Abstract—Cardiac myocytes have been traditionally regarded as terminally differentiated cells that adapt to increased work and compensate for disease exclusively through hypertrophy. However, in the past few years, compelling evidence has accumulated suggesting that the heart has regenerative potential. Recent studies have even surmised the existence of resident cardiac stem cells, endothelial cells generating cardiomyocytes by cell contact or extracardiac progenitors for cardiomyocytes, but these findings are still controversial. We describe the isolation of undifferentiated cells that grow as self-adherent clusters (that we have termed “cardiospheres”) from subcultures of postnatal atrial or ventricular human biopsy specimens and from murine hearts. These cells are clonogenic, express stem and endothelial progenitor cell antigens/markers, and appear to have the properties of adult cardiac stem cells. They are capable of long-term self-renewal and can differentiate in vitro and after ectopic (dorsal subcutaneous connective tissue) or orthotopic (myocardial infarction) transplantation in SCID beige mouse to yield the major specialized cell types of the heart: myocytes (ie, cells demonstrating contractile activity and/or showing cardiomyocyte markers) and vascular cells (ie, cells with endothelial or smooth muscle markers). (Circ Res. 2004;95:911-921.)

Key Words: adult stem cell ■ myocardial regeneration and angiogenesis

Cardiac myocytes have been traditionally regarded as terminally differentiated cells that adapt to increased work and compensate for disease exclusively through hypertrophy.1 In the past few years, compelling evidence has accumulated suggesting that the heart has regenerative potential.2-5 The origin and significance of the subpopulation of replicating myocytes are unknown; these issues could be relevant to understand the for mechanisms coaxing endogenous cardiomyocytes to reenter the cell cycle and to the search for strategies to transplant cardiac progenitor cells.6 In fact, although embryonic stem cells have an exceptional capacity for proliferation and differentiation, potential immunogenic, arrhythmogenic, and, particularly, ethical considerations limit their current use. Moreover, autologous transplantation of skeletal myoblasts has been considered because of their high proliferative potential, their commitment to a well-differentiated myogenic lineage, their resistance to ischemia, and their origin, which overcomes ethical, immunological, and availability problems. However, even if phase II clinical trials with autologous skeletal myoblasts are ongoing, several problems related to potentially life-threatening arrhythmia (perhaps reflecting cellular uncoupling with host cardiomyocytes7) must be taken into account when this approach is considered. Furthermore, although cardiomyocytes can be formed, at least ex vivo, from different adult stem cells, the ability of these cells to cross lineage boundaries is currently causing heated debate in the scientific community,8 with the majority of reports indicating neoangiogenesis as the predominant in vivo effect of bone marrow or endothelial progenitor cells.9,10

This report describes the identification and preliminary characterization of cells from the adult human and murine heart, which have the properties of cardiac stem cells. Because these cells also have been isolated and expanded from human heart biopsy specimens, they could have a significant impact on future clinical strategies to treat patients with heart disease.

Materials and Methods

Tissue Samples

Human tissue was derived from atrial or ventricular biopsy specimens belonging to patients (1 month to 80 years of age) undergoing heart surgery, in conformation with the guidelines of the Italian
Department of Health. Murine tissue was derived from the hearts of previously characterized homozygous MLC3F-nlacZ\textsuperscript{1} and cTnl-nlacZ\textsuperscript{12} transgenic mice expressing a nuclear lacZ transgene under the transcriptional control of the striated muscle myosin light chain or cTnl promoters, respectively, homozygous B5-eGFP\textsuperscript{ mice,\textsuperscript{13} homozygous GFP-cKit\textsuperscript{14} mice, MLC3F-nlacZ/B5-eGFP, MLC3F-nlacZ/GFP-cKit, and cTnl-nlacZ/B5-eGFP cTnl-nlacZ/GFP-cKit crossed mice, SCID mice, and SCID beige mice (Charles River Italia, Lecco, Italy).

**Processing, Isolation, and Cryopreservation of Sphere-Forming Cells**

Isolated myocardial tissue was cut into 1- to 2-mm\textsuperscript{3} pieces, washed with Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free phosphate-buffered solution (PBS) (Invitrogen), and digested three times for 5 minutes at 37°C with 0.2% trypsin (Invitrogen) and 0.1% collagenase IV (Sigma, Milan, Italy). The obtained cells were discarded, and the remaining tissue fragments washed with complete explant medium (CEM) (Iscove’s Modified Dulbecco’s Medium [IMDM] supplemented with 10% fetal calf serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 0.1 mmol/L 2-mercaptoethanol) were cultured as explants in CEM at 37°C and 5% CO\textsubscript{2}. After a period ranging from 1 (embryo) to 3 (adult) weeks, a layer of fibroblast-like cells was generated from adherent explants over which small, phase-bright cells migrated. These phase-bright cells were collected by pooling two washes with Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free PBS, one wash with 0.53 mmol/L EDTA (Versene, Invitrogen) (1 to 2 minutes), and one wash with 0.5 g/L trypsin and 0.53 mmol/L EDTA (Invitrogen) (2 to 3 minutes) at room temperature under visual control. The cells obtained (from 10\textsuperscript{5} to 4\times10\textsuperscript{5} cells/explant) were seeded at ~0.5 to 2×10\textsuperscript{5} cells/mL in poly-D-lysine-coated multwell plates (BD Biosciences, Milan, Italy) in cardiosphere-growing medium (CGM) (35% complete IMDM/65% DMEM–Ham F-12 mix containing 2% B27, 0.1 mmol/L 2-mercaptoethanol, 10 ng/mL epidermal growth factor [EGF], 20 ng/mL basic fibroblast growth factor [bFGF], 40 nmol/L cardiotrophin-1, 40 nmol/L thrombin, antibiotics, and t-Glu, as in CEM). Isolation of the cardiosphere-forming cells could be performed at least 4 times at 6- to 10-day intervals from the same explant. Cardiospheres (CSs) were passaged every 2 to 3 days by trypsinization and were allowed to grow on poly-D-lysine–coated culture surfaces in a low-serum (3.5% fetal calf serum) medium supplemented with a serum substitute (B27), growth factors (EGF and bFGF), cardiotrophin-1 (CT-1), and thrombin. During the first week of culture, the last factor led to a 7-fold increase in the number of spheres with respect to that obtained using the medium supplemented with the other factors, either alone or in combination. Time-course observations of cells derived from human and murine explants showed that early after their seeding (30 minutes), some of these cells began to divide while still in suspension. Most cells became loosely adherent, whereas others remained in suspension, and some contaminating fibroblast-like cells attached firmly to the poly-d-lysine coat. Cellular divisions also were evident from the loosely adherent cell population and produced clusters of small, round, phase-bright cells (that we termed CSs) after 10 to 12 hours (Figure 1a). Within 24 to 36 hours of their appearance, CSs increased in size and some of them detached from the culture surface; after 48 to 72 hours, most CSs were between 20 and 150 μm in size, and, when not subjected to mechanical dissociation, the largest contained dark zones within the inner mass (Figure 1a).

Murine CSs started beating spontaneously soon after their generation (Supplementary Movie: mouse CSs movie 1a) and maintained this function during their life span (Supplementary Movie: mouse CSs movie 1b), whereas human CSs did so only when cocultured with rat cardiomyocytes (Supplementary Movie: human CSs movie 1a and 1b). To be sure that contraction was a new trait acquired by the CSs cells, GFP-labeled human CSs (partially or totally dissociated) were cocultured with cardiomyocytes prestained (Supplementary Human CSs Movie 2a through 2d) or not prestained (Supplementary Human CSs Movie 3a through 3d) with Dil. Contracting GFP-labeled cells were observed after 48 hours of coculture; furthermore, Cx-43 immunostaining performed on the cocultures of human GFP-transduced CSs with unla- beled neonatal rat cardiomyocytes showed the typical punctuate fluorescence pattern of the main gap junction protein of the heart along the cytoplasmatic membrane of the human cells (Figure 2d and Supplementary Figure VIII), suggesting that a functional connection is created between the two cellular populations.

**Clonogenesis**

CSs were found to be composed of clonally derived cells and did not simply represent cellular aggregates. In fact, when human GFP-transduced CSs or murine CSs (derived from eGFP/MLC3F or eGFP/cTrI mice) were dissociated and plated as single cells on mitomycin-treated STO fibroblast-coated 96-well plates (or clonally diluted on 10-cm Petri dishes), fluorescent spheres were generated with a 1% to 10% efficiency (Figure 2a). These spheres could be subcloned on poly-d-lysine-coated surfaces, showing the same functional and phenotypic behavior in culture as the nonclone-derived CSs. In fact, 3 days after their appearance, some of the MLC3F-nlacZ/B5-eGFP or cTnl-nlacZ/B5-eGFP mice clone–derived CSs started to beat (supplementary clone movie), and, after 48 hours of culture with CEM, the majority (6 of 7) of these showed expression of the lac-Z transgene within the nuclei after specific histochemical staining (Figure 2b, and 2b, and Supplementary Figure I). Moreover, human clones derived from a single GFP-labeled cell started a synchronous beating and expressed cTnl after 48 hours of coculture with rat cardiomyocytes (Supplementary Movie human CSs 2a and 2a, and Supplementary Figure II).
Furthermore, when BrdUrd was added to the culture medium, virtually all cells in the small CSs and those of the inner part of the largest CSs were labeled (Figure 3a), indicating that these cells were newly generated (Supplementary Figures III through Va).

Proliferation

Human CS-generating cells were capable of self-renewal. With periodical dissociation, together with partial substitution of CGM every 2 to 3 days, a log-phase expansion of spheres was obtained (Figure 1b). Mouse CS growth was slower (probably because of the more differentiated features assumed in culture, such as beating) and serum-dependent as for the human CSs (Figure 1b).

As shown in Figure 3a and Supplementary Figure V, confocal immunofluorescence analysis of BrdUrd-labeled human CSs with anti-BrdUrd (green) and cardiac-troponin I (cTnI) or atrial natriuretic peptide (ANP) (red) revealed BrdUrd-positive cells, particularly in the inner of the spheres, whereas cTnI-positive or ANP-positive cells were mainly localized in the external layers. Similar features are shown in Supplementary Figures III and IV. BrdUrd-labeled cells (red) mostly localized in the center of a CS and colocalize with the Hoechst-labeled nuclei, whereas cardiac myosin heavy chain (MHC)-expressing cells (green) were preferentially located in the boundary layers. Furthermore, several CS cells expressed cardiac differentiation markers (cTnI, ANP) while still dividing, as indicated by BrdUrd incorporation (Figure 3a and Supplementary Figure Va), suggesting that early cardiac differentiation already occurred during the proliferation phase of their growth. Usually within 10 days, some spheres became adherent,
showing a more flattened morphology. Some small cells eventually migrated out from these “sun-like” spheres in the form of adherent (differentiated) or small, round cells that could generate new spheres. After thawing from cryopreservation, CSs proliferated again, maintaining their ability to beat (Supplementary Movie: human CSs movie).

**Immunophenotype Characterization and Fluorescence-Activated Cell Sorting Analysis**

Phenotypic analysis of newly developing human and mouse CSs revealed expression of endothelial (KDR (human)/flk-1 [mouse], CD-31) and stem cell (CD-34, c-kit, sca-1) markers. As shown in Figure 3b, CSs at the 2- to 10-cell stage strongly
Figure 3. CSs BrdUrd incorporation and CSs characterization. a, Fluorescence confocal analysis of BrdUrd-labeled human CSs for cardiac differentiation markers: 6-μm scans (from the periphery to the center of the sphere) and final pictures (small and large images, respectively) of BrdUrd (green) and cTnI (red) (see Supplementary Figures III through V). b, Confocal analysis of human CSs after 12 hours of culture: CD-34, CD-31, KDR, and c-Kit labeling of CS-generating cells at the beginning of sphere formation. c, fluorescence-activated cell sorting analysis of postnatal mouse CSs-derived cells. A time course at 0 and 6 days was used, and the phenotype profile for CD34, cKit, Cd31, and sca-1 expression was analyzed and shown as a percentage of positive events. Data are presented as mean ± SD (n=3). *Statistically significant difference from 0 days. See the graphics in the Table and in Figure 6.
reacted with antibodies against these antigens. In larger spheres, the expression pattern of some of these markers (particularly cKit) was similar to that of the BrdUrd-labeling (positive staining in the center and in some peripheral zones, generating satellite spheres; data not shown).

A time course (0 and 6 days) of the quantitative characterization of CS cells with these stem and endothelial markers was performed by fluorescence-activated cell sorting analysis (Figure 3c and Supplementary Figure VI). As shown at the beginning of their formation (0 days), the phenotype of these cells seems to reflect the epifluorescent microscopy analysis with \( \approx 10\% \) of positive staining for all four phenotypes. However, at 6 days, cKit appears to be the only conserved marker, suggesting that the cKit\(^{+}\) cells could be the main ones contributing to the maintenance of proliferation. The initial cell-labeling may reflect an early activation state, as has been suggested for CD-34 in several systems.\(^{17}\) Fluorescence microscopy analysis performed on cryosectioned human CSs revealed expression of cardiac differentiation markers (cTnI, MHC) and endothelial markers (von Willebrand factor) (Supplementary Figure Vc1 through Vc5). When totally or partially dissociated into single cells and cultured on collagen-coated dishes in the same medium as the explants, mouse and human CS-derived cells assumed a typical cardiomyocyte morphology, phenotype (Supplementary Figures Vb1 through Vb5 and VIIc and VIIId), and function documented (in the mouse only) by spontaneous contraction (Supplementary Movie: mouse CSs movie 2a and 2b).

Human CSs did not beat spontaneously; however, these began to beat within 24 hours when cocultured with postnatal rat cardiomyocytes, losing their spherical shape and assuming a “sun-like” appearance. Markers of cardiac differentiation were coexpressed within GFP in labeled human CSs cells (Figure 2c).

**Transgenic Mice**

To follow the differentiation process of CSs during the prenatal and postnatal age, MLC3F-nlacZ and cTnI-nlacZ mice were used.\(^{11,12}\) These mice express a form of lacZ transgene that localizes within the nucleus under the skeletal and cardiac muscle myosin light chain or cardiac troponin I promoter, respectively. CSs obtained from embryonic day 9 to 12, fetal day 17 to 18, and from neonatal and adult mice showed spontaneous expression of the reporter gene in variable percentages (10\% to 60\%) of spheres in the different culture conditions used (Figure 4a through 4a and Supplementary Figure VIIa, VIIa, VIIb, and VIIb). Moreover, regarding the human ones, CS-generating cells from mice expressed stem (CD-34, sca-1, cKit) and endothelial cell markers (flk-1, CD-31) (data not shown).

On this basis, we used transgenic mice expressing GFP under the control of the c-kit promoter\(^{14}\) to further clarify the cellular origin of these spheres and to follow the pattern of their growth process. As shown in Figure 4c, GFP-positive cells were present from the beginning of the formation of the CSs and, albeit with reduced fluorescence intensity, also later within the mass of cells of the CSs and in cells migrating from old adherent “sun-like” CSs (Figure 4c). Moreover, as suggested by the growth pattern of human CSs, when satellite secondary CSs appeared to detach from the primary ones, GFP-positive cells localized on the margins of the latter and in the inner part of the former.

We studied this process in double-heterozygous mice obtained from GFP-cKit/MLC3F-nlacZ or GFP-cKit/cTnI-nLacZ crossings. As shown in Figure 4c and 4c, \( \beta \)-Gal positivity did not colocalize with GFP in cells present within the growing areas.

**In Vivo Survival and Morpho-functional Potential of the Cardiospheres**

To investigate the survival and morpho-functional potential of the CSs in vivo, two sets of experiments were performed. In the first, CS cells were injected in the dorsal subcutaneous region of SCID mice. In the second, they were injected into the hearts of SCID beige mice, acutely after myocardial infarction. The objective of ectopic transplantation experiments was to study the pattern and the behavior of growth of CSs in a neutral milieu (ie, without specific cardiac induction) to verify their unique potential of generation of the main cardiac cell types and to exclude the potential of neoplastic transformation. For these experiments, \( \approx 60 \) pooled spheres/inoculum/mouse from prenatal and postnatal MLC3F-nlacZ/B5-eGFP mice, TnI-nlacZ/B5-eGFP mice, MLC3F-nlacZ/CD-1 mice, and cTnI-nlacZ/CD-1 mice were used. During the first 10 days, beating was appreciable through the skin over the injection site, distant from large blood vessels. On day 17, animals were euthanized and the inoculum recognized as a translucent formation, grain-like in size, wrapped in ramified vessel-like structures. Observation of unfixed crossections by fluorescence microscopy (Figure 5a, through 5a) revealed the presence of open spheres from which cells appeared to have migrated. Clusters of “black holes,” particularly in the periphery of the structure, were evident. The tissue contained tubular formations, surrounded by nuclei (Hoechst-positive), identified as cardiac sarcomeres by cTnI-nlacZ and sarcomeric myosin immunostaining (Figure 5b, through 5b). \( \alpha \)-Smooth muscle actin (\( \alpha \)-SMA)-positive structures (known to be transiently expressed during cardiomyogenesis)\(^{12,18}\) were present in the remainder of the spheres and associated with the vasculature (the clusters of “black holes”) (Figure 5a, through 5a). This exhibited well-differentiated structures with a thin endothelium expressing vascular endothelial–cadherin (Figure 5b) and a relative large lumen containing erythrocytes (Figure 5a), indicating the establishment of successful perfusion by the host. Light microscopic observation of the inoculum, after X-gal staining, showed strong nuclear expression of striated muscle-specific lacZ in the remainder of the spheres and in some cells close to them (Figure 5b). No multidifferentiated structures suggesting the presence of tumor formation were observed.

To test the acquisition of functional competence and the cardiac regenerative potential of the CSs when challenged into an infarcted myocardium, orthotopic transplantation experiments with human CSs were performed. To perform these, thawed (cryopreserved) adult human CSs from three atrial (one male and two female) and one ventricular (one female) biopsy specimens were injected into the viable myocardium bordering a freshly produced infarct. Each...
mouse received CSs from a single passage of an explant (derived from a single subject). Four control infarcted animals were injected with an equal volume of PBS. Eighteen days after the intervention, the animals were euthanized and infarct size was determined. Infarct size was 34.9 ± 7.1 (SEM, 3.6) and 31.9 ± 6.9 (SEM, 3.5) in the CS-treated group and PBS-injected group, respectively (P=NS). However, echocardiography showed better preservation of the infarcted anterior wall thickness in the CS-treated group compared with the PBS-injected group (0.80 ± 0.29 [SEM, 0.15] versus 0.60 ± 0.20 [SEM, 0.08]) (P=NS), particularly of percent fractional shortening (36.85 ± 16.43 [SEM, 8.21] versus 17.87 ± 5.95 [SEM, 2.43]) (P<0.05) (Figure 6 and the Table).

At the time of evaluation, bands of regenerating myocardium were present (with different degrees of organization and thickness) throughout most of the infarcted areas, as evaluated with hematoxylin–eosin histochemistry (data not shown) and MHC immunofluorescence (Supplementary Figure IXa and IXb). In the regenerating myocardium, cells expressing lamin A/C (a specific human nuclear marker) also colocalize with cardiomyocytes stained positive for MHC (Figure 6a and 6e and Supplementary Figures IXb, IXb, and X), newly generated capillaries stained for α-SMA (Figure 6b, 6b, and 6d) and platelet endothelial cell adhesion molecule (Figure 6c), and with connexin-43–expressing cells (data not shown).

Discussion

CSs appear to be a mixture of cardiac stem cells, differentiating progenitors, and even spontaneously differentiated car-
Figure 5. In vivo analysis (ectopic CSs inoculum). a1 to a5. Ectopic transplantation of CSs from MLC3F-nlacZ/B5-eGFP mouse to SCID mouse (upper left panels). Fluorescence analysis of unfixed cryosections (a1, a2, and a4) from the subcutaneous dorsal inoculum (day 17). GFP cells seemed to have migrated from the spheres, whereas clusters of vessel-like structures (a5) could be observed mainly in the external area. Staining for SMA of one of these cryosections showed positive immunoreaction of the sphere and some cells within the inoculum (a5). b1 to b6. Fluorescence (b3 to b4) and phase analysis (b5 to b6) of fixed and immunostained cryosections from dorsal inoculum of CSs from MLC3F-nlacZ/CD-1 and cTnI-lacZ/CD-1 mice. Tubular structures were stained for sarcomeric myosin (b3 to b5) and cTnI (b6). X-Gal staining labeled the cells within and those migrating from CS (b2). Endothelial markers (SMA and vascular endothelial–cadherin) stained the vasculature (“black holes”) (a3 and b1).
Figure 6. In vivo analysis (orthotopic transplantation of human CSs). Orthotopic transplantation performed in a SCID-beige mouse. Cryopreserved human CSs were transplanted into the viable myocardium bordering a freshly produced infarct. Confocal analysis of cryosectioned left ventricular heart 18 days after the coronary ligation shows that (a) cardiomyocytes expressing MHC (red) in the regenerating myocardium (particularly those indicated by the two central arrows) also stain positive for lamin A/C (green) (a specific human nuclear marker). In these cells, MHC expression is evident mainly in the perinuclear area (see Supplementary Figure X). Lamin A/C-labeled cells (red) are present in newly generated capillaries staining for α-SMA (b, through d), and platelet endothelial cell adhesion molecule (c). d, Confocal analysis of colocalization of lamin A/C-labeled cells (red) with the newly generated capillaries staining for α-smooth muscle actin. e, Low-magnification image shows viable lamin A/C-expressing cells (green) in regenerating myocardium expressing MHC (red).
diomyocytes. Vascular cells were also present, depending on the size of the sphere and time in culture. It is possible that, as for neurospheres,\textsuperscript{19} differentiating/differentiated cells stop dividing and/or die, whereas stem cells continue to proliferate in an apparently asymmetric way, giving rise to many secondary spheres and to exponential growth in vitro. Mechanical dissociation favors this process. Death, differentiation, and responsiveness to growth factors of the different cells within the CSs could depend on the three-dimensional architecture and on localization within the CSs.\textsuperscript{20} The spontaneous formation of spheres is a known prerogative of neural stem cells, some tumor cell lines (LIM),\textsuperscript{21} endothelial cells,\textsuperscript{22} and fetal chicken cardiomyocytes.\textsuperscript{23} All these models (ours included) that mimic the true three-dimensional architecture of tissues consist of spheroids of aggregated cells that develop a two-compartment system composed of a surface layer of differentiated cells and a core of unorganized cells that first proliferate and then disappear over time (perhaps through apoptotic cell death). As well-documented in fetal chick cardiomyocytes and endothelial cell spheroid culture, three-dimensional structure affects the sensitivity of cells to survival and growth factors.\textsuperscript{21,22} In particular, central spheroid cells do not differentiate and are dependent on survival factors to prevent apoptosis, whereas the cells of the surface layer seem to differentiate beyond the degree that can be obtained in two-dimensional culture and become independent of the activity of survival factors.\textsuperscript{23} Furthermore, cell–cell contact and membrane-associated factors, known to be important for the division of neural precursor cells,\textsuperscript{24} could be involved in our system. This is in accordance with the notion that stem cells (or cells with stem cell function) will only retain their pluripotency within an appropriate environment, as suggested by the “niche” hypothesis.\textsuperscript{25}

Thus CSs can be considered clones of adult stem cells, maintaining their functional properties in vitro and in vivo after cryopreservation.

While the experiments performed for this article were ongoing, two articles were published concerning the isolation of cardiac stem cells or progenitor cells from adult mammalian hearts.\textsuperscript{26,27} Isolation of these cells was based exclusively on the expression of a stem cell-related surface antigen: c-kit in the first article and Sca-1 in the second one. In the first study,\textsuperscript{26} freshly isolated c-kit\textsuperscript{+} Lin\textsuperscript{−} cells from rat hearts were found to be self-renewing, clonogenic, and multi-potent, exhibiting biochemical differentiation into the myogenic cell, smooth muscle cell, or endothelial cell lineage but failing to contract spontaneously. When injected into an ischemic heart, these cells regenerated functional myocardium. In the second study,\textsuperscript{27} Sca-1\textsuperscript{+} cKit\textsuperscript{−} cells from mice hearts were induced in vitro to differentiate toward the cardiac myogenic lineage in response to 5′-azacytidine. When given intravenously after ischemia/reperfusion, these cells targeted injured myocardium and differentiated into cardiomyocytes, with and without fusion with the host cells. Our data obtained on GFP-cKit transgenic mice also suggest that the adult cardiac stem cell is cKit\textsuperscript{+}. It is possible that CSs enclose a mixed population of cells that, as in the niche, could promote the viability of cKit progenitors and contribute to their proliferation. The data obtained in the present article confirm the existence of adult cardiac stem cells/progenitor cells. More importantly, they demonstrate for the first time to our knowledge that it is possible to isolate cells from very small fragments of human myocardium and expand these cells in vitro many-fold (reaching numbers that would be appropriate for in vivo transplantation in patients) without losing their differentiation potential. Previously unforeseen opportunities for myocardial repair could now be identified.

**Acknowledgments**

This study was supported by Italian Ministry of the University and of the Scientific and Technological Research (MURST) and by specific funds of the University of Rome “La Sapienza” from stem...
cell research. We thank Gianluigi Condorelli for continuous encouragement and valuable discussion. We thank Roberto Latini and Lidia Staszewsky in particular for their precious contribution in conducting and analyzing in vivo experiments. We thank Sergio Ottolenghi (Dipartimento Biotecnologie e Bioscienze, Università Milano-Bicocca) for kindly providing transgenic mice. We are also grateful to Francesco Musumeci and Fabio Miraldi for kindly supplying some of the heart biopsy specimens. Marta Alessandrini, Dario Sirabella, Lucia Ricci Vitiani, and Ilaria Falciatori (PhD students), University la Sapienza of Rome, Italy, are also gratefully acknowledged for their availability during the course of this work.

References

Isolation and Expansion of Adult Cardiac Stem Cells From Human and Murine Heart
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Circ Res. 2004;95:911-921; originally published online October 7, 2004;
doi: 10.1161/01.RES.0000147315.71699.51

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Isolation and expansion of adult cardiac stem cells from human and murine heart

Supplemental material

Materials and Methods.

Tissue samples. Human tissue was derived from atrial or ventricular biopsies belonging to patients (aged from 1 month to 80 years) undergoing heart surgery, in conformation with the guidelines of the Italian Department of Health. Murine tissue (was derived from the hearts of previously characterized homozygous MLC1/3F-nlacZ (1) and cTnI-nlacZ (2) transgenic mice (expressing a nuclear lacZ transgene under the transcriptional control of the striated muscle myosin light chain or of cTnI promoters, respectively), homozygous B5-eGFP mice (3), homozygous GFP-cKit (4) mice, MLC3F-nlacZ/B5-eGFP, MLC3F-nlac-Z/GFP-cKit, cTnI-nlacZ/B5-eGFP cTnI-nlac-Z/GFP-cKit crossed mice, SCID mice, and SCID beige mice (Charles River Italia., Lecco, Italy). The last two animals lines were employed as recipient for murine and human cardiospheres because of their non-inbred background. Samples were stored in ice cold serum-free IMDM (Invitrogen, Milan, Italy) and processed within two hours.

Processing, isolation, and cryopreservation of sphere-forming cells. Isolated myocardial tissue was cut into 1-2 mm³ pieces, washed with Ca²⁺/Mg²⁺-free phosphate buffered solution (PBS, Invitrogen) and digested trice for 5 min at 37 °C with 0.2% trypsin (Invitrogen) and 0.1% collagenase IV (Sigma, Milan, Italy). The obtained cells were discarded and the remaining tissue fragments, washed with complete explant medium (CEM) [IMDM supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT),
100 Units/ml penicillin G, 100 µg/ml streptomycin, 2 mmol/L L-glutamine (Invitrogen), 0.1 mmol/L 2-Mercaptoethanol (Sigma-Milan, Italy)], were cultured as explants in CEM at 37°C and 5% CO2. After a period ranging from 1 (embryo) to 3 weeks (adult), a layer of fibroblast-like cells was generated from adherent explants over which small, phase-bright cells migrated. These phase-bright cells were collected by pooling two washes with Ca++-Mg++-free/PBS, one wash with 0.53 mmol/L EDTA (Versene, Invitrogen) (1-2 min) and one wash with 0.5 g/L Trypsin- 0.53 mmol/L EDTA (Invitrogen) (2-3 min) at room temperature, under visual control. The cells thus obtained (from 10^4 to 4 x 10^5 cells/explant) were seeded at about 0.5-2 x 10^5 cells/ml in poly-D-lysine-coated multi-well plates (BD, Biosciences, Milan, Italy), in cardiospheres-growing medium (CGM) [35% complete-IMDM/65% DMEM-Ham’s F-12 mix containing 2% B27 (Invitrogen)], 0.1 mmol/L 2-mercaptoethanol, 10 ng/ml EGF, 20 ng/ml bFGF (PeproTech, London, UK), 40 nmol/L Cardiotrophin-1 (RD), 40 nmol/L thrombin (Sigma), antibiotics and L-Glu as in CEM]. Isolation of the cardiospheres-forming cells could be performed for at least 4 times at 6-10 day intervals from the same explant. CSs were passaged every 2-3 days by partially changing of medium and mechanical trituration of the larger clusters. Movies of cultured CSs were recorded utilizing a Nikon-COOLPIX-4500 digital-camera connected to a Leitz inverted microscope (Germany). For cryopreservation, we utilized CEM/DMEM-Ham-F12 (Invitrogen) 50: 50, 5% B27, 10% DMSO (Sigma) as the freezing-medium.

BrdU labeling. CSs were labeled (0.01 mmol/L BrdU overnight), fixed and screened according to the kit’s instructions (Roche, IN, USA).
**Clonal analysis.** Human CSs were transduced with a third-generation lentiviral vector, pRRLsin.PPT-PGK.GFP expressing green fluorescent protein (GFP), as described elsewhere (15-5). Murine CSs employed for clonal assay were derived from eGFP- or eGFP/MLC3F-mice. After washing, GFP-labeled CSs were dissociated into single cells by trituration in Ca\(^{++}\)/Mg\(^{++}\)-free PBS, Versene, and 1x trypsin-EDTA solutions in sequence, resuspended in CGM, and then seeded at a presumed concentration of 1 cell/well in a 96-well plates or 100-500 cells/10 cm Petri dishes coated with a feeder layer of mitomycin-C (Sigma) (3 µmol/L)-treated STO fibroblasts.

**Differentiation on substrate-coated surface.** Ca\(^{++}\)/Mg\(^{++}\)-free PBS-washed and partially dissociated CSs were seeded in a small volume of CEM (200-300 µl) on type-I collagen-(Sigma) or Matrigel- (BD) coated dishes and cultured for 3-6 days.

**Co-culture experiment.** Isolated primary neonatal rat cardiomyocytes were used as described (3, 16-6, 7) for co-culture with human CSs. About 10 CSs/1x10\(^5\) cardiomyocytes were employed (before and after 10 days of culture from thawing), cultured in CEM, observed at defined times (1, 2, 4, 8 days) for movie recording and then processed for immunocytochemical analysis. As required, GFP-labeled human CSs were co-cultured with neonatal rat cardiomyocytes pre-stained with a red fluorescent dye (Dil, Molecular Probes, Eugene, Oregon USA), according to Serbedzija GN et Al. (17-8) with slight modifications. Briefly, washed cardiomyocytes were covered with a diluted solution (1:80) of Dil (stock of 1mg/ml absolute ethanol) in 0.3 M saccharose (Sigma) pre-warmed at 37°C, and allowed to incubate for 5 min. at 37°C with 5% CO\(_2\). Then, cells were extensively washed in PBS, re-feuded in their culture medium and re-
incubated at 37°C with 5% CO₂. Cardiomyocytes were ready for co-cultures the day after.

**Immunocytochemistry.** This was performed as described (18-9) using the following antibodies: monoclonal (mAb) anti-human-cTnI, anti human-cardiac-MHC, anti-human connexin-43 (Cx-43) and polyclonal (pAb) anti-human ANP (Chemicon, CA); mAb anti-CD-31, CD-34 (BD), mAb anti-Ve-cadherin, anti-sca-1, mAb anti-mouse-cKit (Pharmigen, BD), mAb anti-human-c-Kit (DAKO, Milan, Italy); pAb anti-human-von-Willebrand-factor and mAb anti-human-KDR (Sigma); mAb and pAb anti-mouse/human MHC (18-19/9-10), and anti-Smooth-Muscle-Actin (Sigma), mAb anti-human/mouse-cTnI (20-11), donated by S. Schiaffino (Dep. Of Pathology, Univ. of Padua), pAb anti-mouse-flk-1 (Santa Cruz, USA).

Light-microscopic analysis of \( \beta \)-Galactosidase activity was performed as described (16-7).

Confocal analysis was performed with a FV500 Olympus microscope for the mice heart cryosections, and with a Leica TCS NT for double-fluorescence of human BrdU –labeled CSs and co-cultures of human GFP-labeled CSs with rat cardiomyocytes.

**Immunofluorescence and Flow Cytometric Analysis.** The mAbs used were biotin-conjugated anti-mouse CD31, FITC- conjugated anti-mouse Sca-1, Biotin-conjugated anti-mouse CD34, R-PE -conjugated anti-mouse CD117 (c-Kit) from Pharmingen (BD Biosciences, San Diego, CA); FITC -, R-PE -and PE-Cy5-Streptavidin conjugated were from Caltag (Burlingame, CA).

Briefly, \( 5 \times 10^5 \) cells were harvested from culture, washed with PBS and stained with the appropriate dilution of biotin- or fluorescence-conjugated mAb or a negative control for
30 min at 4°C, then washed twice with cold PBS. Then, cells were incubated for an additional 30 min with fluorescence-conjugated streptavidin or for double immunofluorescence with a second directly fluorescence-conjugated mAb. Those cells incubated with biotin-conjugated mAb and fluorescence-conjugated streptavidin were further incubated with a fluorescence-conjugated mAb and washed with PBS. The percentage of positive cells determined over 10,000 events acquired, was analyzed by a FACScalibur cytofluorimeter equipped with a 488-nm argon laser and CellQuest software (BD Biosciences, San Diego, CA).

In vivo analysis.

Heterotopic transplantation. About 60 washed and pooled cardiospheres, obtained from pre- and post-natal MLC3F-nlacZ/B5-eGFP, cTnI-nlacZ/B5-eGFP or MLC3F/nLacZ/CD1 and TnI-nLacZ/CD1 mice, were suspended in 100 µl of Matrigel (BD) and sub-cutaneously injected into the dorsal region of anesthetized (ketamine, 35 mg/kg i.m) adult SCID mice. Transplanted-CSs survival and function were in vivo monitored by direct palpation of beating through the skin. After three weeks, mice were sacrificed and the isolated inoculum was embedded in OCT (Miles, IN, USA) for immunocytochemical analysis.

Orthotopic transplantation. Myocardial Infarction. SCID/beige mice (Taconic Europe, Denmark) were anesthetized with Avertin (250 mg/kg i.p.), and the trachea intubated with a 22G venous catheter. Artificial ventilation (stroke volume, 1.0 to 1.5 mL; ventilation rate, 120 per minute) was initiated, and a left-sided thoracotomy in the fourth intercostal space was performed. the LAD was ligated immediately distal to the bifurcation of the left main coronary artery, using an un-traumatic needle and a 7-0 silk thread. After
ligation, successful infarction was immediately evident by a pale discoloration of LV myocardium due to ischemia. A suspension containing cardiospheres or PBS only was injected a few minutes after infarction into the LV wall bordering the infarct using a 32G needle and syringe. Each animal received four 2.5μl injections of the CS-suspension (a mean of 10 spheres/animal) or PBS. At the end of the operation, the thorax was closed, pneumothorax reduced and tracheal tubes disconnected from the ventilator, thus allowing free breathing. For sham operation, a control group of mice underwent an identical surgical procedure with the exception that the LAD was not ligated. Survival rates of mice after LAD ligation and sham operation were 71% and 100%, respectively. Animals were sacrificed 18 days post-surgery.

Echocardiography. Two-dimension and M-mode echocardiography was performed on conscious previously trained mice 18 days after surgery with a 13MHz linear probe connected to an Aloka 5500 echocardiograph. (34-11).

Infarct size. Infarct size was calculated using computer-based planimetry. OCT embedded hearts were cut from apex to base in 10 μm thick serial sections. Sections spacing 500 μm were H&E stained and used for infarct size measurement. Infarct size was defined as the sum of epicardial and endocardial infarct circumference divided by the sum of total left ventricular epicardial and endocardial circumferences.

Statistics. Results are mean ± standard deviation. Differences between groups for functional studies were tested for significance by one-way analysis of variance (ANOVA) corrected by Bonferroni test. A value of p<0.05 was considered significant.
References


**SUPPLEMENTARY MOVIES (1a-b, 2a-b)**

1. **Mouse CSs. 1a.** CSs start to beat at the beginning of their formation (after 24 h of culture in CGM). 1b. At 10 days of culture most of the CSs continue beating.

2a-b. Spontaneous differentiation of one week old prenatal CS: cells derived from the sphere show a synchronous contraction and an ordered arrangement.

2. **Co-culture of human CSs with neonatal rat cardiomyocytes. 1a.** The “sun-like” feature of the CSs evident after 96h of co-culture; beating can be observed mainly in differentiated cells (external layers), as better shown at 40x magnification (1b). 2a-2a1 2a-transmission. Co-culture with GFP-labeled human single-cell-CSs-derived clone are shown: contraction is mainly present in the external cells migrating out from the center of the sphere and in those pleated after dissociation of the clone (2a1). The same features can be observed in the 3a (transmission)-b (Hoechst labeling)-c (green light and transmission) –d (green light alone) series of movies, were co-culture of GFP-labeled human CSs with rat cardiomyocytes are shown.. 2b, 2c, 2d. Cells derived from partially dissociated human GFP-labeled CSs are co-cultured with 6 days old rat cardiomyocytes labeled with DiL (see methods). Records are performed in green light, so that the rat cells, red stained, show a brown appearance. In some human cells the dye is gone through (yellow color) suggesting that a kind of connection has been created
between the two cell populations. In the movie human GFP-labeled cells show a lined arrangement (as in prenatal mouse CSs in the “mouse CSs movies” 2a-b).

3. **Clone movies.** In this series of movies, the spontaneous contraction of a clone generated from the dissociation at single cell level of GFP-labeled mouse CSs and cultured on growth-arrested STO-fibroblasts (see methods) is shown at different magnifications, in transmission and green light.

**SUPPLEMENTARY FIGURES**

**Fig. 1.** Fluorescent, phase contrast, and merged images of eGFP/MLC3F-CSs-derived clones: **a,** a fluorescent clone appears after 48 h of culture in mytomicin-arrested STO fibroblast (fluorescence, phase, low and high magnification merged images are shown); **b,** blue nuclear staining of a spontaneously differentiated clone with clustered, round shaped cells and, **c,** same histochemical stain of an other clone grown in growth factors-free medium, showing a more flattened feature of the cells that seem to migrate from the center of the cluster toward the feeder layer.

**Fig. 2.** Fluorescence analysis of partially dissociated eGFP-labeled human CSs-derived clone at 96 h of co-culture with rat cardiomyocytes: the same green cells that showed a synchronous contraction with cardiocytes (see also human CSs movies 2a in the supplementary materials), express cTnI.
**Fig. 3.** Fluorescence-confocal analysis of BrdU-labeled (red) human CSs for MHC expression. As in Fig. 2a this high magnification picture shows a preferential localization of BrdU labeled cells in the central area of the sphere.

**Fig. 4.** Fluorescent analysis of BrdU-labeled (red) human CSs stained for MHC (green) and Hoechst (blue). Co-localization of BrdU labeling with the blue staining of the nuclei is shown in the merged image (4e).

**Fig. 5. a,** Fluorescence-confocal analysis of BrdU-labeled human CSs for cardiac differentiation markers: 6 µm scans (from the periphery to the center of the sphere) and final pictures (small and large images respectively). BrdU (green), cANP (red).  **b-b1,** Fluorescence phenotype analysis of human partially dissociated-CSs, after four days of culture on collagen coat in CEM: cTnI (red) and MHC expression appears in the cytoplasm of the human cells (migrated from the sphere) showing a triangular or polygonal shape with a row arrangement.  **c-1-2,** Fluorescence phenotype analysis of human CSs (cryosections): (c) cTnI (red), (c1) sarcomeric myosin and (c2) vWF (green).

**Fig. 6.** FACS analysis of post-natal mouse CSs-derived cells. A time course at 0 and 6 days was performed and the phenotype profile for CD34, cKit, Cd31 and sca-1 expression markers was analyzed and showed as percentage of positive events. The table data are presented as mean ± SD (n=3). *Indicates a statistically significant difference from T 0. Representative graphics for each time points are also shown.

**Fig. 7.** MLC3F-nLacZ (a-a1) and cTnI-nLcZ (b-b1) mice derived CSs. Most of the cells, derived from partially dissociated CSs and cultured for 5 days on collagen-coated surface, show a blue staining both in the adult and embryo mice.  **c, d,** Fluorescence
analysis of differentiating mouse CSs: MHC expression in the cells inside the sphere and in those starting their migration is shown (c). d, Large version of Fig. 2b.

**Fig. 8.** Fluorescent analysis of connexin-43 expression (red) in eGFP-labeled human CSs co-cultured with rat cardiomyocytes (as in fig. 1f): the punctuate red fluorescence in the cell membrane of human cells is shown at high magnification.

**Fig. 9.** Orthotopic transplantation on a SCID-bg mouse, of cryopreserved human CSs into the viable myocardium bordering a freshly produced infarct. a-a1, Fluorescence analysis of myocardial repair. Images, acquired from the center of the left ventricular anterior well, show the MHC positive stain (green) in the regeneration area of treated heart (a), that is absent in the control (a1). Low (b) and high (b1) magnification confocal analysis of cryosectioned left ventricular heart after 18 days from the coronary ligature, shows viable lamin A/C expressing cells (green) in regenerating myocardium expressing MHC (red) (b); b1, a human cell (green nucleus) is migrated and become integrated with healthy myocardium were the sarcomers red-stained for MHC are evident.

**Figure 10.** High magnification of manuscript Figure 6a (In vivo analysis (ortotopic transplantation of human CSs). The thin red stained (MHC) cytoplasmatic rings (more evident in panels b1-b2), surround the green nuclei (Lamin A/C) of newly generated myocytes.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
CSs-derived cells (FACS analysis)

Data are expressed as mean ± SD (n=3)
* Indicates a statistically significant difference (cKit: P=0.015)
5° day on collagen-coat

MLC3F-nlacZ

embryo

a1

10 µm

embryo

a2

20 µm

adult

cTnl-nlacZ

embryo

b1

10 µm

embryo

b2

10 µm

adult

Mouse

Fig. 7
Figure 8

GFP Connexin-43/hoechst merged

5 µm

a b c
d e f
Fig. 9