Supporting Materials and Methods

Animals. Ninety-four FVB/N male mice at 3 months of age were used in the entire study. For the evaluation of cardiac stem cell (CSC) number, volume, and cellular composition of cardiac niches, BrdU-labeling assay, and symmetric and asymmetric division of CSCs, 62 animals were included. With mice under tribromoethanol anesthesia, the abdominal aorta was cannulated with a polyethylene catheter, PE-50, filled with a phosphate buffer, 0.2 M, pH 7.4, and heparin, 100 units/ml. In rapid succession, the heart was arrested in diastole by the injection of 0.15 ml of $CdCl_2$, 100 mM, through the aortic catheter; the thorax was opened; perfusion with phosphate buffer was started; and the vena cava was cut to allow the drainage of blood and perfusate.

The aortic catheter was connected to a pressure reservoir to adjust perfusion pressure to mean arterial blood pressure measured *in vivo*. Simultaneously, the left ventricular chamber (LV) was filled with fixative, 4% formaldehyde in phosphate buffer from a pressure reservoir set at a height equivalent to end-diastolic pressure determined *in vivo*. This was accomplished by inserting a 25G3/4 needle into the LV through the apex. The needle was connected to a pressure reservoir. After perfusion with buffer for 2 min, the coronary vasculature was perfused for 15 min with fixative. Subsequently, the heart was excised, and weights were recorded. The volume of the myocardium in each anatomical region of the heart was determined by dividing the weight by the specific gravity of muscle tissue, 1.06 g/ml (1). Samples were obtained from the atria, base–midregion, and apex of the LV. The base–midregion included 80% of the LV and 20% of the apex. After paraffin embedding, sections 5- to 6-μm in thickness were collected and immunolabeled.

Immunohistochemistry: Lineage-negative (Lin^-) CSCs and early committed cells were identified by mixtures of antibodies labeled directly with fluorochromes (Table 1). The epitopes used to classify CSCs, cardiac progenitors, and myocyte smooth muscle cell (SMC) and endothelial cell (EC) progenitors and precursors are listed in Table 1. The expression of $\alpha_4\beta_1$-integrin (Pharmingen), α_2-chain of laminin (AG Scientific, San Diego), laminin-8/9 and -10/11, and fibronectin was searched for in cardiac niches to

characterize the integrin receptors and extracellular ligands of this structural compartment. Immunolabeling was analyzed by confocal microscopy (2-5).

Volume, Number, and Cellular Composition of Cardiac Niches. The volume of the niches was measured morphometrically following a methodology described in ref. 1. This determination, together with the measurements of the volume and number of CSCs and LCCs within the niches, was determined in eight mice. To measure the average volume of cardiac niches, the long, L, and short, S, diameters of the niches were evaluated. The volume of a niche, V_N, was calculated assuming an ellipsoid shape. The number of CSCs, N_c, and average diameter of CSCs, D_c, within the niches was measured after the staining protocol described above. Similarly, the number of LCCs, N_l, and average diameter of LCCs, D_l, was determined:

$$V_N = 4/3 \times \pi \times (L/2) \times (S/2) \times ((L + S)/4).$$

To obtain the number of CSCs in a niche, the number of these cells per unit area, A, of the niche, $N_{c(A)}$, was first measured:

$$N_{c(A)} = N_c/(\pi \times (L/2) \times (S/2)).$$

Subsequently, the number of CSCs per unit volume of a niche, $N_{c(V)}$, was computed from the following:

$$N_{c(V)} = N_{c(A)}/D_c.$$

Finally, the aggregate number of CSCs in a niche, $N_{c(niche)}$, was obtained from the product of $N_{c(V)}$ and V_N:

$$N_{c(niche)} = N_{c(V)} \times V_N.$$

A similar procedure was followed for the determination of the aggregate number of LCCs in the niche, $N_{l(niche)}$.

For the acquisition of the number of CSCs and LCCs per unit volume of myocardium, the procedure of Schwartz–Saltykov (6, 7) was used. These cell categories were identified according to the labeling protocols indicated in Table 1. The Schwartz–Saltykov method categorizes cell diameters into 12 classes, differing by 1.0 μm each. The distribution of the number of cells of each size class ($N_1 \ldots N_{12}$) can be transformed to yield the actual number of cells per unit volume:

$$N_{(V)k} = (1/D_k) \times [1.6461\ N_{(A)k} - 0.4561\ N_{(A)k+1} - 0.1162\ N_{(A)k+2} - 0.0415\ N_{(A)k+3} - 0.0173\ N_{(A)k+4} - 0.0079\ N_{(A)k+5} - 0.0038\ N_{(A)k+6} - 0.0018\ N_{(A)k+7} - 0.0010\ N_{(A)k+8} - 0.0003\ N_{(A)k+9} - 0.0002\ N_{(A)k+10} - 0.0002\ N_{(A)k+11}].$$

$N_{(V)k}$ is the number of cells in class k per 1 mm^3 of myocardium. The number of cells per unit volume of myocardium, N_V, is given by the following:

$$k = 12$$

$$N_V = \sum N_{(V)k}$$

$$k = 1,$$

where the average diameter, D, is obtained from

$$D = N_V / N_A.$$

The magnitude of sampling consisted of 770 mm^2, 3,920 mm^2, and 430 mm^2 of myocardial tissue in the atrial, base–midregion, and apex, respectively (1).

Isolation of c-kit$^+$ CSCs-LCCs, Myocytes, Fibroblasts, and ECs for *in Vitro* Studies. Hearts were excised and cannulated for retrograde perfusion through the aorta. The cell isolation procedure included three main steps. (*i*) Low calcium perfusion: Blood washout in the presence of EGTA was followed by collagenase perfusion of the myocardium with Hepes-minimal essential medium gassed with 85% O_2 and 15% N_2. ECs were collected at the end of this step from the perfusate. (*ii*) Mechanical tissue dissociation: After removal of the heart from the cannula, the myocardium was cut into small pieces and shaken in collagenase solution. (*iii*) Separation of intact cells: Intact small cells and myocytes were separated and enriched by centrifugation. The fraction of small cells was then incubated with microbeads coated with c-kit antibody (Miltenyi Biotec, Auburn, CA). Fibroblasts were obtained from the remaining fraction of small cells and cultured. Separate cultures of myocytes and ECs were also prepared. These methodologies have been published in refs. 5, 8, and 9. CSCs–LCCs were also used for Western blotting (5, 9). Twenty-eight mice were used.

Calcein Dye Transfer Assay. Isolated c-kit$^+$ CSCs–LCCs were loaded with 5 μM calcein-acetoxymethyl ester (Molecular Probes) for 30 min at 37°C. Cells were then washed in Ca^{2+}/Mg^{2+} containing Hank's balanced salt solution. Cells were subsequently labeled with 1–2 μM 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) (Molecular Probes) for 5 min at 37°C and for 15 min at room temperature in Hank's solution (10). Labeled CSCs–LCCs were cultured overnight in the presence of cardiomyocytes, fibroblasts, and ECs. This approach was followed for the detection of functional gap junctions between CSCs–LCCs and myocytes, fibroblasts, or ECs. Because DiI cannot leave the cells, the presence of green fluorescence in the absence of red fluorescence was considered indicative of the transfer of calcein through gap junctions to myocytes, fibroblasts, or ECs. This analysis was performed by two-photon microscopy (n = 11). Subsequently, the same preparations were fixed, stained for connexins, and examined by confocal microscopy. In additional experiments, the gap junction-blocker heptanol (11) was added at a concentration of 0.6–1.5 mM to preparations of c-kit$^+$ CSCs–LCCs and myocytes before their coculture. This was done to inhibit the transfer of calcein through the forming gap junctions (n = 4).

Long-Term BrdU-Retaining Studies. A group of animals (n = 24) was injected i.p. with BrdU (Sigma), 50 mg/kg body weight, at 12-h intervals for 2 and 6 days. Mice were then killed at 2 days (n = 8), 6 days (n = 8), and 10 weeks after a 6-day period of BrdU administration (n = 8). By this approach, bright and dim $BrdU^+$ CSCs were counted within the niches. Levels of fluorescence >4,000 and <2,000 units (pixel × average intensity) were considered representative of bright and dim cells, respectively. Lin^- CSCs with intermediate levels of fluorescence, >2,000 but <4,000, were excluded from the analysis. These criteria were introduced to score the long-term label-retaining Lin^- CSCs. The autofluorescence of the section, together with the signal generated by the irrelevant antibody used as a negative control for BrdU staining, was <50 units. Levels of labeling >200 units were included. $BrdU^-$ CSCs were also counted. An identical approach was used to evaluate the fraction of myocyte nuclei labeled by bright and dim BrdU at 6 days and after 10 weeks of chasing. However, myocyte nuclei with intermediate fluorescence intensity (>2,000 and <4,000) were included in the analysis.

The number of CSC profiles counted in the niches and the number of myocyte nuclei counted in each region of the heart is listed in Table 2. Moreover, the volume of myocytes in the different parts of the heart measured by confocal microscopy in isolated myocytes is included in Table 4, together with the number of atrial and ventricular myocytes.

Asymmetric and Symmetric Division of CSCs. Asymmetric and symmetric division of CSCs within the niches (see Table 2 for sampling) was identified *in situ* by the localization of Numb, α-adaptin, and GATA-4. Mitotic CSCs were recognized by phosphohistone-H3. This part of the work included 30 animals. This *in situ* evaluation was complemented with the analysis of markers of cell commitment. c-kit^+ CSCs–LCCs were cultured in F12K medium supplemented with 15% FBS to induce a growth response. The expression of Numb, α-adaptin, and GATA-4 was determined (n = 17).

Myocyte Life Span. The life span of myocytes was determined by implementing the equations developed for hierarchically structured cell populations (13). Six primary measurements were required for this computation, which are listed in Table 3. They included the number of CSCs per unit volume of myocardium, the percentage of cycling CSCs, the number of CSCs committed to the myocyte lineage per unit volume of myocardium (LCCs, myocyte progenitors and precursors together), and the number of myocytes per unit volume of myocardium (Table 3).

Additionally, the duration of the cell cycle in CSCs was derived from the measurement of CSCs labeled by MCM5 (Table 3). MCM5 was selected because this nuclear protein is expressed throughout G1, S, and mitosis (12), and, among several potential markers, MCM5 is present for most of the duration of the cell cycle (3, 14, 15). CSCs in mitosis were also measured (Table 3) by the localization of phosphohistone-H3 (3, 16). Observations from our laboratory indicate that mitosis of CSCs *in vitro* lasts ≈30 min, confirming previous results (17). On the basis of these primary data, the duration of the cell cycle in CSCs was derived as follows: (fraction of $MCM5^+$ CSCs)/(fraction of phosphohistone-$H3^+$ CSCs) × 0.5 h. This value is listed in Table 3.

The variables indicated thus far allowed us to determine the aggregate rate of entry of CSCs, R_s, into the cell cycle. This is given by

$$R_s = f \times (N_s/T_s),$$

where f corresponds to the fraction of cycling CSCs, N_s to the number of CSCs per unit volume of myocardium, and T_s to the average time of CSC cycle. The birth rate of differentiated cells, r, can be calculated from:

$$r = ((f \times N_s)/T_s) \times 2^{Gt}. \qquad [\mathbf{1}]$$

In this equation, Gt corresponds to the number of transit generations, i.e., the number of divisions that a LCC goes through before it reaches the phenotype of a young

differentiated myocyte (see below). Because a myocyte number does not change during adulthood in the mouse heart (9, 18), ongoing parenchymal cell death, d, has to be, by necessity, equal to the generation of new myocytes:

$$d = r.$$

The half-life, $T_{1/2}$, of myocytes can then be calculated from the following:

$$T_{1/2} = N_m/2d$$

and Eq. **1**:

$$T_{1/2} = N_m/(2 \times ((f \times N_s)/T_s) \times 2^{Gt}), \qquad [\mathbf{2}]$$

where N_m is the number of myocytes per unit volume.

The number of transit generations, Gt, was derived from the ratio of LCCs to CSCs. After asymmetric division of a CSC, the daughter cell destined to be committed gives rise to two LCCs. Thus, if one generation is in transit, two LCCs are present. If two generations are in transit, four LCCs are found. Thus, for n generations in transit, 2^n LCCs are seen:

$$N_c = N_s \times 2^{Gt},$$

where Gt is the number of generations in transit, and N_s and N_c represent the number of CSCs and LCCs per unit volume of myocardium, respectively. Gt can be calculated from the following:

$$Gt = (\log(N_c/N_s))/\log 2.$$

These computations are complicated by the fact that CSCs can divide symmetrically, forming two LCCs, or asymmetrically, creating one LCC. To account for this

phenomenon, a symmetry factor, S_f, was introduced and calculated from the frequency of symmetric, N_{symm}, and asymmetric, N_{asymm}, divisions measured in the current study (Table 3):

$$S_f = (N_{asymm} + (N_{symm} + N_{asymm}))/N_{asymm}.$$

In this model, S_f ranges from a value of 2 (if all divisions of CSCs are asymmetric) to infinity (if the number of asymmetric divisions tends to 0). Accordingly,

$$Gt = (\log((N_c \times S_f)/N_s))/\log 2. \quad [\mathbf{3}]$$

Gt in Eq. **2** can be substituted by Eq. **3**:

$$T_{1/2} = Nm/2 \times ((f \times N_s)/T_s) \times 2^{(\log(Nc \times Sf)/Ns)/\log 2}. \quad [\mathbf{4}]$$

Eq. **4** was used to establish the half-life of myocytes in the atria, base–midregion, and apex of the mouse heart.

Statistical Analysis. Results are presented as means ± SD. Differences were determined by the two-tailed unpaired Student's *t* test, analysis of variance and Bonferroni method, and χ^2 (1).

1. Anversa, P. & Olivetti, G. (2002) in *Handbook of Physiology*, eds. Page, E., Fozzard, H. A. & Solaro, R. J. (Oxford Univ. Press, New York), Sect. 2, Vol. 1, pp. 75-144.

2. Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., *et al.* (2003) *Cell* **114,** 763-776.

3. Urbanek, K., Torella, D., Sheikh, F., De Angelis, A., Nurzynska, D., Silvestri, F., Beltrami, C. A., Bussani, R., Beltrami, A. P., Quaini, F., *et al.* (2005) *Proc. Natl. Acad. Sci. USA* **102,** 8692-8971.

4. Linke, A., Muller, P., Nurzynska, D., Casarsa, C., Torella, D., Nascimbene, A., Castaldo, C., Cascapera, S., Bohm, M., Quaini, F., *et al.* (2005). (2005) *Proc. Natl. Acad. Sci. USA* **102,** 8966-8971.

5. Urbanek, K., Rota, M., Cascapera, S., Bearzi, C., Nascimbene, A., De Angelis, A., Hosoda, T., Chimenti, S., Baker, M., Limana, F., *et al.* (2005) *Circ. Res.* **97**, 663-673.

6. Olivetti, G., Anversa, P., Rigamonti, W., Vitali-Mazza, L. & Loud, A. V. (1977) *J. Cell Biol.* **75,** 573-585.

7. Olivetti, G., Anversa, P., Melissari, M. & Loud, A. V. (1979) *Kidney Int.* **17,** 438-454.

8. Limana, F., Urbanek, K., Chimenti, S., Quaini, F., Leri, A., Kajstura J., Nadal-Ginard, B., Izumo S. & Anversa, P. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 6257-6262.

9. Torella, D., Rota, M., Nurzynska, D., Musso, E., Monsen, A., Shiraishi, I., Zias, E., Walsh, K., Rosenzweig, A., Sussman, M. A., *et al.* (2004) *Circ. Res.* **94,** 514-524.

10. Cancelas, J. A., Koevoet, W. L., de Koning, A. E., Mayen, A. E., Rombouts, E. J. & Ploemacher, R. E. (2000) *Blood* **96,** 498-505.

11. Suadicani, S. O., Vink, M. J. & Spray, D. C. (2000) *Am. J. Physiol. Heart. Circ. Physiol.* **279,** H3076-H3800.

12. Savill, N. J. (2003) *Cell Prolif.* **36,** 1-26.

13. Tanaka, T., Knapp, T. & Nasmyth, K. (1997) *Cell* **90,** 649-660.

14. Chimenti, C., Kajstura, J., Torella, D., Urbanek, K., Heleniak, H., Colussi, C., Di Meglio, F., Nadal-Ginard, B., Frustaci, A., Leri, A., *et al*. (2003) *Circ. Res.* **93**, 604-613.

15. Urbanek, K., Quaini, F., Tasca, G., Torella, D., Castaldo, C., Nadal-Ginard, B., Leri, A., Kajstura, J., Quaini, E. & Anversa, P. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 10440-10445.

16. Oh, H., Bradfute, S. B., Gallardo, T. D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L. H., Behringer, R. R., Garry, D. J. & Schneider, M. D. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 12313-12318.

17. Antonio, C., Ferby, I., Wilhelm, H., Jones, M., Karsenti, E., Nebreda, A. R. & Vernos, I. (2000) *Cell* **102,** 425-435.

18. Reiss, K., Cheng, W., Ferber, A., Kajstura, J., Li, P., Olivetti, G., Homcy, C. J., Baserga, R. & Anversa, P. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8630-8635.