the heart. A total of 47,321 cell nuclei were counted.

*All counts are represented as the corrected count, which incorporated the hybridization efficiency obtained from male control subjects, in this case, 47.7 ± 2.7%.

†P<0.001 vs large epicardial arteries and veins; ‡P<0.01 vs with large intramyocardial vessels; §P<0.05 vs large epicardial arteries and veins and large intramyocardial vessels.

**Figure 1.** Bright-field microscopic examples of endothelial cell chimerism (A, B), vascular smooth muscle cell chimerism (C, D), and Schwann cell chimerism (E, F) in autopsy specimens. Endothelial cells, smooth muscle cells, and Schwann cells were stained for CD31, smooth muscle α-actin, and S-100, respectively, with a red chromogen. Insets show magnification of Y chromosome–positive nuclei, shown as a dark, punctate signal within the nucleus. Multiple Y-positive endothelial cells (arrows) are visible in this intramyocardial venule (A) and this tangentially sectioned epicardial vein (B). A single Y-positive smooth muscle cell is present in an intramyocardial arteriole (C) and in a thick-walled muscular artery (D). E, A single Y-positive Schwann cell in a cardiac nerve fiber. F, A pair of Y-positive Schwann cells. Scale = 20 μm for A through D, 10 μm for E and F.
biopsy, there was no consistent increase or decrease over time. Indeed, biopsies from patients at 10 years after transplantation revealed 19.2 ± 8.8% of endothelial cells to be of extracardiac origin (Table 5). Thus, endothelial chimerization appears to be established at high levels within the first month after transplantation and is stable thereafter.

**Discussion**

In the present study, we have demonstrated that extracardiac progenitor cells are capable of repopulating most cell types in the human heart. We found that the frequency of chimerism varies widely by cell type in the following order: endothelial cells (24.3%) > Schwann cells (11.2%) > smooth muscle cells (3.4%) ≥ cardiomyocytes (0.04%) (Figure 4). Comparable values were obtained with a FISH and confocal microscopic technique. Endothelial cell chimerism has a predilection for small epicardial and intramyocardial vessels, which had a notable 3- to 5-fold-greater chimerism than their larger counterparts. Surprisingly, the degree of endothelial cell chimerism plateaus as early as 1 month and maintains that level even 10 years after transplantation. It should be noted that multiple studies in experimental animals have demon-

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**TABLE 4. Smooth Muscle Cell Chimerism by Location in Autopsy Specimens**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Large Epicardial Arteries, %</th>
<th>Large Epicardial Veins, %</th>
<th>Small Epicardial Veins, %</th>
<th>Large Intramyocardial Vessels, %</th>
<th>Intramyocardial Arteries, %</th>
<th>Other Intramyocardial Vessels, %</th>
<th>Total, Average, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6</td>
<td>4.6</td>
<td>2.4</td>
<td>2.6</td>
<td>5.3</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>9.9</td>
<td>4.2</td>
<td>2</td>
<td>2.9</td>
<td>3.5</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>4.2</td>
<td>1.5</td>
<td>3.7</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>0.9</td>
<td>8.8</td>
<td>6.2</td>
<td>8.6</td>
<td>6.6</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>2.9</td>
<td>7.3</td>
<td>1.1</td>
<td>2.9</td>
<td>2.9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Corrected mean*</td>
<td>3.5</td>
<td>3.8</td>
<td>5.4</td>
<td>2.7</td>
<td>4.7</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>SD</td>
<td>1.1</td>
<td>4.5</td>
<td>2.6</td>
<td>2</td>
<td>2.4</td>
<td>1.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Each of the autopsy specimens was divided into regions of the epicardium, intramyocardium, and endocardium during counting. Large epicardial arteries and veins were defined as vessels >250 μm in diameter. Vessels <250 μm in the epicardial region were classified as small epicardial veins. Large intramyocardial vessels were defined as vessels >250 μm in diameter. Intramyocardial arterioles are vessels ranging from 30 to 100 μm in diameter. Other intramyocardial vessels were <30 μm, which predominantly represented capillaries. A total of 38,212 cell nuclei were counted.

*All counts are corrected for the hybridization efficiency, which was determined by male control subjects, in this case, 45.4 ± 1.3% for vascular smooth muscle.

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**Figure 2.** Confocal microscopic examples of endothelial cell chimerism (A, B), vascular smooth muscle cell chimerism (C, D), and Schwann cell chimerism (E, F) in autopsy specimens using FISH for the Y chromosome (green) and specific cell markers (red). Endothelial cells, smooth muscle cells, and Schwann cells were stained for Ulex, smooth muscle α-actin, and S-100, respectively. Insets show magnification of the Y chromosome showing a fluorescent green, punctate signal within the nucleus surrounded by cytoplasm of the specific cell type. Note autofluorescence of erythrocytes, cardiomyocytes, and arterial internal elastic lamina. Scale – 30 μm for A, 50 μm for B, and 20 μm for C through F.
strated that circulating progenitor cells are capable of repopulating cells in the heart, possibly contributing to coronary atherosclerosis, transplant arteriopathy, and angiogenesis. Although a detailed review of these animal studies is beyond the scope of this article, we refer interested readers to the recent excellent review by Hillebrands et al.19

Although our study addressed most cell populations in the heart, we were, unfortunately, unable to study chimerism in fibroblasts. Despite many attempts, we could not identify an antibody that marks this cell population selectively in formalin-fixed, paraffin-embedded tissue. We would expect, however, that fibroblast chimerism might play an important role in the cardiac reparative process because fibroblasts produce the extracellular matrix and scar tissue of the heart.

Endothelial Chimerism in Human Transplants

Quaini et al14 studied the degree of chimerism in 8 sex-mismatched transplant recipients using fluorescent Y probe to detect cells from recipient origin. Like us, they restricted their analyses to structurally normal coronary vessels. Unlike the present study, however, they counted the frequency of Y-positive vessels (defined as 30% or more Y-positive cells) rather than Y-positive cells. They reported significant levels of chimerism in capillaries (14% extracardiac derived) and arterioles (21% to 50% extracardiac-derived endothelial cells in vessels having at least 30% Y-positive smooth muscle cells). Despite these methodological differences, their findings seem to be in the same range as the present study. Simper et al20 investigated the levels of circulating endothelial cells and endothelial progenitor cells in 5 female-to-male heart transplant patients who had angiographic evidence for transplant atherosclerosis. In that study, comparison of endothelial cell chimerism between diseased and nondiseased segments of the coronary arteries also was performed with FISH. In the diseased segments, chimerism of luminal microvascular endothelium ranged from 1% to 24%, whereas chimerism of adventitial microvascular endothelium ranged from 1% to 11%. If we adjust these data for their hybridization efficiency (41%), chimerism more than doubles and hence overlaps significantly with our own data set. Surprisingly, when these investigators performed a smaller subgroup analysis, they found that structurally normal regions of arteries (with disease elsewhere) showed only 0.2% endothelial chimerism. This is significantly lower than what we observed in nondiseased segments. Finally, Hocht-Zeisberg et al21 compared endothelial chimerism in gender-mismatched transplants with (n=5) and without (n=9) myocardial infarction. They reported >50-fold-increased endothelial chimerism in the infarcted hearts, although their baseline levels of chimerism (0.01%) were substantially lower than what we observed in our patients. The reason for this difference is not entirely clear. One contributing factor may be their use of von Willebrand factor to define endothelium. Von Willebrand factor is heterogeneously expressed in endothelium, with a predilection toward large vessel endothelium (where we observed lower chimerism). In our hands, both Ulex europaeus and CD31 afford more uniform staining of macrovascular and microvascular endothelium. Hocht-Zeisberg et al21 proposed that conventional microscopy overestimates the degree of chimerism

TABLE 5. Endothelial Cell Chimerism by Time Points in Endomyocardial Biopsy Specimens From 7 Gender-Mismatched Post–Cardiac Transplantation Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>At 1 mo, %</th>
<th>At 4 mo, %</th>
<th>At 6 mo, %</th>
<th>At 12 mo, %</th>
<th>At 5 y, %</th>
<th>At 10 y, %</th>
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<tr>
<td>1</td>
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<td>32.4</td>
<td>36.4</td>
<td>20.3</td>
<td>32.4</td>
<td>18.8</td>
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<td>3</td>
<td>NA</td>
<td>19.4</td>
<td>25.1</td>
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<tr>
<td>4</td>
<td>19.4</td>
<td>24</td>
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<td>15.9</td>
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<td>5</td>
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<td>14.6</td>
<td>37.6</td>
<td>30.5</td>
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<tr>
<td>6</td>
<td>23.6</td>
<td>32.6</td>
<td>28</td>
<td>25.1</td>
<td>28.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>17.1</td>
<td>16.9</td>
<td>28.6</td>
<td>NA</td>
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</tr>
<tr>
<td>Corrected mean*</td>
<td>21.7</td>
<td>22.9</td>
<td>30.2</td>
<td>29</td>
<td>28.9</td>
<td>19.2</td>
</tr>
<tr>
<td>SD</td>
<td>6.6</td>
<td>8.5</td>
<td>6.5</td>
<td>6.3</td>
<td>9.2</td>
<td>8.8</td>
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*All counts were corrected by the hybridization efficiency, which was determined by dividing Y chromosome–positive endothelial cells by the total nuclei counted in male control subjects. In this case, hybridization efficiency was 47.7 ±2.7%. A total of 40 406 nuclei were counted. Endocardial endothelium was excluded from this analysis.

Figure 3. Bright-field microscopic examples of early (A) and late (B) biopsy specimens using Ulex europaeus to identify endothelial cells (Vector Red chromagen). Arrows point to the Y chromosome–positive nuclei shown as dark brown, punctate nuclei stained with diaminobenzidine. Insets show magnification of Y chromosome–positive nuclei. The Y chromosome–positive nuclei not surrounded by an endothelial cell cytoplasm in the same plane were not counted. Scale =20 μm for A and B.
resulting from the overlap of cells in which a Y-positive leukocyte nucleus might be mistaken for that of a resident cell. We minimized this artifact by using strict criteria to assign nuclei to cells or in situ signals to nuclei and showed that these criteria gave data comparable to those obtained by confocal microscopy.

**Smooth Muscle Cell Chimerism in Human Transplants**

Recently, Glaser et al. reported a range of 0.8% to 5.6% recipient-derived smooth muscle cells in coronary arteries (corrected range, 2.3% to 16%) from 6 male heart transplant recipients with various degrees of transplant vasculopathy who received a female donor heart. These data are comparable to our observation of 3.4% recipient-derived smooth muscle cells derived by bright-field microscopy and 5.9% by confocal microscopy. Quaini et al. also studied smooth muscle chimerism in gender-mismatched hearts, although like their endothelial data, they counted the frequency of Y-positive arterioles (defined as containing ≥30% Y-positive smooth muscle cells) rather than Y-positive cells. They reported that 10% of arterioles were Y positive, which, although not directly comparable, seems within the range of our approach.

A recent study by Caplice et al. suggests that a source of these smooth muscle progenitor cells may be bone marrow. Studying gender-mismatched bone marrow transplant patients, they reported that 9.4% of smooth muscle cells in atherosclerotic coronary intimas were marrow derived versus 0.1% in nondiseased intimas. This relatively low chimerism in nondiseased segments after bone marrow transplantation versus relatively high chimerism (3.5%) after cardiac transplantation suggests that vessels in the allograft may be subject to injury and repair cycles not present after bone marrow transplantation. For example, ischemic injury in the peri-transplantation period or more chronic immune injury may recruit progenitor cells to participate in vascular repair.

**Schwann Cell Chimerism**

Cardiac denervation occurs after transplantation, and the consequences include diminished exercise capacity as a result of chronotropic incompetence and lack of sympathetic and parasympathetic regulation. Reinnervation has been reported in some patients, as evidenced by improvement in hemodynamic response to exercise and the presence of chest pain during activity or with cardiac biopsies. Our finding that 11.6% of Schwann cells arise from extracardiac progenitors is, to the best of our knowledge, the first structural evidence for cardiac reinnervation after transplantation. Interestingly, the Y-positive Schwann cells were typically isolated or in small cell clusters rather than extending contiguously along a fiber tract. This suggests that the Y-positive Schwann cells did not arise by direct extension from the transected host nerve fibers. Their isolated distribution is more consistent with seeding from a circulating source.

**Clinical Implication of Chimerism**

Hematopoietic chimerism has been proposed as a method for graft protection and as a method to ameliorate allograft vasculopathy. Orloff et al. created bone marrow chimeras using marrow from Lewis rats to F-344 rats and performed heterotopic heart transplantation. These animals demonstrated tolerance for 120 days after transplantation and a lack of graft vasculopathy. Control groups without marrow chimerism developed rejection despite cyclosporine and consistently developed graft vasculopathy. Similarly, Shirwan et al. induced donor-specific tolerance to cardiac allografts by injecting donor blood or T or B cells 7 days before transplantation and found graft acceptance by recipients. We hypothesize that, similar to hematopoietic chimerism, high levels of endothelial cell chimerism may have a protective effect and potentially minimize graft failure. Additional studies are required to understand the mechanism, regulation, and ultimately the long-term consequences of chimerism and assist in predicting clinical outcome. If we could learn to control endothelial chimerism, it might be possible to delay or prevent transplant arteriopathy, which is the most common cause for failure of transplanted solid organs.

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