

Adult Cardiac Stem Cells Are Multipotent and Support Myocardial Regeneration

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Summary

The notion of the adult heart as terminally differentiated organ without self-renewal potential has been undermined by the existence of a subpopulation of replicating myocytes in normal and pathological states. The origin and significance of these cells has remained obscure for lack of a proper biological context. We report the existence of Lin⁻ c-kit^{POS} cells with the properties of cardiac stem cells. They are self-renewing, clonogenic, and multipotent, giving rise to myocytes, smooth muscle, and endothelial cells. When injected into an ischemic heart, these cells or their clonal progeny reconstitute well-differentiated myocardium, formed by blood-carrying new vessels and myocytes with the characteristics of young cells, encompassing ~70% of the ventricle. Thus, the adult heart, like the brain, is mainly composed of terminally differentiated cells, but is not a terminally differentiated organ because it contains stem cells supporting its regeneration. The existence of these cells opens new opportunities for myocardial repair.

Introduction

Until recently, the accepted paradigm in cardiac biology considered the adult mammalian heart to be a postmitotic organ without regenerative capacity. It has been assumed that from shortly after birth to adulthood and senescence the heart has a relatively stable but slowly diminishing number of myocytes. This static view of the myocardium implied that both myocyte death and myocyte regeneration had little role in cardiac cellular homeostasis. Although stem cells have been isolated from many adult tissues including the blood, skin, central nervous system, liver, gastrointestinal tract, and skeletal muscle (see Rosenthal, 2003), the search for a cardiac stem cell has been considered futile given the accepted lack of regenerative potential of this tissue.

Evidence challenging the accepted wisdom has been slowly accumulating (McDonnell and Oberpriller, 1984; Romyantsev and Broisov, 1987). In the past few years, we have documented the existence of cycling ventricular myocytes in the normal and pathologic adult mammalian heart of several species, including humans (Kajstura et al., 1998; Beltrami et al., 2001; Quaini et al., 2002). Although these data provided an alternative view of cardiac homeostasis, they also raised questions because it required reconciliation of two apparent contradictory bodies of evidence: the well-documented irreversible withdrawal of cardiac myocytes from the cell cycle soon after birth on one hand (MacLellan and Schneider, 2000; Chien and Olson, 2002), and the presence of cycling myocytes undergoing mitosis and cytokinesis on the other. These results raised the question as to the origin of the cycling myocytes and their dramatic increase in response to an acute work overload.

In cases of sex-mismatched cardiac transplants in humans, the female hearts in the male hosts had a significant number of Y positive myocytes and coronary vessels (Quaini et al., 2002). Most likely due to technical differences (Anversa and Nadal-Ginard, 2002a), there are some discrepancies among groups about the degree of cardiac chimerism (Muller et al., 2002; Glaser et al., 2002; Laflamme et al., 2002). It is likely that these male cells colonized the female heart after the transplant and subsequently differentiated, although alternative explanations have been raised. These male cells in the female heart presuppose the existence of mobile stem-like cells able to differentiate into the three main cardiac cell types: myocytes, smooth, and endothelial vascular cells.

Primitive cells of donor and recipient origin that express stem cell-related surface antigens—c-kit, Sca-1, and MDR1—were identified in the recipient hearts. More importantly, identical cells were found in human control hearts (Quaini et al., 2002; Anversa and Nadal-Ginard, 2002b). It is well known that in early fetal life, c-kit^{POS} cells colonize the yolk sack, liver, and probably other organs. The colonized organs express stem cell factor (SCF), the ligand of the c-kit receptor (Teyssier-Le Discorde et al., 1999); SCF mRNA is also present in fetal and neonatal myocardium (Kunisada et al., 1998), raising the possibility that stem-like cells could have been in the heart from fetal life. The rapid induction of SCF during myocardial ischemia (Frangogiannis et al., 1998) could be involved in the activation of these cells and explain the significant increase in new myocyte formation (Beltrami et al., 2001). However, the origin of these primitive cells, their presence in normal and pathological hearts, together with the identification of some of them having initiated the cardiomyocyte gene expression program, is suggestive that they might be true cardiac stem cells that give rise to the cycling myocytes detected in the adult heart. If this were the case, their manipulation might provide the opportunity to stimulate myocardial regeneration with endogenous cells. For this reason, we endeavored to establish a precursor-product relationship between these primitive cells and the fully differenti-

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ated cardiac cells and to determine, in vitro and in vivo, whether they behave like true adult cardiac stem cells.

Results

Cells with the Characteristics of Myogenic Stem/Progenitor Cells Are Present in the Adult Myocardium

To determine whether the putative cardiac stem cells detected in human heart transplants and their controls are bona fide stem cells with cardiogenic potential, we isolated them to test their differentiation potential in vivo and in vitro. For experimental convenience, we chose the rat as the animal model system. We first analyzed whether cells with the cell surface markers commonly expressed by other stem cells could be identified in the adult rat myocardium. Based on the postulated higher number of proliferating stem and precursor cells with age (Morrison et al., 1996), we analyzed the myocardium from older animals. Histological sections of myocardium from Fisher rats 20–23 months of age were examined by confocal microscopy for the presence of cells negative for the expression of blood lineage markers (Lin^-) but positive for the common stem cell markers c-kit (Kondo et al., 2003), Sca-1 (Morrison et al., 1997), and MDR-1 (Sellers et al., 2001). Small Lin^- cells with a very high nucleus/cytoplasm ratio and positive for each of the above markers were distributed throughout the ventricular and atrial myocardium with a higher density in the atria and the ventricular apex. Because of the role of bone marrow-derived Lin^- c-kit^{POS} cells in myocardial regeneration (Orlic et al., 2001), the mesodermal origin of both the heart and the bone marrow, and the use of c-kit as a hematopoietic stem cell marker (Morrison et al., 1997; Weissman et al., 2001; Kondo et al., 2003), we decided to concentrate on the cardiac cells expressing this marker, the receptor for SCF. Although the density of these cells varied among different regions of the heart, on average we identified one Lin^- c-kit^{POS} cell every $\sim 1 \times 10^4$ myocytes. It should be noted that most, if not all, of the detected c-kit^{POS} cells were negative for the pan leukocyte marker CD45 and the endothelial/hematopoietic progenitor marker CD34.

As shown in Figures 1A and 1B, the Lin^- c-kit^{POS} cells are found in small clusters in the intersticia between well-differentiated myocytes and have an appearance compatible with the clonal expansion of an activated primitive cell. Accordingly, many of these cells express Ki67 (not shown), a marker for cells that either are or have recently been in the cell cycle (Scholzen and Gerdes, 2000). Many of these clusters contain cells at several early stages of cardiac myogenic differentiation, as demonstrated by the expression of the transcription factors GATA4, Nkx2.5, and MEF2, together with small amounts of sarcomeric proteins in the cytoplasm.

This phenotype strongly suggests that these cell clusters represent amplifying myogenic precursors and/or progenitors derived from the activation of a more-primitive stem cell. The expression of transcription factors associated with early cardiac development, such as GATA4 (Molkentin et al., 1997) and Nkx2.5 (Kasahara et al., 1998), and particularly, the expression of cardiac-specific sarcomeric proteins by some of the cells in each

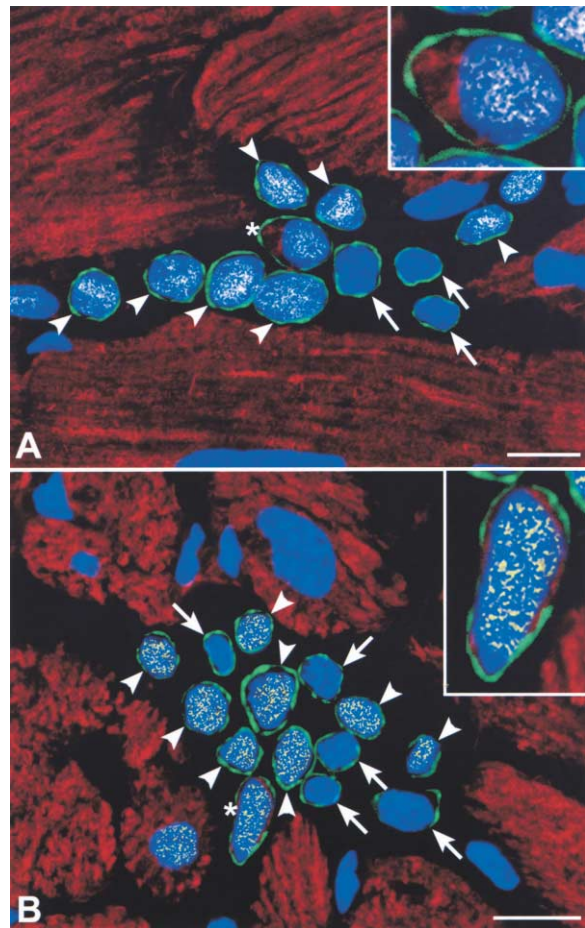


Figure 1. Clusters of Primitive and Early Committed Cells in the Heart

(A) Cluster of 11 c-kit^{POS} cells (green) with three expressing c-kit only (arrows), seven expressing Nkx2.5 (white dots; arrowheads) in nuclei (blue, propidium iodide, PI), and 1 Nkx2.5 and α -sarcomeric actin in the cytoplasm (red; asterisk, see inset).

(B) Cluster of 15 c-kit^{POS} cells with five c-kit^{POS} cells only (arrows), eight expressing MEF2C (yellow dots; arrowheads), and one expressing MEF2C and α -sarcomeric actin (asterisk, see inset). Bars, 10 μm .

cluster (see Figure 1), is strong evidence in support of their cardiac myogenic potential and, most likely, of their cardiac myogenic fate. That cells with similar characteristics have also been identified in the mouse, dog, and pig (P.A., unpublished data), in addition to the human (Quaini et al., 2002; Urbanek et al., 2003), is further indication of their likely biological significance.

Isolation and Characterization of Lin^- c-kit^{POS} Cells from the Adult Myocardium

Cardiac cells were isolated from female Fischer rats at 20–25 months of age. Small cells, containing most of the Lin^- c-kit^{POS} population, were separated from the differentiated myocytes by differential centrifugation and incubated with an anti-c-kit antibody, which recognizes the N-terminal epitope located at the external aspect of the membrane (Broudy, 1997). The c-kit^{POS} cells were separated either by repeated panning, using immu-

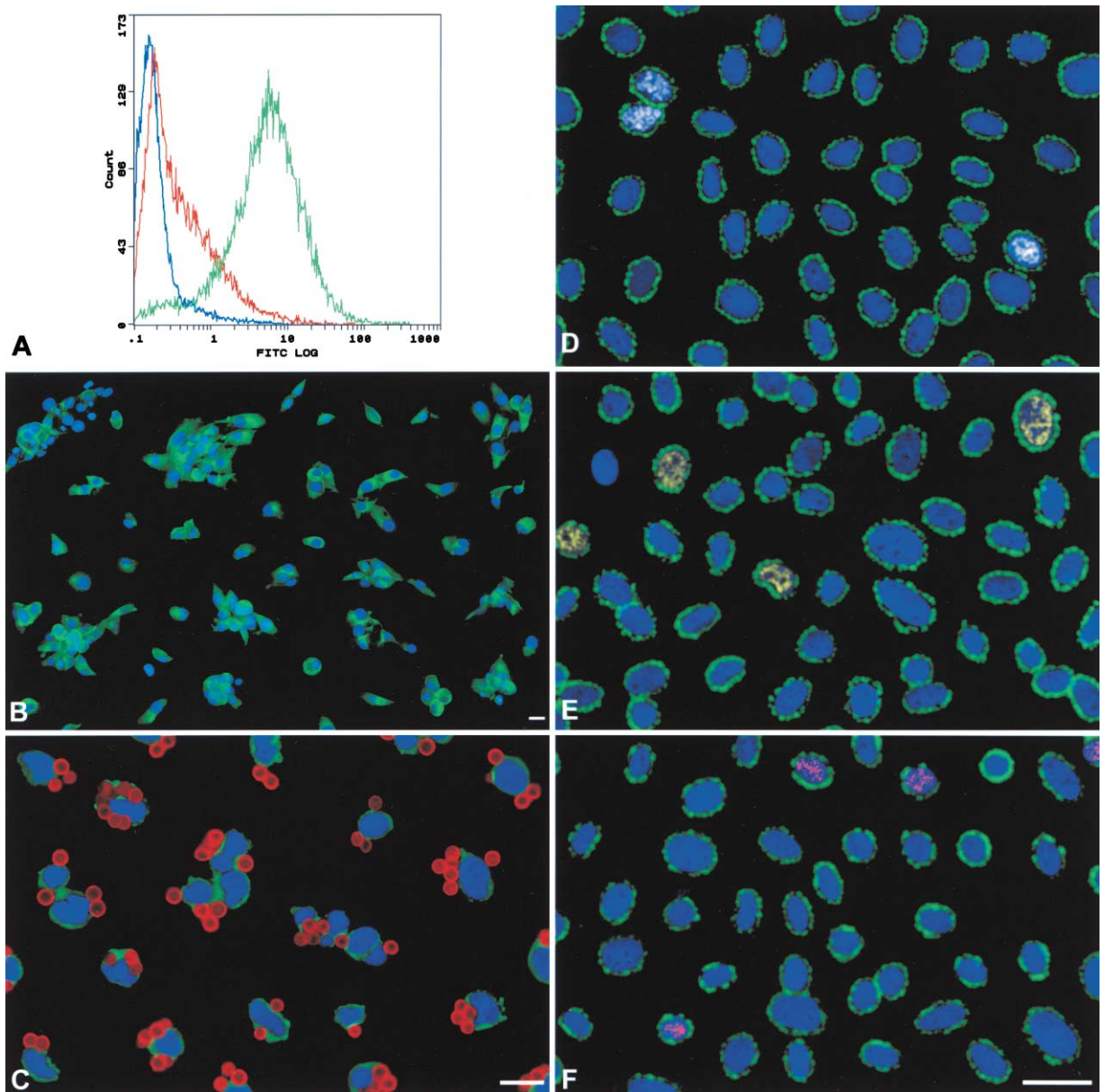


Figure 2. FACS Analysis and Culture of $\text{Lin}^- \text{c-kit}^{\text{POS}}$ Cells

(A) Profiles of cells labeled with c-kit (red), secondary-antibody only (blue), and beads-c-kit (green). $\text{c-kit}^{\text{POS}}$ cells were $7\% \pm 3\%$ of purified myocardial small cells.

(B) $\text{c-kit}^{\text{POS}}$ cells isolated by FACS cultured in mNSCM: c-kit, green; PI, blue.

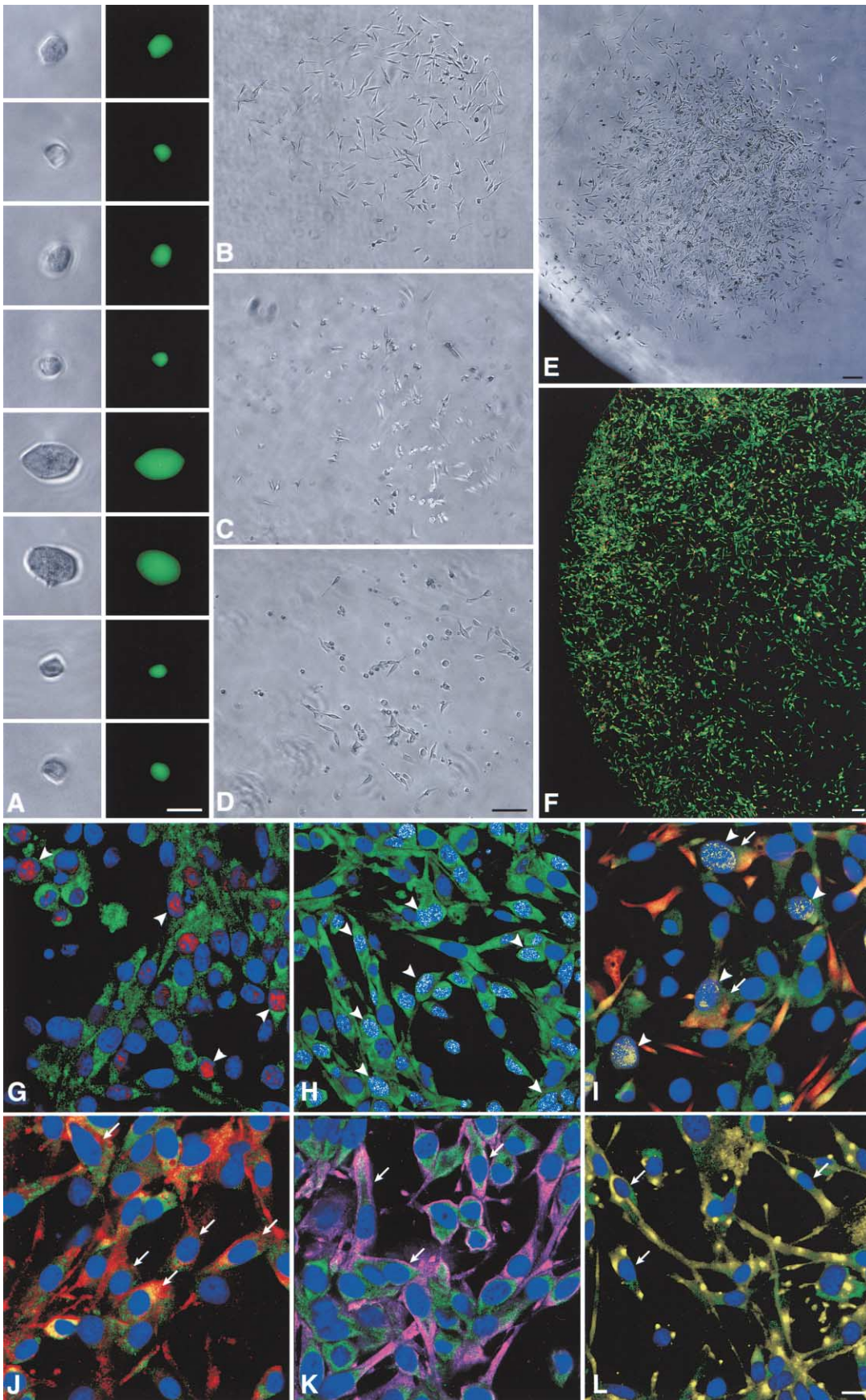
(C) Freshly isolated $\text{c-kit}^{\text{POS}}$ cells with adherent beads. c-kit, green; beads, red; PI, blue.

(D–F) Smears of purified $\text{c-kit}^{\text{POS}}$ cells (green) expressing Nkx2.5 (D), white), MEF2C (E), yellow), and GATA-4 (F), magenta). PI, blue. Bars, $10 \mu\text{m}$.

nomagnetic microbeads or by FACS sorting (see Experimental Procedures). There was no appreciable difference between the cells isolated by these two methods, each resulting in $\sim 90\%$ $\text{c-kit}^{\text{POS}}$ cells (Figures 2A–2C).

With few exceptions, freshly isolated $\text{c-kit}^{\text{POS}}$ cells scored negative for myocyte (α -sarcomeric actin, cardiac myosin, desmin, α -cardiac actinin, and connexin 43), endothelial cell (EC; von Willebrand factor, CD31, and vimentin), smooth muscle cell (SMC; α -smooth muscle actin and desmin), and fibroblast cytoplasmic proteins (F; fibronectin, procollagen I, and vimentin). How-

ever, transcription factors expressed early in the myocyte lineage (Nkx2.5, GATA-4, and MEF2) were each detected in 7%–10% of the freshly isolated $\text{c-kit}^{\text{POS}}$ cells (Figures 2D–2F), with occasional ($<0.5\%$) $\text{c-kit}^{\text{POS}}$ cells expressing sarcomeric proteins, as would be expected from their phenotype shown in Figure 1. These results further document that the $\text{c-kit}^{\text{POS}}$ cell population is heterogeneous as to its stage of differentiation and that some of them are already committed to the cardiac myogenic lineage. Skeletal muscle transcription factors (MyoD, myogenin, and Myf5) were not detected in this



population either by immunohistochemistry or Western blot analysis (see Supplemental Figures S1A–S1F online at <http://www.cell.com/cgi/content/full/114/6/763/DC1>). It should be noted that, despite that isolation of these cells was based exclusively on c-kit expression with no negative selection for the blood cell lineages, the cells were uniformly negative for markers of the myeloid, lymphoid, and erythroid lineages (CD34, CD45, CD20, CD45RO, CD8, and TER-119) (Kondo et al., 2003).

The phenotype of the cardiac c-kit^{POS} cells raises the question of their origin. Clearly, they are different from endothelial progenitor cells (EPCs) characterized from different species. EPCs, in addition to expressing c-kit, are also positive for CD34, while the cells isolated from the myocardium are CD34 negative. The data available, however, does not distinguish between intrinsic cardiac cells present in the myocardium since fetal life and cells of extracardiac origin, perhaps from the bone marrow, which have homed to the myocardium through the circulation. CD45 expression has been considered as a marker of hematopoietic origin for even the most primitive stem cells (Nadin et al., 2003). Because most c-kit^{POS} cells of bone marrow origin are also positive for CD45 and other blood lineage markers, the cells isolated here either represent a different subpopulation or they have resided in the myocardium long enough to have lost the epitopes of the blood cell lineages.

The Cardiac c-kit^{POS} Cells Are Self-Renewing, Clonogenic, and Multipotent, Giving Rise to a Minimum of Three Different Cardiogenic Cell Lineages

Freshly isolated c-kit^{POS} cells were plated in enriched F12K medium (see Experimental Procedures) for 2–3 days, when most of the contaminating c-kit^{NEG} cells attached to the plastic while the c-kit^{POS} remained unattached. The nonattached cells were transferred to modified neural stem cell medium (mNSCM) lacking neurogenic factors (Tropepe et al., 1999). After 7–10 days in mNSCM, most cells attached and were continuously grown for more than 1 year at subconfluent densities. Under these conditions, the cells maintained a stable phenotype, most remained c-kit^{POS}, and had a doubling time of ~40 hr. Cycling cells in culture, as determined by the expression of Ki67, ranged from 74% ± 12% to 84% ± 8% at passages (P)1–P5 and remained 72% at P23. Cells have continued to proliferate until the present without reaching growth arrest or senescence more than 19 months after their isolation, as indicated by the diploid chromosomal complement of >90% of the cells. Cells frozen at P23 and thawed out 6 and 12 months later have retained the characteristics of the original population.

To test the differentiation characteristics of these cells in vitro, subconfluent cultures at different passages ranging from P0–P23 were placed in differentiation medium (DM). 7–10 days later, many of the cells had adopted a flatter morphology, and most of them exhibited signs of biochemical differentiation into the myogenic, SMC, or EC lineage (see Experimental Procedures; Supplemental Figures S2A–S2D). At P0 (n = 7), P3 (n = 10), P10 (n = 13), and P23 (n = 13), myocytes were 29%–40%, EC 20%–26%, SMC 18%–23%, and F represented 9%–16%. At P0 and P1, when grown in DM, 30% of the cells had detectable GATA-4 in the nucleus and 55% expressed GATA-5; 50% were positive for Nkx2.5 and 60% for MEF2. Conversely, skeletal muscle (MyoD, myogenin, and Myf5), blood cell lineage (CD45, CD20, CD45RO, CD8, and snf TER-119) and neural (MAP1b, neurofilament 200, and snf GFAP) markers were not detectable (Supplemental Figures S3A–S3F). Cells tested at later passages (P19 and P26) exhibited similar differentiation profiles.

Despite the clear evidence of biochemical differentiation in the main myocardial cell types, the phenotype of the in vitro differentiated c-kit^{POS} cells was morphologically and functionally immature. Myocytes had a disorganized structure with no identifiable sarcomeres. Myocytes and SMC failed to contract spontaneously and in response to angiotensin II, isoproterenol, norepinephrine, and electrical stimulation. EC did not express eNOS. This spontaneous biochemical differentiation in vitro falls short from progressing to a recognizable morphological and functional differentiated phenotype.

Despite the aborted differentiated phenotype, the very high frequency of expression of biochemical markers specific for each of the three main myocardial cell types, at each passage tested, is a strong indication of their self-renewal potential but does not address the issue of their apparent multipotentiality. The data cannot distinguish between the presence of a multipotent precursor cell able to give rise to different cell types and a mixed culture containing two or more precursors, each differentiating into one or two cell lineages. A clonal analysis can distinguish between these alternatives.

To test the growth and differentiation potential of single cells, clones were isolated by two different methods. For cloning by the dilution plating technique, c-kit^{POS} cells were first separated from the bulk of small cells of the dissociated myocardium by immunomagnetic beads and/or by FACS (see Experimental Procedures). The purified population was seeded in 100 mm culture dishes at a density of 100 cells per plate in mNSCM (Tropepe et al., 1999). After 2–3 weeks, 156 small clones were obtained from a total of ~2,000 single cells. Fibronectin, procollagen I, and vimentin were absent in 30 tested clones, excluding fibroblast contamination (Supplemen-

Figure 3. Cloning, Growth, and Differentiation of c-kit^{POS} Cells

(A) Wells of a Terasaki plate with one sorted c-kit^{POS} cell (green) in each well. Left, phase contrast; right, epifluorescence.

(B–E) Clones in mNSCM shown by phase contrast.

(F) c-kit^{POS} clone (green). A few c-kit negative cells are visible.

(G–L) c-kit^{POS} cells (green) in DM express GATA-4 ([G], magenta, arrowheads; 45% ± 10%), Nkx2.5 ([H], white, arrowheads; 39% ± 8%), MEF2C ([I], yellow, arrowheads; 36% ± 6%) and α -sarcomeric-actin ([J], red, arrows; 27% ± 6%), cardiac myosin ([J], orange, arrows; 29% ± 7%), α -smooth-muscle-actin ([K], magenta, arrows; 21% ± 4%), and von Willebrand factor ([L], yellow, arrows; 17 ± 4%). Bars: 100 μ m in (B)–(F) and 10 μ m in (A) and (G)–(L).

tal Figures S4A–S4F). These clones were also negative for cytoplasmic markers of myocytes, SMC, and EC. Of 35 clones expanded in mNSCM, 31 grew successfully and were expanded to mass cultures.

A single, freshly isolated $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cell was placed in each well of a 96-well Terasaki plate in mNSCM using a cell sorter (see Experimental Procedures). From two separate platings of 5×10^3 cells each, not a single expandable clone was isolated. For this reason, freshly isolated $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells were first allowed to recover for 7–10 days in F12K enriched medium and for an additional week in mNSCM. Cells were then sorted as above. Over 90% of the individual cells in the wells were $\text{c-kit}^{\text{POS}}$ (Figure 3A) without nuclear or cytoplasmic signs of differentiation (not shown; see Experimental Procedures). Five different cell isolations from five rats were used to seed 1536 wells. 2–3 weeks later, 54 clones were collected. 25 of these clones, fixed and analyzed by immunocytochemistry, expressed c-kit (Figures 3B–3F) and were negative for nuclear and cytoplasmic markers of biochemical differentiation (Experimental Procedures). Of the remaining 29 clones, 12 did not grow, and the rest were expanded and frozen. All the clones analyzed were undifferentiated without lineage-committed cells.

Individual clones from both preparations (automated cloning = 17, dilution cloning = 31) were characterized. In DM, 76% of the clones developed cells expressing cytoplasmic markers specific for the cardiac lineages as well as $\text{c-kit}^{\text{POS}}$ cells expressing GATA-4, Nkx2.5, or MEF2. Aggregates of small, spindle-shaped cells containing nestin alone or nestin and other structural proteins were also present. c-kit was detected in early differentiating myocytes, EC, and SMC (Figures 3G–3L). Myocytes, SMC, and EC with a more advanced phenotype were occasionally identified.

Cloned cells in mNSCM grew in suspension in bacteriological dishes and generated spherical clones (Figure 4A) similarly to other stem cells (Suslov et al., 2002). Spheroids consisted of clusters of $\text{c-kit}^{\text{POS}}$ and $\text{c-kit}^{\text{NEG}}$ cells surrounded by large amounts of nestin (Figures 4B–4D). They readily attached when transferred to culture dishes and grown in DM; cells spread on the dish and differentiated (Figures 4E–4H).

Subclones of primary clones confirmed the stability of the phenotype and further demonstrated their clonogenicity, self-renewal, and multipotentiality. The phenotype of most subclones was indistinguishable from that of the primary clones and produced myocytes, SMC, and EC. In the cases tested, >80% of the cells had a diploid karyotype. However, 2 of 14 subclones (automated cloning = six; dilution cloning = eight) generated only myocytes and one only EC.

These results indicate that the $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells isolated from the adult myocardium are self-renewing, clonogenic, and multipotent. The progeny of single cells gives rise to the main myocardial cell types: myocytes, SMC, and EC (Supplemental Figures S5A–S5D). Thus, these cells have the in vitro properties expected from multipotent cardiac progenitor cells. However, given the heterogeneity of the starting cell sample and the paucity of cell markers discriminating among developmental stages of the myogenic pathway, it is not possible to determine whether these cells are true stem cells or

multipotent cells that have moved further along in the differentiation pathway.

The Cardiac-Derived $\text{Lin}^{\text{NEG}} \text{c-kit}^{\text{POS}}$ Cells Regenerate Functional Myocardium In Vivo

The abortive differentiation in vitro of the isolated cells, their clones, and subclones suggested that DM was deficient in factors required for complete differentiation. To determine whether these cells were capable of acquiring functional competence when properly stimulated, we tested their ability to fully differentiate and reconstitute the myocardium when injected into the hearts of syngeneic rats acutely after myocardial infarction. To this end, 1×10^5 cells at P2, grown in DM supplemented with 1 mg/ml of BrdU replenished daily for 6 days, were injected at each of two sites of the border of a 5-hr-old infarct in two groups of animals. Similarly, infarcted animals injected with equal volumes of PBS and sham-operated animals injected with the same number of cells as the experimental ones were used as controls.

Infarct size was determined at the time of sacrifice by comparing the number of spared myocytes in the left ventricle (LV) of control and experimental animals with those of the sham-operated animals in the same group, as determined by morphometry (see Experimental Procedures). Infarct size was $53\% \pm 7\%$ and $49\% \pm 10\%$ (NS) of the LV in treated and untreated rats in the group followed for 10 days, and $70\% \pm 9\%$ and $55\% \pm 10\%$ ($p < 0.001$) in treated and untreated rats in the 20 days group, respectively. The differences in infarct size between control and experimental group is due, at least in part, to the survival of animals with larger infarcts in the treated as compared to the control groups.

A band of BrdU-labeled regenerating myocardium was found in 9 of 12 treated infarcts at 10 days and in all 10 treated infarcts at 20 days. At the early time point, the regenerating band was thin and incompletely covered the infarcted area but was thicker and present throughout the infarct at 20 days (Figures 5A–5F). The total volume of the new myocardium generated at 10 and 20 days was 30 and 48 mm^3 , respectively. This regeneration had reduced infarct size from $53\% \pm 7\%$ to $40\% \pm 5\%$ ($p < 0.001$) in the 10 days group, and from $70\% \pm 9\%$ to $48\% \pm 7\%$ ($p < 0.001$) in the 20 days group. At 10 days, the proportion of myocytes, capillaries, and arterioles was lower, and collagen was higher than at 20. Cell growth evaluated by the fraction of BrdU-positive cells expressing Ki67 was greater at 10 than at 20 days.

The regenerated myocardium was constituted by BrdU-positive small myocytes, capillaries, and arterioles that appeared to mature over time. The myocytes expressed cardiac myosin heavy chain, α -sarcomeric actin, α -cardiac actinin, N-cadherin, and connexin 43 (Figures 5D and 5E). At 10 days, the regenerating band contained $14 \pm 2.5 \times 10^6$ myocytes with a volume of $1500 \pm 150 \mu\text{m}^3$, while at 20 days the band had $13 \pm 3 \times 10^6$ larger myocytes with an average volume of $3,400 \pm 560 \mu\text{m}^3$, as compared to the average volume of 20,000–25,000 μm^3 for adult rat myocytes. While at 10 days sarcomeres were rarely detectable in the small myocytes, at 20 days these cells were closely packed

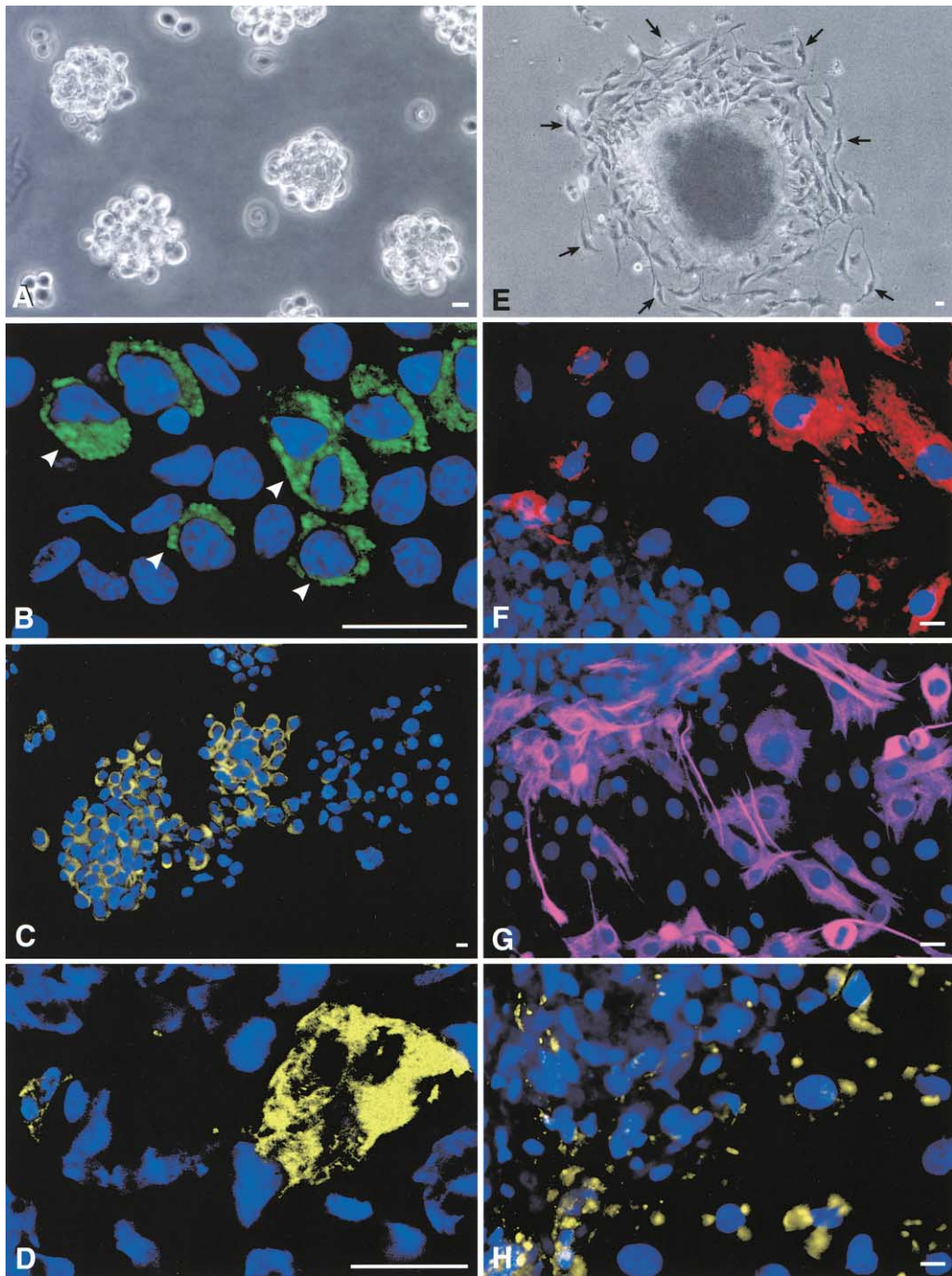


Figure 4. Proliferation and Differentiation of Clonogenic Cells in Spherical Clones

- (A) Spherical clones in cell suspension.
- (B) c-kit^{POS} cells (green, arrowheads) within the spheroid.
- (C) Cell clusters in the spheroid surrounded by nestin (yellow).
- (D) Accumulation of nestin (yellow) in spheroids.
- (E) Cells in DM migrate out of the spheroid.
- (F) Myocytes express cardiac myosin (red), (G) SMC, α -smooth-muscle-actin (magenta), (H) EC, von Willebrand factor (yellow). Bars: 10 μ m in (A)–(H).

with more abundant myofibrils and visible striations; N-cadherin and connexin 43, which define the fascia adherens and nexuses in intercalated discs, were well developed at the two time points. The degree of amplifi-

cation of the injected cells measured here requires a minimum of six to nine cell cycles, depending on their survival rate at the injection site. The high frequency of BrdU signal in the regenerating cells is in agreement

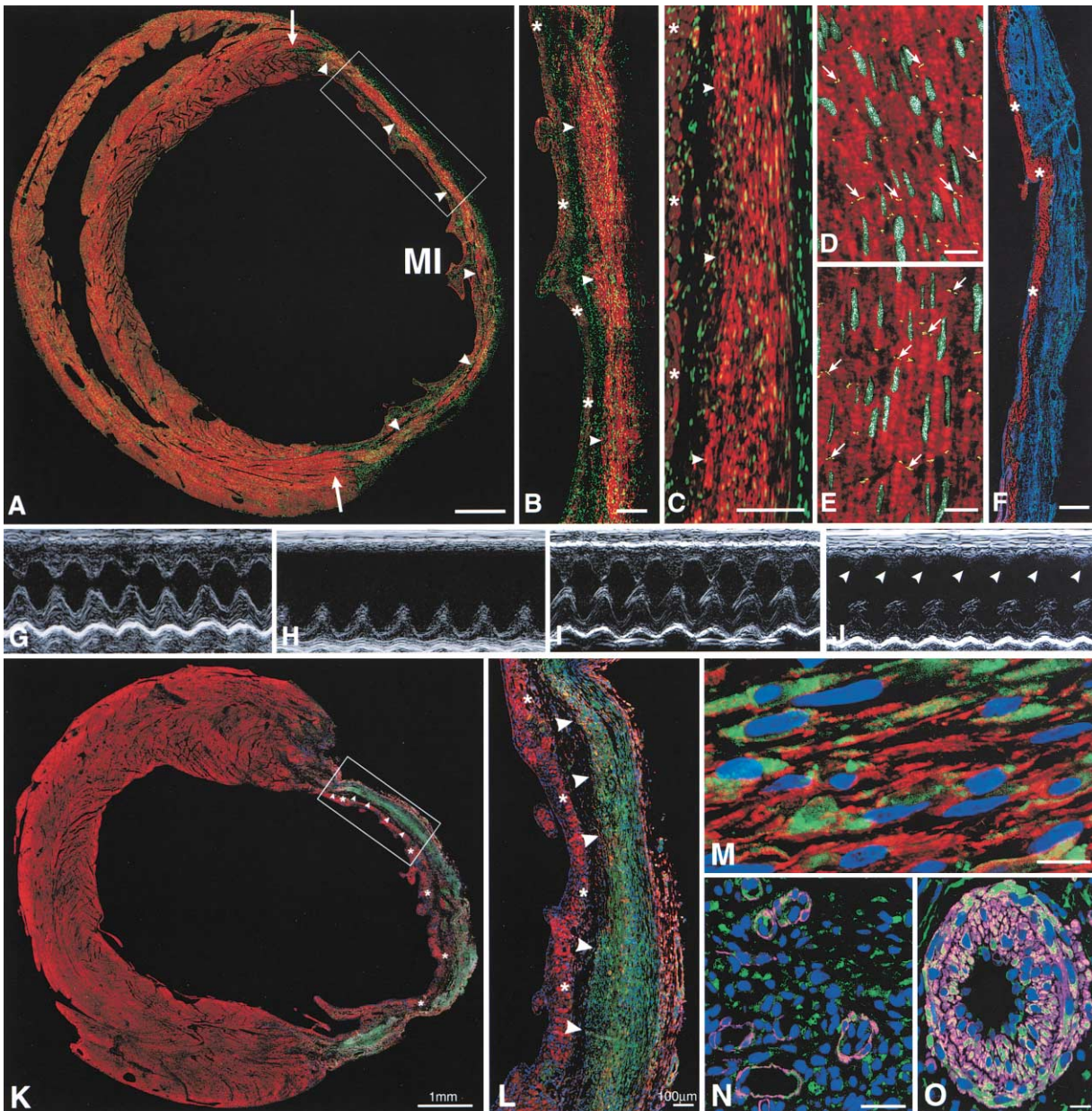


Figure 5. Myocardial Repair

(A–E) Bands of regenerating myocardium in infarcted treated hearts at 20 days. (A) Sites of cell injection (arrows); (A–C) regenerated myocardium (arrowheads). The rectangle in (A) is shown at higher magnification in (B). Cardiac myosin, red; PI, green. (D) Connexin 43 (yellow; arrows). (E) N-cadherin (yellow; arrows). (D and E) BrdU-PI labeled nuclei, white-green. (F) Scarring in a nontreated infarct. Collagen type I–III, blue. (B, C, and F) Asterisk indicates spared endomyocardium of infarcted LV. (G–J) M mode tracings of untreated (G and H) and treated infarcted rats (I and J). (G and I) Baseline conditions before coronary artery occlusion. (J) Reappearance of contraction at 19 days (arrowheads). (K and L) Regenerating myocardium (green, arrowheads) at 10 days after injection of EGFP-clonogenic cells. The rectangle in (K) is shown at higher magnification in (L). Asterisk indicates spared endomyocardium of infarcted LV. (M) New myocytes within regenerating myocardium: cardiac myosin, red; EGFP, yellow-green; PI, blue. (N and O) Arterioles, thin (N) and thick (O) walled: α -smooth-muscle-actin, magenta; EGFP, yellow-green; PI, blue. Bars: 1 mm in (A) and (K); 100 μ m in (B), (C), (F) and (L); and 10 μ m in (D), (E), and (M)–(O).

with reports that in similarly labeled cells, BrdU can be detected for up to 15 generations (Mahmud et al., 2001).

The plateau in myocyte number from 10–20 days was consistent with a decrease in Ki67-expressing cells and an increase in apoptosis ($0.33\% \pm 0.23\%$ to $0.85\% \pm 0.31\%$, $p < 0.001$) between the two time points. Addi-

tionally, the numerical density of capillaries and arterioles increased from 10 to 20 days, paralleling the increase in cardiac tissue. At 10 days, there were 49 ± 11 arterioles and 595 ± 103 capillaries per mm^2 that increased to 85 ± 28 and 946 ± 191 per mm^2 , respectively, at 20 days. Thus, although arteriolar density has

Table 1. Effects of Myocardial Regeneration on Myocardial Performance

	SO	MI		MI-T	
		10 Days	20 Days	10 Days	20 Days
Echocardiography					
Chamber diameter (mm)	5.4 ± 0.4	7.4 ± 0.6 ^a	8.1 ± 0.3 ^a	6.6 ± 0.5 ^{a,b}	6.9 ± 0.5 ^{a,b}
Viable WT (mm)	1.11 ± 0.11	0.78 ± 0.08 ^a	0.88 ± 0.05 ^a	0.87 ± 0.11 ^a	0.91 ± 0.04 ^{a,b}
Infarcted WT (mm)	1.03 ± 0.14	0.46 ± 0.11 ^a	0.45 ± 0.07 ^a	0.56 ± 0.06 ^a	0.62 ± 0.09 ^{a,b}
Ejection fraction (%)	82 ± 5	33 ± 7 ^a	34 ± 3 ^a	41 ± 8 ^{a,b}	45 ± 10 ^{a,b}
Hemodynamics					
LV EDP (mmHg)	6.5 ± 2.4	18.7 ± 2.6 ^a	18.8 ± 3.3 ^a	14.6 ± 4.7 ^a	10.5 ± 6.3 ^b
LV DP (mmHg)	107 ± 5	68 ± 12 ^a	67 ± 9 ^a	77 ± 12 ^a	79 ± 6 ^{a,b}
LV + dP/dt (mmHg/s)	10870 ± 1250	5350 ± 520 ^a	4830 ± 570 ^a	6400 ± 250 ^{a,b}	6410 ± 980 ^{a,b}
LV - dP/dt (mmHg/s)	9940 ± 1200	4930 ± 870	4590 ± 440 ^a	5730 ± 470 ^a	5470 ± 740 ^{a,b}
Wall stress (dynes/mm ²)	100 ± 64	590 ± 130	560 ± 100 ^a	370 ± 150 ^{a,b}	270 ± 160 ^{a,b}
Regenerating myocardium (mm ³)				30 ± 5	48 ± 6 ^b

Data are present as mean ± SD. Abbreviations: SO, sham-operated; MI, myocardial infarction; MI-T, myocardial infarction treated; WT, wall thickness; LV, left ventricle; EDP, end diastolic pressure; and DP, developed pressure.

^aIndicates a statistically significant difference from SO.

^bIndicates a statistically significant difference from MI.

reached mature values (55–60/mm²) in these animals, capillary density is more comparable to the neonatal (1130/mm²) than the adult (4000/mm²) heart, even at the later time point. Taken together, these characteristics reinforce the conclusion that at 20 days, the regenerated myocardium resembles that of the neonatal heart.

The new myocardial cells positively affected cardiac performance. Contraction reappeared in the infarcted ventricular wall (Figures 5G–5J). Cell implantation reduced infarct size and cavitory dilation, increased wall thickness, and ejection fraction. End-diastolic pressure, developed pressure and dP/dt significantly improved at 20 days. Diastolic stress was 52% lower in treated rats (Table 1). Despite that treated surviving animals had an infarct size 27% larger than controls, they had a decreased diastolic load and improved overall pump function.

Clearly, after many doublings in culture, cardiac Lin⁻ c-kit^{POS} cells remain differentiation competent and generate myocytes, SMC, and EC when placed into a proper context. The structure and cellular composition of the formed myocardial band are conclusive proof that these cells rapidly replicate and differentiate when implanted into the infarcted tissue. Proliferation is predominant at the earlier time point while hypertrophy is prevalent later. Interestingly, the cells exhibited a tropism for the ischemic zone because all the regenerating cells were located in the necrotic area and not in the spared myocardium despite the fact that cells were injected at the border between ischemic and healthy myocardium. The stimulating role of ischemia is highlighted by the rapid disappearance of the BrdU-labeled cells when injected into healthy hearts of mock-infarcted animals (not shown).

The Cardiac Lin⁻ c-kit^{POS} Cells Are Multipotent In Vivo

The in vivo results described above do not address whether the Lin⁻ c-kit^{POS} cells injected, although able to regenerate a functional ventricular wall, are truly

multipotent and capable of generating the three main cardiac cell types. It is possible that the injected population is composed of a mixture of cells, with each having a more restricted developmental potential. Also, despite the very significant expansion of the injected cells, the results do not provide a measure of the proliferative potential of these cells. To gain a better insight on these questions, 2 × 10⁵ cells grown from a single clone in mNSCM (dilution technique), genetically marked by a retroviral vector expressing enhanced green fluorescent protein (EGFP), were locally injected in the border zone of a 5-hr-old myocardial infarction in six rats, as above. 10 days later, a band of newly formed myocardium was identified in all six hearts. Most of the myocytes, SMC, and EC in the regenerating bands expressed EGFP and, therefore, had originated from the injected cells. In general structure, size, and cellular composition these bands were indistinguishable from those obtained with the mixed cell population.

Cloning by dilution technique does not assure a single cell origin of the clones harvested. For this reason, we tested two different clones and one subclone obtained by directly depositing single cells into wells containing mNSCM (see Experimental Procedures) and harvested from wells that we had ascertained by visual inspection were seeded with a single EGFP-labeled cell. Each clone and the subclone were injected into two infarcted hearts each, as described above. 10 days after injection, all six animals had similar myocardial regenerating bands (Figures 5K and 5L) composed of EGFP-positive myocytes, arterioles, and capillaries (Figures 5M–5O). These regenerating bands were not distinguishable from either those produced by the dilution technique clones or the mixed cell population.

From these three independent myocardial regeneration assays, it is fair to conclude that Lin⁻ c-kit^{POS} cells isolated from the adult rat heart behave in vivo like stem cells since they are self-renewing, clonogenic, and multipotent. Their proliferation potential is very robust since the progeny of a single cell can, at a minimum, regenerate two functional ventricular walls, each com-

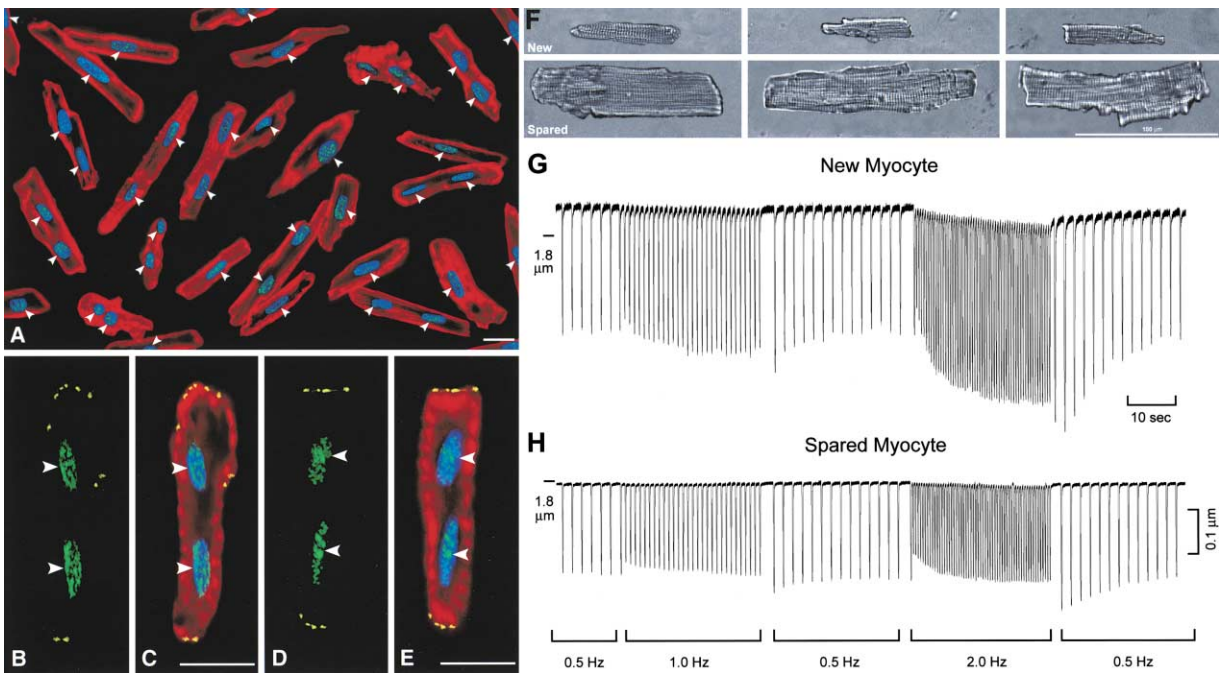


Figure 6. Myocyte Mechanics

(A–E) Regenerated BrdU-positive myocytes ([A], [B], [D]; green dots in blue nuclei, arrowheads), connexin 43 ([B] and [C]; yellow), and N-cadherin ([D] and [E]; yellow). Bars: 10 μm in (A)–(E).

(F–H) Small new and large spared myocytes (F) in treated rats. Sarcomere mechanics in a new (G) and a spared (H) myocyte.

posed of $\sim 14 \times 10^6$ myocytes and approximately two to three times as many of the other cell types.

The Myocytes Produced by the Cardiac Lin^- $\text{c-kit}^{\text{POS}}$ Cells Are Structurally and Functionally Competent

The improved functional performance of the postinfarcted hearts injected with Lin^- $\text{c-kit}^{\text{POS}}$ cells is strongly suggestive that the newly generated myocytes are functional and contribute to the overall contractility of the treated hearts. This point of view is further supported by the reappearance of synchronous motility of the treated ventricular wall as detected by echocardiography. Yet, none of these parameters conclusively proves that the generated myocytes have normal contractile properties. Therefore, to unambiguously determine whether these myocytes are truly functional, they were isolated at 20 days after injection. As shown in Figures 6A–6E, the BrdU-labeled myocytes had their myofibrillar proteins mostly distributed at the periphery of the cell in the subsarcolemmal region, with visible sarcomeric striations in a pattern very similar to fetal and neonatal cardiocytes. N-cadherin and connexin 43 are located at the expected position for myocytes that are functionally integrated and electrically and mechanically coupled.

Figures 6F–6H compare the contractility parameters of the new cells with those of the spared myocytes. Despite the dramatic size difference between new and surviving myocytes, they exhibit similar time to peak shortening, with higher values of peak shortening and velocity of shortening for sarcomeres of the new myocytes. These data clearly establish that *in vivo*, the Lin^-

$\text{c-kit}^{\text{POS}}$ cells generate bona fide cardiac myocytes from a structural and functional point of view.

The Regenerating Myocytes Are Not the Result of Cell Fusion between Injected Cells and Spared Myocytes

The purported plasticity and multipotency of some stem cells, such as those from the bone marrow, and their capacity to differentiate into cell types other than those of their tissue of origin, as reported by us and many others, has been thrown into doubt by the recent demonstration that under some situations certain stem cells have a tendency to fuse *in vitro* and with the differentiated cells of the host organ *in vivo* (see Alison et al., 2003; Rosenthal, 2003). This phenomenon can give the appearance of transdifferentiation when in reality only hybrid cells are exhibiting the differentiated phenotype. Although the results described here do not address the issue of transdifferentiation because cells of cardiac origin are shown to differentiate into cardiac cell types, it is still necessary to determine whether the regenerating myocytes, expressing the biochemical tags specific of the injected cells, could have originated by the fusion of a donor cell with a spared myocyte instead of undergoing true lineage commitment.

Five lines of evidence conclusively argue against cell fusion as a biologically significant phenomenon for the observations described here, although we cannot rule out that a very small fraction of new myocytes are produced by cell fusion. (1) The number of new myocytes is orders of magnitude higher than the injected cells, which in addition also give rise to SMC and EC. (2)

The myocardial infarct model used here leaves very few spared myocytes in the necrotic area, and those are mainly located in the subendo- and/or subepicardium (see Figures 5B and 5C), while the regenerating myocytes are mainly located in the center of the wall. (3) No small myocytes with the biochemical tags of the injected cells (BrdU or EGFP) are found in the spared myocardium where differentiated fusion partners are in great abundance. (4) The size of newly formed myocytes at the stages analyzed here is more than an order of magnitude smaller than that of the spared myocytes (see Figure 6F), while the product of cell fusion would be expected to be at least as large as the differentiated partner. (5) Regenerating myocytes have a 2n DNA content and not the 4n expected for fusion products as determined from the analysis of four different cell preparations in which the DNA content of cycling (Ki67 positive) and noncycling (Ki67 negative) myocytes was determined using lymphocytes as controls. In all cases, >99% of the noncycling myocyte population had a 2n DNA content. Higher values were always associated with cycling lymphocytes and myocytes (not shown).

From these data, we conclude that the regenerating myocytes result from proliferation and differentiation of the injected cardiac Lin⁻ c-kit^{POS} cells and not from cell fusion.

Discussion

The Adult Heart Contains Undifferentiated Cells with the Characteristics of Cardiac Stem Cells

The results presented here document the existence in the adult rat heart of a population of Lin⁻ c-kit^{POS} cells that in vitro and in vivo exhibit all the properties expected for cardiac stem cells. They are self-renewing, clonogenic, and multipotent, giving rise to a minimum of three differentiated cell types: myocytes, smooth muscle, and endothelial vascular cells. Moreover, when injected into an ischemic heart, a population of these cells or the clonal progeny of one of them reconstitute a well-differentiated myocardial wall that encompasses up to 70% of the LV. This regenerated ventricular wall is constituted by blood carrying new vessels and myocytes that, although smaller in size, have the anatomical, biochemical, and functional properties of young myocytes. To the best of our knowledge, this is one of the most extensive solid organ tissue regenerations using stem cells reported so far. In a matter of a few weeks, the injected cells regenerate more than 50% of the contractile myocytes and vascular cells normally present in the myocardium.

Over the past 2 years, a high level of skepticism has met many claims on the behavior of adult stem cells, particularly their multipotency and transdifferentiation properties (see Alison et al., 2003; Rosenthal, 2003). Also, there have been attempts to establish criteria for the analysis of the developmental potential of putative adult stem cells (Anderson et al., 2001) modeled on the known behavior of hematopoietic stem cells. These criteria require tracking and phenotyping the progeny in vivo of a single putative stem cell that has not been cultured in vitro and documenting the stability of the phenotype by successive passages in vivo. Unfortu-

nately, these assays are not possible in most solid organs and particularly in the heart where the tested cells have no selective advantage. If judged by the criteria mentioned above, our data fall short of establishing the stem cell nature of the cardiac Lin⁻ c-kit^{POS} cells. Moreover, because we have not yet identified antigenic markers to follow the progression of these cells along the cardiac lineages, we cannot distinguish between true stem, amplifying, and progenitor cells. Yet, given the limitations of the biology of this organ, the cloning and subcloning of these cells, their in vitro and in vivo phenotype, together with the organ regenerative capacity exhibited by the progeny of a single cell make a convincing argument that these cells have the characteristics expected of a cardiac stem cell and/or their immediate progeny.

Origin and Nature of the Resident Cardiac Stem Cells

One of bewildering findings of the past few years has been the variety of cell types that under different experimental conditions have shown the capacity to give rise to cardiac myocytes. In addition to the cells reported here, cardiogenic potential has been demonstrated for bone marrow cells—presumably Lin⁻ c-kit^{POS}—cells mobilized with the systemic administration of cytokines, human mesenchymal stem cells, liver-derived stem cells, endothelial cells, cells from the embryonic dorsal aorta, and multipotent adult progenitor cells (MAPCs) from the bone marrow (see Nadal-Ginard et al., 2003). Surprisingly, the markers used to identify these “cardiogenic” cells are very different and at times mutually exclusive. While the bone marrow (Orlic et al., 2001) and the cardiac cells used here were isolated using c-kit as a marker, lack of expression of this receptor is one of the identifying characteristics of the MAPCs (Jiang et al., 2002). Other confounding aspects of the data are that the identity of the myocardium-generating cells has only been established in a few cases by the use of single cells or their progeny (this work; Krause et al., 2001; Jiang et al., 2002) and, with the exception of this report, the differentiated phenotype remains functionally uncharacterized.

The origin of the Lin⁻ c-kit^{POS} cells is not known. Data from the sex-mismatched heart transplants (Quaini et al., 2002) show that cells of recipient origin with similar characteristics reach the transplanted heart and differentiate into the three types of cardiac cells in a matter of days. These cardiac cells, however, are different from circulating EPCs, which are CD45⁺, CD34⁺, and participate in the regeneration of smooth muscle and endothelial cells, but not myocytes (see Nadal-Ginard et al., 2003). If the cardiogenic cells presented here reached the myocardium through the circulation, then they have resided in the myocardium long enough to adopt their specific phenotype, because no cells with their characteristics have been identified in the bone marrow or peripheral circulation.

Cardiac Organogenesis versus Cardiac Regeneration

During cardiac development, the myocyte lineage is derived from the cardiogenic plates, which differentiate

into contractile cells that rapidly divide until the perinatal stage. Coronary vasculature does not appear until later in development. Cell lineage marking experiments during cardiogenesis in the chicken, zebrafish, and mammals show that myocytes, SMC, and EC originate each from a separate lineage (see Mikawa, 1999). This developmental pattern contrasts with the pluripotent nature of the cardiac $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells, which produce myocytes and vascular cells both *in vitro* and in the ischemic myocardium. Because the lineage marking experiments do not test the developmental potential of the cells tracked, the different outcomes might represent another example of the differences between normal developmental fate and developmental potential. Alternatively, it is possible that cardiogenesis and cardiac regeneration might result from distinct differentiation programs, as is the case of Wolffian regeneration where a new lens is formed from the iris, while the original lens is formed from the epidermis (Kosaka et al., 1998). Experiments to distinguish between these alternatives and elucidate the origin of the adult cardiogenic cells are in progress.

The Adult Heart Is Not a Terminally Differentiated Organ

Despite the increasing body of data documenting the formation of new cardiac myocytes in the adult normal and diseased mammalian heart, the idea that normal and pathological cardiac homeostasis involves myocyte cell death and renewal has met with significant resistance in the cardiovascular community. This resistance has been due to the fact that until now, there has been a lack of biological understanding about the origin and fate of the cycling myocytes detected in the normal and pathological heart. The existence of myocardial stem cells capable of committing to the myogenic lineage conclusively dispels the notion of the heart as a terminally differentiated organ without self-renewal potential and provides an explanation, as well as a biologically satisfactory context, for the existence of the cycling myocytes. The finding of $\text{c-kit}^{\text{POS}}$ cells that have already activated the myogenic program *in situ*, their capacity to generate a progeny of millions of myocytes when injected into the ischemic myocardium as reported here, together with the small size of the cycling myocytes in the normal, pathological, and regenerating heart indicates that these cycling cells are the progeny of the cardiac stem or amplifying cells that have not yet reached a mature and terminally differentiated state. Long-term labeling and chase experiments suggest that once committed to the myocyte lineage the progeny of a stem cell can undergo three to four rounds of cell division before permanently withdrawing from the cell cycle (A.L., unpublished). All these data strongly suggest that throughout adult life, the stem cells characterized here regenerate the myocytes lost by the normal wear and tear and go on overdrive in response to significant myocyte loss. Thus, the adult heart, which like the brain (Gage, 2002) is composed of mainly terminally differentiated parenchymal cells that do not reenter the cell cycle, is not a terminally differentiated organ because it also contains stem cells that support its regeneration.

Prospects for Myocardial Regeneration

Despite the significant advances over the past two decades in the management of human acute myocardial infarction, there remains a large population of postinfarct survivors who will develop cardiac failure, leading to early death (Hellermann et al., 2002). Cardiac transplantation remains the only therapy available to these patients. Because of the ingrained concept of the myocardium as a terminally differentiated organ, it has been assumed that any attempt to replace the lost myocytes using cellular therapy would require the introduction of exogenous cells into the myocardium. Recently, there has been a flurry of reports of human myocardial therapy using bone marrow cells (Strauer et al., 2002; Assmus et al., 2002; Tse et al., 2003; Perin et al., 2003) based, in part, on our early results with myocardial regeneration with bone marrow derived cells (Orlic et al., 2001). The demonstration that the heart harbors stem cells capable of regenerating large amounts of functional myocardium explains our earlier observation of a robust regenerative response in the acute postinfarct (Beltrami et al., 2001) while raising the question of why this regenerative response stops before the completion of the repair. At the same time, the identification of these cells opens the tantalizing possibility that they might be coached *in vivo* to home within the damaged myocardium, subsequently promoting functional cardiac repair without the need of introducing exogenous cells. The extraordinary clinical potential of myocardial regeneration makes the dissection of the biology of these cardiac stem cells a challenging and exciting endeavor.

Experimental Procedures

$\text{c-kit}^{\text{POS}}$ Cells

Cardiac cells were isolated from female Fischer rats at 20–25 months of age, and myocytes were discarded. Small intact cells were resuspended and aggregates removed with a strainer. Cells were incubated with a rabbit c-kit antibody (H-300, Santa Cruz) (Broudy, 1997). $\text{c-kit}^{\text{POS}}$ cells were collected with magnetic beads (Dyna) coated with anti-rabbit IgG ($n = 13$). For FACS ($n = 4$), cells were stained with r-phycoerythrin-conjugated rat monoclonal anti- c-kit (Pharmingen). Purity of sorted cells was determined by FACS.

Cell Culture and Cloning

$\text{c-kit}^{\text{POS}}$ cells were plated for 5–7 days at $1\text{--}2 \times 10^4$ cells/ml F12K medium containing 5% FCS, bFGF, and LIF. After recovery, they were moved to mNSCM (Tropepe et al., 1999) without heparin and neurotrophic growth factors: Dulbecco's MEM and Ham's F12 (ratio 1:1), bFGF (10 ng/ml), EGF (20 ng/ml), LIF (10 ng/ml), HEPES (5 mM), and insulin-transferrin-selenite. For cloning by plating dilution technique, cells were seeded at 1–5 cells/cm² in mNSCM. For cloning by plating dilution technique, cells were seeded at 1–5 cells/cm² in mNSCM. After one week, individual colonies were collected with cloning cylinders and plated. One clone from each preparation was chosen for characterization. MEM containing 10% FCS and 10^{-8} M dexamethasone was employed to induce differentiation (DM). For subcloning, cells from a clone were plated at 1–5 cells/cm² in mNSCM. At each subcloning step, an aliquot was grown in suspension to develop clonal spheres.

In a separate experiment, single cell cloning was employed. Isolated $\text{c-kit}^{\text{POS}}$ cells were collected with Miltenyi immunomagnetic microbeads ($n = 5$). Before sorting, bead-coated cells were treated first in enriched F12K medium and then in mNSCM for 5–7 days each. Subsequently, ~20,000 cells were sorted (MoFlo High Performance Cell Sorter, Cytomation), and single cells were deposited in Terasaki plates. The individual cells were grown in F12K medium for 1–2 weeks when clones were identified and expanded. Clono-

genic cells were then grown in mNSCM for an additional week before plating in DM or retroviral infection in mNSCM.

Retroviral Infection

Clonogenic cells obtained by dilution or single cell sorting were infected with a retrovirus expressing EGFP. The vector was collected from the supernatant of cultures of E86-Mieg3 packaging cells. Viral titer was 10^6 cfu/ml. Clonogenic cells were transduced three times over 7 days; $61\% \pm 7\%$ cells were EGFP positive (rabbit GFP antibody, Molecular Probes).

Cell Immunocytochemistry

Cells were fixed and labeled with c-kit antibody. Markers for myocytes included Nkx2.5, MEF2, GATA-4, GATA-5, nestin, α -sarcomeric actin, α -cardiac actinin, desmin, and cardiac myosin heavy chain; for SMC were α -smooth muscle actin and desmin; for EC von Willebrand factor, CD31 and vimentin; and for F vimentin, fibronectin and procollagen type I. Skeletal muscle cells were excluded by MyoD, myogenin, and Myf5; hematopoietic lineages by CD34, CD45, CD20, CD45RO, CD8, and TER-119; and neural lineages by MAP1b, neurofilament 200, and GFAP. Cycling cells were identified by BrdU and Ki67 (Urbanek et al., 2003).

Myocardial Infarction and Cell Implantation

BrdU-labeled cells ($P2$; positive cells = $88\% \pm 6\%$) were implanted. Myocardial infarction was produced in female Fischer rats at 2 months of age (Orlic et al., 2001). 5 hr later, 22 rats were injected with 2×10^5 cells in two opposite regions bordering the infarct; 12 rats were sacrificed at 10 days and 10 at 20 days. At each interval, eight to nine infarcted and ten sham-operated rats were injected with saline and five with $\text{Lin}^- \text{c-kit}^{\text{NEG}}$ cells and used as controls. Under ketamine anesthesia, echocardiography was performed at 9 and 19 days. From M mode tracings, LV end-diastolic diameter and wall thickness were obtained. Ejection fraction was computed. At 10 and 20 days, animals were anesthetized and LV pressures and $+$ and $-$ dP/dt were evaluated in the closed-chest preparation. Mortality was lower, but not significantly different, in treated rats, averaging 35% in all groups. Protocols were approved by the institutional review board.

Tissue Fixation, Infarct Size, and Newly Formed Myocytes

Hearts were arrested in diastole, fixed, and infarct size was determined by the fraction of myocytes lost from the ventricle. The volume of 400 new myocytes was measured in each heart. Cell length and diameter across the nucleus were measured in longitudinally oriented myocytes to compute cell volume.

Tissue Immunohistochemistry

Following BrdU and Ki67 labeling, myocytes (M) were identified at the confocal microscope by cardiac myosin, EC by von Willebrand factor, SMC by α -smooth muscle actin, and F by vimentin in the absence of von Willebrand factor (Anversa and Nadal-Ginard, 2002b; Orlic et al., 2001). The number of nuclei sampled for BrdU labeling was: M = 5229, EC = 3572, SMC = 4010, and F = 5529. Corresponding values for Ki67 were: M = 9290, EC = 9103, and SMC = 8392. Myocyte differentiation was established with cardiac myosin, α -sarcomeric actin, α -cardiac actinin, N-cadherin, and connexin 43. Collagen was detected by collagen type I and type III antibodies. Myocyte apoptosis was measured by a hairpin probe. The number of nuclei sampled for apoptosis was 30,464 and 12,760 at 10 and 20 days.

Mechanical Properties of New Myocytes

Myocytes isolated from infarcted treated rats ($n = 6$) were placed in a cell bath ($25^\circ\text{C} \pm 0.2^\circ\text{C}$) containing 1.0 mM Ca^{2+} and stimulated at 0.5–2 Hz by square pulses, 3–5 ms in duration in twice diastolic threshold in intensity. Cell mechanics were measured utilizing a CCD camera (Myocam, IonOptix, Milton, MA) and IonWizard 4.4 software (IonOptix) in SarcLength mode, for sarcomere length measurement by fast Fourier transform analysis.

Statistics

Results are mean \pm SD. Significance ($p < 0.05$) was determined by Student's t test and Bonferroni method.

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