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# Regeneration of the Infarcted Heart With Stem Cells Derived by Nuclear Transplantation

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**Abstract**—Nuclear transfer techniques have been proposed as a strategy for generating an unlimited supply of rejuvenated and histocompatible stem cells for the treatment of cardiac diseases. For this purpose, c-kit–positive fetal liver stem cells obtained from cloned embryos were injected in the border zone of infarcted mice to induce tissue reconstitution. Cloned embryos were derived from somatic cell fusion between nuclei of cultured LacZ–positive fibroblasts and enucleated oocytes of a different mouse strain. We report that regenerating myocardium replaced 38% of the scar at 1 month. The rebuilt tissue expressed LacZ and was composed of myocytes and vessels connected with the coronary circulation. Myocytes were functionally competent and expressed contractile proteins, desmin, connexin43, and N-cadherin. These structural characteristics indicated that the new myocytes were electrically and mechanically coupled. Similarly, the formed coronary arterioles and capillary structures contained blood and contributed, therefore, to tissue oxygenation. Cardiac replacement resulted in an improvement of ventricular hemodynamics and in a reduction of diastolic wall stress. These beneficial effects were obtained by stem cell transdifferentiation and commitment to the cardiac cell lineages. Myocardial growth was independent from fusion of the injected stem cells with preexisting partner cells. In conclusion, c-kit–positive stem cells derived by nuclear transfer cloning restore infarcted myocardium. Although problems currently plague nuclear transplantation, including the potential for epigenetic and imprinting abnormalities, stem cells derived from cloned embryos are sufficiently normal to repair damaged tissue in vivo. Importantly, the magnitude of myocardial regeneration obtained in this study is significantly superior to that achieved with adult bone marrow cells. (*Circ Res.* 2004;94:●●●-●●●.)

**Key Words:** fetal stem cells ■ cardiac repair ■ new coronary arterioles ■ cardiomyocytes

In the last few years, effort has been made to regenerate acutely infarcted myocardium. To restore function within the dead portion of the heart, fetal, and adult myocytes, skeletal myoblasts and bone marrow–derived immature myocytes have been implanted in the infarct or delivered to the ischemic tissue through the coronary circulation.<sup>1,2</sup> Unfortunately, these approaches failed to develop healthy myocardium integrated structurally and functionally with the unaffected region of the heart. More successful was the injection in the border zone of bone marrow cells<sup>3</sup> or the mobilization of bone marrow cells into the circulation by systemic delivery of cytokines.<sup>4</sup> Both therapeutic modalities resulted in the formation of new working myocardium and vascular structures connected with the primary coronary circulation. Recently, a resident cardiac stem cell has been identified, and after its expansion in vitro and injection in vivo, cardiac regeneration was induced after infarction.<sup>5</sup> However, these

forms of cellular therapy cannot be performed immediately after the acute event and this delay may be critical for the survival of patients. A 46% infarct results in a few days in irreversible congestive heart failure and death in humans,<sup>6,7</sup> indicating how dramatic can be the outcome of the disease and how important is the possibility of rapid intervention, myocardial regeneration, and reduction of infarct size.

An alternative strategy for the generation and storage of cells to be used for the repair of injured myocardium is offered by cloning techniques. The opportunity to reprogram the nucleus of a fully differentiated adult cell and induce the formation of an entirely different cell type constitutes a unique tool that, in the future, could lead to the development of novel treatments for cardiac diseases. Nuclear transfer–generated cells and tissues have been successfully transplanted into animals without immune rejection.<sup>8</sup> In addition, nuclear transfer has been shown to extend cell lifespan and

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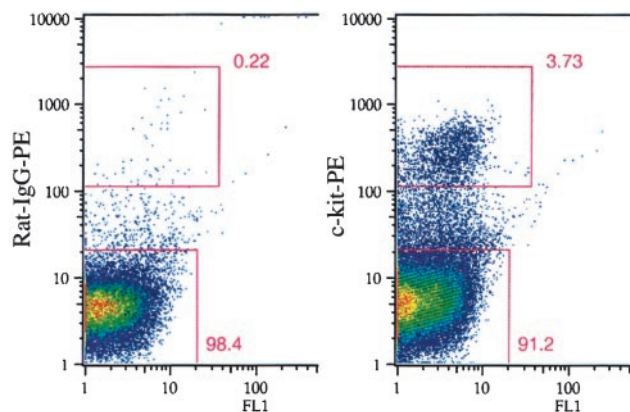
From Advanced Cell Technology (R.L., M.D.W.), Worcester, Mass; Laboratory of Developmental Hematopoiesis (M.A.S.M., J.-H.S., J.H.), Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY; Laboratory for Genomic Reprogramming (T.W.) and Laboratory of Mammalian Molecular Embryology (A.C.F.P.), RIKEN Center for Developmental Biology, Kobe, Japan; and the Cardiovascular Research Institute (A.L., S.C., A.M., D.N., J.K., P.A.), Department of Medicine, New York Medical College, Valhalla, NY.

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**Figure 1.** FACS profile of c-kit-positive cells isolated from livers of cloned embryos. Left, PE-labeled rat anti-mouse IgG (negative control). Right, PE-labeled rat anti-mouse c-kit.

telomere length in animals cloned from senescent somatic cells.<sup>9</sup> The replicative lifespan of terminally differentiated cells can be reversed by cloning, and most importantly, the production of a uniform population of cells can be obtained and its differentiation potential carefully characterized. This approach requires cloning of pluripotent primitive cells able to mature into myocytes and coronary vessels, ultimately leading to the formation of adult contracting myocardium. Bone marrow cells expressing c-kit, the receptor for the Steel/stem cell factor, might give rise to cardiomyocytes, arterioles, and capillaries after infarction as speculated but not proven in previous studies.<sup>3,4</sup> We chose to isolate c-kit-positive cells from the liver of cloned embryos, because this is the major embryonic site of hematopoiesis,<sup>10,11</sup> preceding the development of bone marrow. Cloned embryos were derived from somatic cell fusion between nuclei of cultured LacZ-positive fibroblasts and enucleated oocytes of a different mouse strain. Liver c-kit-positive cells were injected near an infarct in mice exposed to coronary artery ligation. Animals were euthanized 1 month after surgery to determine whether cardiac repair and reappearance of function occurred in the infarcted ventricle at the completion of healing.

## Materials and Methods

An expanded Materials and Methods can be found in an online data supplement at <http://circres.ahajournals.org>.

## Results

### Myocardial Infarction and Cloned c-Kit-Positive Cell Implantation

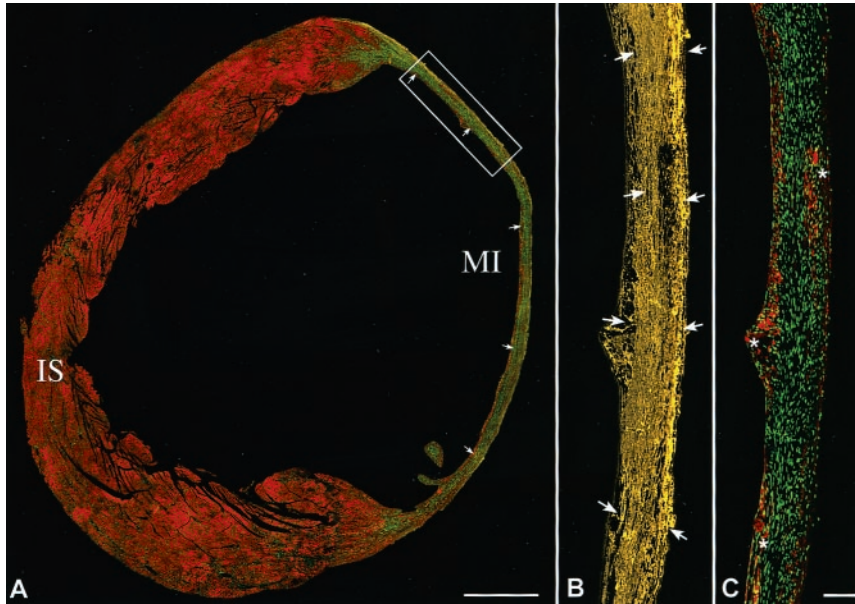
Donor cells were collected from the tail of 129/Sv-ROSA:lacZ mice and their nuclei injected into enucleated oocytes to obtain cloned embryos expressing LacZ. Subsequently, c-kit-positive cells were isolated by cell sorting from the liver of these embryos (Figure 1). Myocardial infarction was induced by occlusion of the left coronary artery in adult syngeneic 129/Sv mice. Four to six hours later, two injections of approximately 10 000 c-kit-positive cells each were made in the opposite regions of the border zone. At this interval, parenchymal and vascular cells in the myocardium supplied by the occluded coronary artery have reached irreversible

injury and death.<sup>2</sup> Control groups consisted of infarcted mice and sham-operated animals injected with physiological saline.

Infarct size was measured by the fraction of myocytes lost by the left ventricle (LV) and septum. In this manner, the number of noninfarcted myocytes provided an anatomical measurement that reflected the degree of cardiac function at euthanasia.<sup>12</sup> The dimension of the infarct was similar in the two groups of mice. Infarct size comprised  $56 \pm 5\%$  (total number of LV myocytes in sham-operated control animals,  $2.72 \pm 0.30 \times 10^6$ ; total number of LV myocytes in infarcted animals,  $1.18 \pm 0.13 \times 10^6$ ; total number of LV myocytes lost in infarcted animals,  $1.54 \pm 0.13 \times 10^6$ ) and  $54 \pm 6\%$  (total number of LV myocytes in sham-operated control animals,  $2.72 \pm 0.30 \times 10^6$ ; total number of LV myocytes in infarcted animals,  $1.24 \pm 0.15 \times 10^6$ ; total number of LV myocytes in sham-operated control animals,  $2.72 \pm 0.30 \times 10^6$ ; total number of LV myocytes in infarcted animals,  $1.18 \pm 0.13 \times 10^6$ ; total number of LV myocytes lost in infarcted animals,  $1.48 \pm 0.15 \times 10^6$ ) of myocytes in treated and untreated animals, respectively. In untreated mice, a compact scarred area substituted the infarcted myocardium at 1 month after surgery (Figures 2A through 2C). Connective tissue accumulation consisted of both collagen types I and III and comprised most of the entire left ventricular free wall. In contrast, myocardial regeneration within the infarct was detected in all mice injected with cloned c-kit-positive cells. Tissue reconstitution involved new myocytes and vessels that together constituted  $7.4 \pm 3.0 \text{ mm}^3$  of myocardium. Tissue regeneration comprised  $38 \pm 11\%$  of the infarcted scarred myocardium. The repairing band of cloned c-kit-positive cell-derived myocardium was present throughout the infarcted portion of the wall. It occupied predominantly the inner and middle layers of the damaged region of the ventricle (Figure 3A). The expression of LacZ was identified in the entire band, confirming the origin of the new myocardium from the implanted c-kit-positive cells (Figures 3B through 3D).

### Newly Formed Myocardium

The repairing myocardium was composed of myocytes ( $76 \pm 5\%$ ), coronary arterioles ( $5.4 \pm 2.6\%$ ), capillaries ( $5.7 \pm 2.9\%$ ), and other interstitium ( $12.9 \pm 3.0\%$ ). The number of arterioles and capillaries per  $\text{mm}^2$  of tissue was  $28 \pm 8$  and  $246 \pm 60$ , respectively. Because these measurements were corrected for sample orientation, they corresponded to 28 mm of arterioles and 246 mm of capillaries per  $\text{mm}^3$  of myocardium, respectively. The length density of arterioles is one of the critical factors of coronary vascular resistance and distribution of blood flow to the myocardium.<sup>13</sup> The large number of arterioles might constitute an angiogenic response attempting to decrease vascular resistance in an area supplied by an occluded coronary artery. Moreover, capillary numerical and length densities are the determinants of oxygen availability and diffusion within the tissue.<sup>12</sup> Red blood cells, which were stained by TER-119 antibody, were detected in arterioles and capillaries (Figures 4A through 4D) distributed throughout the newly formed myocardium. The endothelial lining of arterioles and capillaries was identified by factor VIII or by *Griffonia simplicifolia* lectin labeling. The latter was used

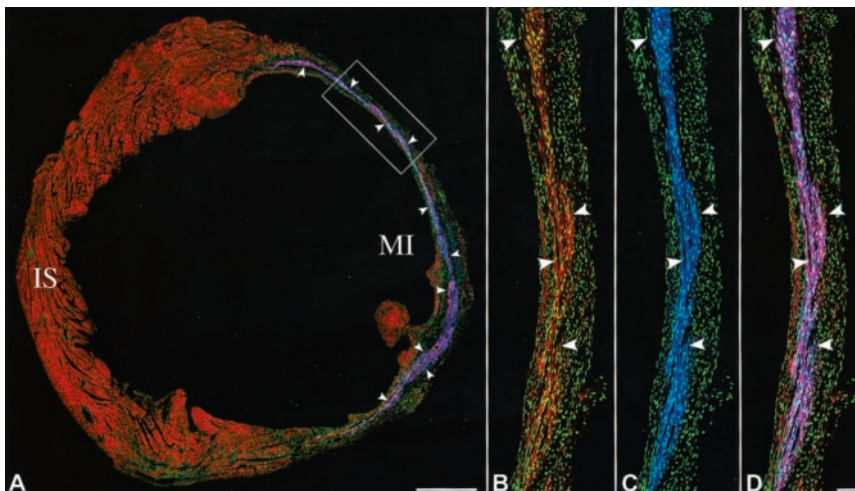


**Figure 2.** Myocardial infarction. A through C, Large infarct of the left ventricle in an untreated mouse. Area defined by a rectangle (A) is shown at higher magnification in the two adjacent panels (B and C). Accumulation of collagen type III and type I (yellow fluorescence) within the infarct (B; arrows) demonstrates the lack of myocardial regeneration. Small layer of viable myocytes (cardiac myosin, red fluorescence) is present in the subendocardium and subepicardium (C, asterisk). Nuclei are stained by PI (green fluorescence). IS indicates interventricular septum; MI, myocardial infarct. A, bar=1 mm; B and C, bars=100  $\mu$ m.

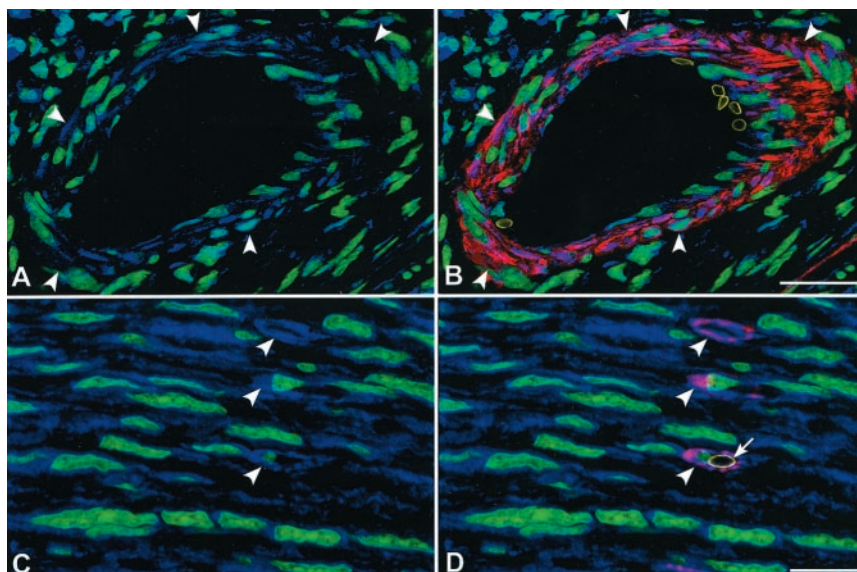
when  $\beta$ -gal was also established. The presence of red blood cells in the lumen of the coronary vasculature after perfusion fixation suggests that the forming vessels were connected with the preexisting coronary circulation. The capillary-to-myocyte ratio in the reconstituted myocardium was only 0.03. This value was markedly lower than in the adult heart where it approaches unity. In the regenerating band, there were 35 myocytes for each capillary, a condition found during late fetal and early postnatal cardiac development.<sup>12</sup>

### Forming Myocytes

Parenchymal cells within the rebuilding myocardium were small in size, cylindrical in shape, and expressed contractile proteins including cardiac myosin heavy chain, troponin I, connexin43, and N-cadherin (Figures 5A through 5J). Connexin43 is expressed at the level of the gap junctions<sup>14</sup> and N-cadherin at the level of the fascia adherens<sup>15</sup> of the intercalated discs. These new myocytes were positive for  $\alpha$ -sarcomeric actin,  $\alpha$ -actinin, and desmin; they were the



**Figure 3.** Cloned c-kit-positive cells and myocardial repair. A through D, Large infarct of the left ventricle in a mouse treated with cloned c-kit-positive cells. Area defined by a rectangle (A) is shown at higher magnification in the 3 adjacent panels (B through D). Myocardial regeneration within the infarct (B) is documented by newly formed myocytes (cardiac myosin, red fluorescence; arrowheads). Expression of  $\beta$ -gal is shown in C by the distribution of blue fluorescence in the same area (arrowheads). Combination of cardiac myosin and  $\beta$ -gal within the regenerating myocardium (D) is demonstrated by magenta fluorescence (arrowheads). IS indicates interventricular septum; MI, myocardial infarct. A, bar=1 mm; B through D, bars=100  $\mu$ m.



**Figure 4.** Vessel formation in the regenerating myocardium of treated infarcted hearts. A and B document in the thick wall a new arteriole the expression of  $\beta$ -gal (A; blue fluorescence, arrowheads) and  $\beta$ -gal and  $\alpha$ -smooth muscle actin in smooth muscle cells (B; red-magenta fluorescence, arrowheads). Erythrocytes within the lumen are stained by TER-119 (B; yellow fluorescence). C and D, Thin wall of 3 capillaries stained by  $\beta$ -gal (C; blue fluorescence, arrowheads) and by  $\beta$ -gal and *Griffonia simplicifolia* lectin and (D; magenta fluorescence, arrowheads). An erythrocyte stained by TER-119 is present in the lumen of one of the capillaries (D; yellow fluorescence, arrow). Nuclei are labeled by PI (A through D; green fluorescence). Bars=10  $\mu$ m.

progeny of cloned c-kit-positive cells because  $\beta$ -gal was identified in all cases.

Quantitatively, myocyte length, diameter, and cross-sectional area averaged  $35 \pm 4$   $\mu$ m,  $4.8 \pm 0.3$   $\mu$ m, and  $18 \pm 2$   $\mu$ m<sup>2</sup>, respectively. Myocyte volume varied from 200 to 2700  $\mu$ m<sup>3</sup> and had a mean value of 690  $\mu$ m<sup>3</sup>. Together,  $8 \times 10^6$  myocytes were accumulated within the infarcted heart in a period of 1 month (Figure 6A); myocyte regeneration exceeded by more than 5-fold the magnitude of myocytes lost with coronary occlusion. These cells were 97% smaller than adult fully matured binucleated myocytes of sham-operated control mice ( $24\,000 \pm 3400$   $\mu$ m<sup>3</sup>). Myocyte hypertrophy did not vary in the surviving myocardium of untreated and treated animals. An average 96% increase in cell size ( $47\,000 \pm 7000$   $\mu$ m<sup>3</sup>) was measured in both cases.

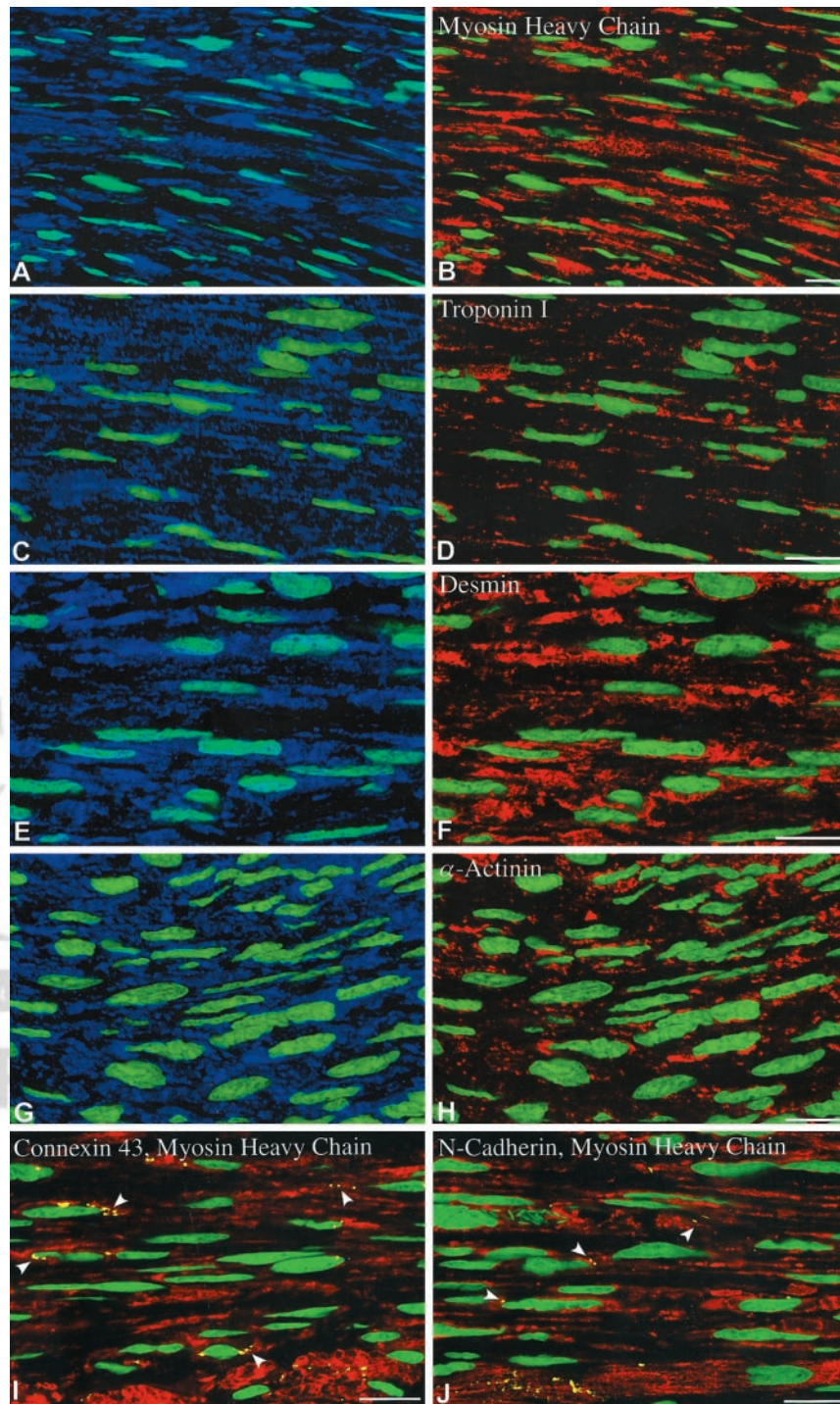
In addition to LacZ, BrdU was administered in the drinking water of treated mice throughout the period of investigation to label the forming myocytes within the infarct (Figures 6B through 6D). Moreover, the fraction of cycling myocytes was measured at euthanasia by Ki67 labeling (Figures 6E through 6G). The objective was to establish whether the level of cell replication was relatively low at 1 month, pointing to a prevailing differentiation phase of myocytes at this time. Quantitatively,  $93 \pm 3\%$  of myocyte nuclei were BrdU-positive, whereas only  $5 \pm 1\%$  expressed Ki67. These results suggest that DNA synthesis and replication were attenuated in these new myocytes at the completion of infarct healing. However, reduction in cell proliferation occurred at a stage of cardiac repair in which the formed myocytes had not reached yet the adult phenotype. Longer intervals after infarction and cell implantation will have to be examined to determine the actual long-term therapeutic potential of cloned c-kit-positive cells.

#### DNA Content in Newly Formed Myocytes

Newly formed myocytes were  $93 \pm 4\%$  mononucleated and  $7 \pm 4\%$  binucleated. Sections of 10  $\mu$ m in thickness of the regenerated myocardium were examined by confocal microscopy and the fluorescence intensity of propidium iodide labeling of myocyte nuclei completely included in the section was measured. Mouse lymphocytes were used for baseline diploid DNA content. Both preparations were stained by Ki67 to evaluate separately DNA content in cycling and noncycling cells (Figure 6H). Noncycling myocyte and lymphocyte nuclei had a 2n DNA content; greater values were restricted to cycling Ki67-positive myocyte and lymphocyte nuclei, excluding the formation of polyploidy during myocyte growth.

#### Echocardiography and Ventricular Hemodynamics

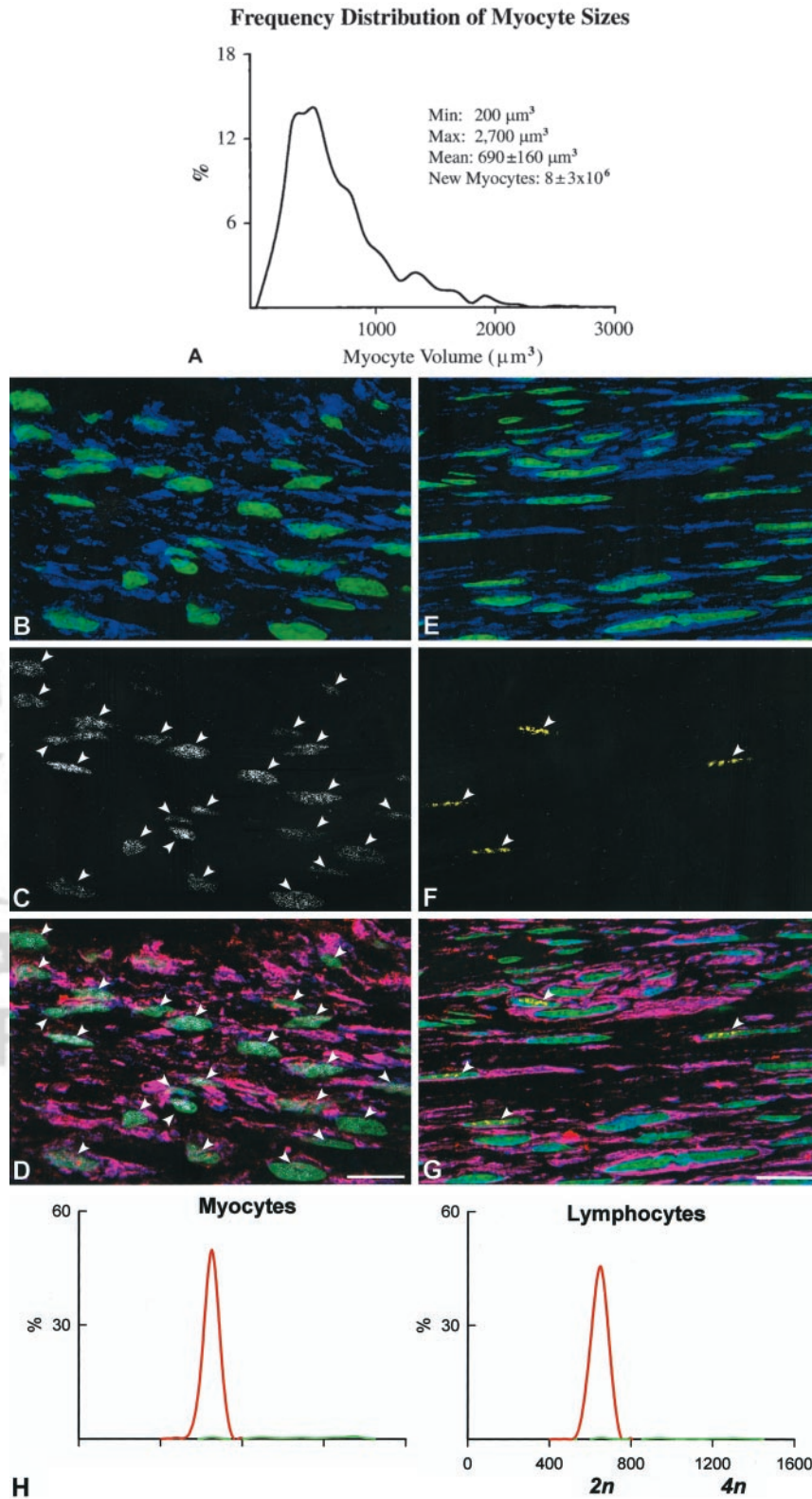
To obtain information concerning the functional competence of repairing myocytes, echocardiographic measurements were collected at 15 and 29 days after coronary occlusion in conscious unanesthetized mice.<sup>4,16,17</sup> In the absence of implantation of cloned c-kit-positive cells, contractile function was not detected in the infarcted region of the wall at either time interval. Conversely, in treated mice, wall motion was minimally detectable at 15 days but became clearly evident at 29 days (Figures 7A through 7F). Therefore, the small myocytes were electrically coupled and integrated mechanically with the remaining portion of the unaffected left ventricle. This was consistent with the expression of connexin43 and N-cadherin and the partial restoration of contractile activity within the infarcted myocardium. However, fractional shortening and EF were not statistically different in the two groups of infarcted animals (data not shown). These observations suggest that the performance of the new tissue



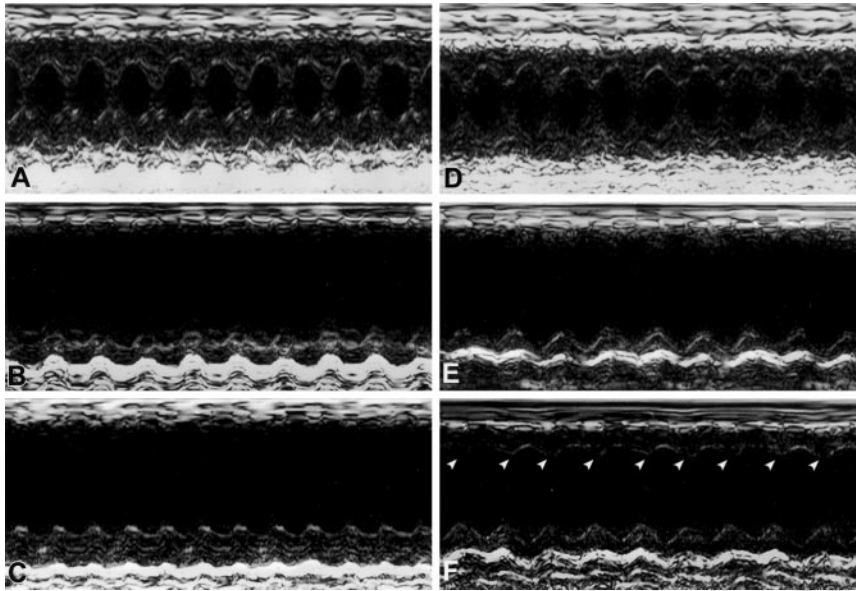
**Figure 5.** Maturing myocytes in the regenerating myocardium of treated infarcted hearts. A through H, Expression of  $\beta$ -gal (A, C, E, and G; blue fluorescence) and specific contractile proteins in new developing myocytes (B, D, F, and H; red fluorescence). I and J, Connexin43 (I; yellow fluorescence, arrowheads) and N-cadherin (J; yellow fluorescence, arrowheads) in forming myocytes (I and J; myosin heavy chain, red fluorescence). Nuclei are labeled by PI (A through J; green fluorescence). Bars=10  $\mu$ m.

was not sufficient to affect echocardiographic parameters of ventricular function in these large infarcts. Conversely, hemodynamic measurements at euthanasia showed an improvement of LV end-diastolic pressure in infarcted treated mice. Diastolic wall stress was also reduced by nearly 30% in this group (Figures 8A through 8D).

The effects of tissue reconstitution on infarct size were evaluated morphometrically. The newly formed tissue had two consequences on the infarcted heart: it increased the amount of functioning myocardium and decreased the extent of tissue loss. The combination of these factors reduced infarct size by 18%, from 56% to 46% of the LV and septum



**Figure 6.** Proliferation of myocytes in the regenerating myocardium. A, Distribution of the volumes of newly generated myocytes. B through D and E through G depict the same 2 fields. B through G, Localization of  $\beta$ -gal in the cytoplasm (B and E; blue fluorescence) and PI in nuclei (B and E; green fluorescence) of the regenerating myocardium of an infarcted treated mouse. C and F, Labeling of BrdU (C; white fluorescence, arrowheads) and Ki67 (F; yellow fluorescence, arrowheads) in myocyte nuclei. D and G, Expression of  $\beta$ -gal and cardiac myosin heavy chain in myocyte cytoplasm (D and G; magenta fluorescence) and the combination of PI and BrdU (D; green and white fluorescence) and PI and Ki67 (G; green and yellow fluorescence) in myocyte nuclei. Bars=10  $\mu\text{m}$ . H, Distribution of DNA content in noncycling (red) and cycling (Ki67-positive; green) myocyte and lymphocyte nuclei.



**Figure 7.** Myocardial repair and echocardiography. A through F, M-mode echocardiograms of untreated-infarcted (A through C) and treated-infarcted (D through F) hearts obtained at baseline (A and D) and at 15 (B and E) and 29 (C and F) days after coronary artery occlusion. Arrowheads indicate partial restoration of contractile activity within the infarcted myocardium of a mouse treated with cloned c-kit-positive cells.

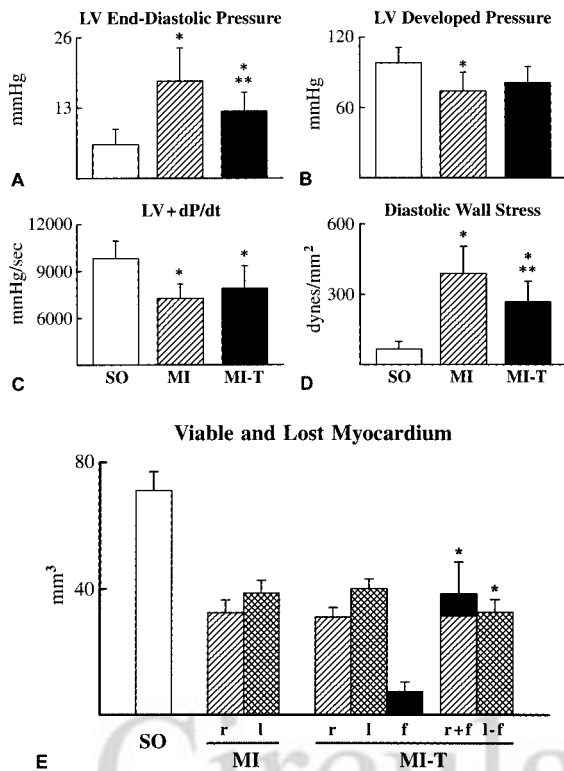
(Figure 8E). The reduction of infarct size was not sufficient to attenuate the remodeling of the postinfarcted heart. Chamber diameter, chamber volume, the wall thickness-to-chamber radius ratio, and the LV mass-to-chamber volume ratio were not statistically different from those in infarcted untreated mice (data not shown). Thus, cloned c-kit-positive cells replaced dead myocardium, ameliorating the diastolic properties and loading condition of the infarcted heart.

### Discussion

The results of the present study demonstrate that a pure population of cloned c-kit-positive cells can repair an infarcted heart. This observation has no precedent and may have important clinical implications. The ability to regenerate the replicative lifespan of cells using cloning may have therapeutic advantages. It allows the expansion of a population of cells from a single cell resulting from gene targeting. This, in turn, permits the production of uniform populations of cells with one or more specific modifications. In addition, reprogramming by nuclear transfer makes feasible the *in vitro* production of embryonic and fetal cell types, such as liver cells. Animal studies suggest that murine fetal liver stem cells can provide long-term hematopoietic repopulation of adult mice, 5-times more effectively than adult bone marrow stem cells.<sup>18,19</sup> Similarly, fetal liver stem cells from cloned cows have 10- to 100-fold selective advantage over adult hematopoietic stem cells in providing long-term hematopoietic engraftment (unpublished data, 2004). As shown in this study, a relatively small number of fetal liver stem cells regenerated a significant portion of dead myocardium. However, there are numerous problems that currently plague cloning, including the potential for epigenetic and imprinting abnormalities in replacement cells created by somatic cell nuclear transfer.<sup>20</sup>

A major question regarding therapeutic cloning is whether stem cells derived from cloned embryos are sufficiently normal to repair damaged tissue *in vivo*. The present findings support this possibility and document for the first time that fetal c-kit-positive cells replaced 38% of scarred tissue chronically after infarction. Adult c-kit-positive bone marrow cells regenerate infarcted myocardium but approximately 200 000 cells had to be injected to restore the dead tissue,<sup>3</sup> whereas only 20 000 cells have been employed here. This critical difference between the two protocols indicates that the magnitude of cardiac repair obtained with stem cells from cloned embryos was significantly superior to that achieved with adult bone marrow cells.

An additional relevant question concerns the mechanisms of tissue growth within the infarcted myocardium. Myocyte replication and reconstitution of healthy contracting ventricular muscle mass could have been the result of fusion of fetal stem cells with existing myocytes and formation of hybrid cells or the consequence of stem cell transdifferentiation and cardiac lineage commitment. These two distinct pathways of myocyte regeneration raise once more the controversial issue of cell fusion versus transdifferentiation.<sup>21–24</sup> However, several lines of evidence argue against cell fusion as the cause for the cardiac phenotype and pattern of gene expression of the injected cells found in the present study. After permanent coronary artery occlusion, all the cells in the supplied region of the myocardium die in less than 5 hours.<sup>2</sup> Essentially there are no partner cells left for fusion. Additionally, adult myocytes have an average volume of  $24\,000 \pm 3400 \mu\text{m}^3$ . If cell fusion occurred in our experimental condition, the newly generated myocytes should have a cell volume of at least  $24\,000 \mu\text{m}^3$  or larger. As illustrated in the distribution of the volume of new myocytes, these cells reached a maximum size of  $2700 \mu\text{m}^3$  and a minimum size of  $200 \mu\text{m}^3$ . If we assume



**Figure 8.** Myocardial repair and ventricular function. A through D, Hemodynamic parameters obtained before euthanasia. Results are mean  $\pm$  SD. Significant difference,  $P < 0.05$  vs SO\* and MI\*\*, respectively. E, Effects of myocardial regeneration on infarct size. On the basis of the volume of LV in sham-operated (SO) mice and infarct size in untreated mice (MI) and in treated mice (MI-T), the volume of myocardium destined to remain (r) and destined to be lost (l) after coronary ligation was computed. Volume of newly formed myocardium (f) was measured quantitatively in treated mice. Myocardial regeneration increased the volume of remaining myocardium (r+f) and decreased the volume of lost myocardium by the same amount of tissue (l-f). Results are mean  $\pm$  SD. \*Significant difference,  $P < 0.05$  vs r and l alone.

that the variability of the volume measurement of the adult ventricular mouse myocytes was three times the standard deviation of the mean value ( $3400 \times 3 = 10\,200 \mu\text{m}^3$ ), the resident cardiomyocytes would have a minimum volume of  $13\,800 \mu\text{m}^3$  ( $24\,000 \mu\text{m}^3 - 10\,200 \mu\text{m}^3$ ), which is 20-fold larger than the mean volume of the newly formed cardiomyocytes,  $690 \mu\text{m}^3$ . Also, the reconstitution of dead myocardium was characterized by the generation of 8 million new myocytes. This number is 3-fold higher than the total number of myocytes in the mouse left ventricle ( $2.7 \times 10^6$ ) and 5-fold higher than the number of myocytes lost after infarction ( $1.54 \times 10^6$ ). Donor-derived cells divide rapidly and extensively, whereas in general, tetraploid cells divide slowly and might not divide at all if one of the partners is a terminally differentiated myocyte. Moreover, 92% of resident adult mouse cardiomyocytes are binucleated and 6% are mononucleated. Conversely, 93% of new myocytes are mononucleated and only 7% binucleated. Cell fusion would imply the generation of myocytes with two nuclei, one tetraploid and the other diploid, or myocytes with three diploid nuclei. This was not the case. Finally and most importantly, regenerating

myocytes had 2n DNA content, excluding unequivocally that the process of cell fusion was implicated in cardiac repair.

An issue to be addressed concerns the structural properties of the rebuilt myocardium. The new myocytes resembled fetal-neonatal cells, which were paralleled by the formation of a proportional number of capillaries and a larger number of arterioles. This concept is based on developmental studies of the heart in rodents.<sup>12,25</sup> If this were a successful recapitulation of prenatal cardiac growth, myocytes would be expected to increase rapidly in size and, over a period of nearly 2 months, reach the adult phenotype. The prevailing hypertrophic growth of myocytes should be accompanied by an intense proliferative response of the capillary microvasculature to preserve oxygen availability, diffusion, and transport in the enlarged heart.<sup>25</sup> This phenomenon has been carefully defined in rodents and, in part, in humans.<sup>12,25</sup> However, it remains an unresolved problem in our experimental conditions. The excessive number of arterioles might have had significant implications in the restoration of blood flow in the infarcted area. Coronary vascular resistance is inversely correlated with the number of arterioles,<sup>13</sup> suggesting that the excessive growth of coronary vessels could have been required to connect the regenerating vasculature with the primary unaffected coronary circulation. Thus, the system needs to be perfected and the number of cells necessary to optimize myocardial reconstitution has to be determined. Importantly, more work has to be done to identify the long-term outcome of this form of cardiac repair.

The cell population within the c-kit–positive subset of fetal liver cells is, like its adult marrow counterpart, heterogeneous. The c-kit receptor is present on hematopoietic stem and progenitor cells and is critical for their function as revealed by the hematopoietic defects seen in loss of function mutations at the mouse *c-kit*/white spotting locus.<sup>26</sup> Endothelial stem cells in fetal liver and adult bone marrow that can participate in neovascularization are also c-kit–positive.<sup>27</sup> Expression of c-kit on mesenchymal stem cells in marrow or fetal liver has been reported in some but not all studies.<sup>28,29</sup> Bone marrow–derived human mesenchymal stem cells have been shown to differentiate into a cardiomyocyte phenotype when injected into the adult murine heart.<sup>30</sup> A more restricted mesodermal stem cell, which was a common precursor of both mesenchymal stem cells and hematopoietic stem cells, has recently been isolated by c-kit sorting of early embryo yolk sac and differentiated embryonic stem cells.<sup>31</sup> The fetal liver also contains hepatic stem cells or oval cell that are c-kit–positive and normally differentiate into hepatocytes and bile epithelial cells,<sup>32</sup> but differentiation into pancreatic endocrine tissue has been reported.<sup>33</sup> The pluripotent mesenchymal stem cell identified by Verfaillie and collaborators<sup>28</sup> is c-kit–negative and is capable of cardiomyocyte differentiation only when injected in the blastocyst.

In spite of these limitations and the caution that has to be exercised with this type of approach, it is remarkable that c-kit–positive cloned cells were capable of reconstituting healthy myocardium with all its components. Transplanted embryonic stem cells can differentiate into a variety of tissues giving rise to cellular aggregates that do not always reproduce the organized structure of an organ or tissue.<sup>34</sup> It is reasonable

to postulate that cellular and/or extracellular factors produced in the stressed myocardium of the border zone or in the hostile environment of the ischemic area have been able to direct and restrict the developmental potential of the implanted pluripotent cells. The cardiac repair obtained here via nuclear transfer cloning represents, therefore, a model system that can be used for the identification of the processes regulating growth and differentiation of myocytes, endothelial cells, and smooth muscle cells in the heart.

The present observations extend the early results of myocardial tissue formation in vitro by nuclear transfer cloning technique<sup>8</sup> and provide an in vivo demonstration of the effectiveness of this procedure. Somatic cell nuclear transfer offers a clear advantage to the direct use of embryonic stem cell-derived tissues.<sup>35</sup> The latter promotes an immune response with the inevitable consequence of graft rejection in the absence of immunosuppressive therapy.<sup>36</sup> However, the approach used in this study cannot be applied clinically because the cells were obtained from fetuses and ethical principles require that preimplantation embryos not be allowed to grow beyond the blastocyst stage.<sup>37,38</sup>

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