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Human Cardiac Progenitor Cell Grafts as Unrestricted Source of Supernumerary Cardiac Cells in Healthy Murine Hearts

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ABSTRACT

Human heart harbors a population of resident progenitor cells that can be isolated by stem cell antigen-1 antibody and expanded in culture. These cells can differentiate into cardiomyocytes in vitro and contribute to cardiac regeneration in vivo. However, when directly injected as single cell suspension, less than 1%-5% survive and differentiate. Among the major causes of this failure are the distressing protocols used to culture in vitro and implant progenitor cells into damaged hearts. Human cardiac progenitors obtained from the auricles of patients were cultured as scaffoldless engineered tissues fabricated using temperature-responsive surfaces. In the engineered tissue, progenitor cells established proper three-dimensional intercellular relationships and were embedded in self-produced extracellular matrix preserving their phenotype and multipotency in the absence of significant apoptosis. After engineered tissues were leant on visceral pericardium, a number of cells migrated into the murine myocardium and in the vascular walls, where they integrated in the respective textures.

The study demonstrates the suitability of such an approach to deliver stem cells to the myocardium. Interestingly, the successful delivery of cells in murine healthy hearts suggests that myocardium displays a continued cell cupidity that is strictly regulated by the limited release of progenitor cells by the adopted source. When an unregulated cell source is added to the system, cells are delivered to the myocardium. The exploitation of this novel concept may pave the way to the setup of new protocols in cardiac cell therapy. STEM CELLS *2011;29:2051–2061*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cardiac diseases represent the first cause of mortality and morbidity in industrialized countries, notwithstanding the continuous progress in early diagnosis and treatments [1]. Therefore, novel therapeutic strategies are urgently needed, featuring safety, sophistication and cost-effectiveness, and ease of production and clinical application. In this context, cellular therapies did not hold initial promises to definitively cure cardiac diseases. Nevertheless, recent evidences suggest that adult progenitor cells can be used to fabricate ex vivo engineered cardiac tissue to be implanted in the injured myocardium. To this aim, progenitor cells can be appropriately grown on polymeric scaffolds [2, 3] or, alternatively, thermo-responsive surfaces [4–6]. Following the latter procedure, scaffoldless tissue sheets fabricated using clonally expanded stem cell antigen-1 positive (Sca-1^{pos}) progenitor cells (CPCs) isolated from murine hearts, prevascularized [7] and transplanted into hearts with experimental myocardial infarction have shown to prevent cardiac

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remodeling and fibrosis, very likely, promoting neoangiogenesis by paracrine mechanisms [8].

Besides an adequate scaffolding procedure, the fabrication of tissue substitutes requires the selection of the most appropriate cell type with respect to the target tissue. Concerning myocardium, muscle and nonmuscle cell population from multipotent progenitors located in the first and second heart fields contribute to cardiogenesis [9, 10], tangling the identification of the resident progenitor cells actually involved in myocardial homeostasis throughout lifetime [11]. Cells expressing c-kit/CD117 have been acknowledged as the "true cardiomyocyte progenitors" [12]; however, Sca-1 has been recently shown to be a marker identifying a murine [13] and a human [14] heart cell population with cardiomyogenic potential, since both could generate new cardiomyocytes in vitro and in vivo on specific treatments [13–16]. Indeed, Sca-1 is a very efficient marker to isolate and to enrich for progenitor cells from various tissues [2, 17] and has been associated with multipotency and self-renewal in bone marrow, skeletal muscle, dermis, heart, and liver [18]. The relative abundance and rapid ex vivo expansion of Sca-1^{pos} progenitor cells after isolation from human specimens candidates these cells as the optimal cellular population for cardiac repair. Therefore, the present study was designed (a) to investigate whether human Sca-1^{pos} cardiac progenitor cells expanded after isolation from patients during cardiac surgery could be used to fabricate scaffoldless cardiac prototissues, while retaining their undifferentiated multipotent status, (b) to assess the potential of Sca-1^{pos} cardiac progenitor cells embedded into the ex vivo engineered tissue to home into the ventricular wall, and (c) to analyze whether, once transplanted, cells could start a differentiation pathway toward cardiac cell phenotypes.

Materials and Methods

Isolation, Characterization, and Culture of Human Cardiac Progenitor Cells

Patients ($n = 13$; seven male, six female, 52-83 years) undergoing cardiac surgery were enrolled in the present study after signing a written consent form according to a joint protocol approved by the Ethic Committees of Ospedale Maggiore della Carità, Novara and University Hospital "Le Molinette," Turin, Italy.

Human cardiac progenitor cells (hCPCs) were obtained from right auricular biopsies. Briefly, 1-3 cm^3 specimens were harvested and treated under sterile hood. Samples were mechanically minced and incubated at 37°C for 2 hours in 0.05% trypsin, 0.02% EDTA in phosphate-buffered solution (PBS), followed by collagenase II (1500 U, Worthington Biochemical Corporation) in Leibovitz medium (Worthington Biochemical Corporation, NJ, USA) for 60 minutes at 37°C. Reactions were blocked in cold medium. Fragments were centrifuged at 4°C for 10 minutes at 800*g*, pellets were resuspended in Dulbecco's modified Eagle medium (Lonza Group Ltd, Basel, CH), 10% fetal bovine serum (FBS, Lonza Group Ltd), 100 IU/ml penicillin and 100 μg/ml streptomycin, insulin-transferrin-selenium $\times$1, 300 ng/ml retinoic acid, 0.8 μg/ml linoleic acid, 2 mM L-glutamine, 0.1 ng/ml insulin-like growth factor 1, and 0.1 ng/ml endothelial growth factor (hereafter referred to as "complete medium"), recovered by filtration through a 100-μm cell strainer (BD Biosciences, NJ, USA), and incubated at 37°C, 5% CO_2. The following day, medium and nonadhering fragments were removed and replaced with fresh complete medium, which was then changed every second day. After 10-15 days, fibroblastoid cells migrated from the fragments and reached confluence. Cells were expanded in complete medium or analyzed for c-kit expression by flow cytometry. Cells were selected by magnetic immunobeads with anti-c-kit antibody (Miltenyi Biotec, Bergisch Gladbach, DE) and plated on fibronectin (2 μg/ml, Sigma-Aldrich, MO, USA) precoated dishes in complete medium or analyzed in flow cytometry for immunophenotype characterization (see below). For coculture experiments with murine neonatal cardiomyocytes (nCMs), hCPCs were prestained with the viable red fluorescent dye Vybrant DiI (Molecular Probes, Invitrogen, CA, USA) following the manufacturer's instructions.

Isolation of Murine nCMs and Coculture Experiments

nCMs were isolated from hearts of 1-3-day-old C57/Bl/6 mice following the manufacturer's instructions (kit by Worthington Biochemical Corporation). Immediately after isolation, cells were preplated for 30 minutes to recover the non-adherent-enriched fraction of nCMs, which were then seeded on fibronectin (2 μg/ml), laminin (0.2%), gelatin (0.02%, Sigma-Aldrich) precoated plates or precoated glass chamber slides (BD Biosciences), in case of coculture experiments [19]. After 24 hours, Vybrant-labeled hCPCs were seeded directly onto cardiomyocytes (1:5) in complete medium. Cocultures were prolonged up to 7 days. hCPC and nCMs alone were used as negative controls.

Assessment of hCPC Multilineage Ability

hCPCs (2×10^5 cells per dish) were plated onto 35-mm dishes and cultured in adipogenic or osteogenic (Lonza Group Ltd) media. Media were changed every second day. After 14 days, Adipored (Lonza Group Ltd) and Alizarin Red S (Sigma-Aldrich) decoration were used to detect the adipogenic and the osteoblastic differentiation, respectively. The presence of lipid vacuoles was visualized under fluorescence microscope, while the production of calcium deposits was confirmed using light microscopy. Chondrogenic differentiation was induced by chondrogenic medium containing transforming growth factor β3 (TGF β3) (Lonza Group Ltd) on a cell pellet and assessed by Alcian Blue (Sigma-Aldrich) staining after 4-week stimulation. Cells cultured in the absence of induction media were used as negative controls.

Preparation of Human CPC Cell Sheets Using Temperature-Responsive Dishes

Human CPCs (5×10^5 cells per dish) were cultured in standard conditions and, once at passage 7-12, were detached by trypsin, stained or not with Vybrant DiI, plated onto 60-mm temperature-responsive poly-*N*-isopropyl-acrylamide (PnIPAAm)-coated dishes (Thermo Fisher Scientific, MA, USA) pretreated for 6 hours with FBS at 37°C, and cultured at 37°C, 5% CO_2. After 3 days, confluent cell monolayers were detached by incubation at 20°C for 45 minutes. Cell sheets were gently aspirated using 10-ml pipettes and transferred onto standard cell culture dish (BD Biosciences). The adherent sheets were cultured up to 7 days at 37°C for further analyses.

TUNEL Assay

The number of apoptotic cells in the hCPC sheets was determined 1, 3, and 7 days after sheet preparation in snap-frozen, thin cross-sections and was expressed as the number of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive cells (R&D Systems, MN, USA)/total nucleated cells in at least 12 independent fields. Stem cells grown in monolayer, treated or not with 5 μM H_2O_2, were used as controls, as described elsewhere [20]. The experiment was repeated three times.

hCPC Cytofluorimetric Analysis

Cells were incubated for 20 minutes with anti-Sca-1-fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled or anti-c-kit-PE (BD Biosciences) and washed twice with ice-cold PBS. Alternatively, cells were stained for 20 minutes with anti-CD90, CD105, ABCG2 PE-labeled or CD34, CD44 FITC-labeled

antibodies (Biolegend, CA, USA). Cells were then fixed in buffered 1% paraformaldehyde (PFA), 2% FBS for 15 minutes at 4°C and analyzed in a FACScalibur flow cytometer (BD Biosciences) within 48 hours.

hCPC Immunofluorescence

Cells at passages 5 (p5), 35 (p35), and 50 (p50) were seeded at the concentration of 2×10^4 per cm^2 on glass chamber slides (Thermo Scientific) and used when 60%-70% confluent. They were washed in PBS, fixed in 3% PFA in PBS containing $CaCl_2$ for 30 minutes at 4°C and permeabilized with 0.1% Triton X-100 for 2 minutes. hCPC sheets were fixed, embedded in octamer-binding transcription factor (OCT) solution (Bio-Optica, Milano SpA, Milano, Italy) and snap-frozen in liquid nitrogen. They were cross-sectioned (5-μm slices) with a Microm HM550 (Bio-Optica). Cells, sheets, and cardiac tissue sections were incubated with antibodies specific for Sca-1, c-kit (BD Biosciences), CD31, fetal liver kinase-1 (FLK-1), GATA binding protein-4 (GATA-4), NK2 transcription factor related, locus 5 (Nkx-2.5) (Santa Cruz Biotechnology, Inc., CA, USA), connexin 43, α-smooth muscle actin (αSMA), caveolin 3, α-actinin, vinculin, laminin (Sigma-Aldrich), cardiac troponin T (Abcam, Cambridge, UK), collagens I, IV, and XVIII, human nuclei (Millipore, MA, USA), followed by the appropriate 488-Alexa fluorochrome-conjugated secondary antibodies (Invitrogen). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI, Invitrogen). Positive cells were counted in at least 12 independent fields against total nucleated cells. Images were taken with a Leica DMRB microscope equipped with a digital camera or with a Leica TCS-SP5 confocal microscope.

hCPC Sheets Gene Expression Analysis

Analysis of gene expression in hCPC cell sheets (in quadruplicate), when compared with ex vivo isolated cells seeded onto standard culture plates (80% confluent), was performed using the PIQOR Human Stem Cell Microarray (Miltenyi Biotec). Briefly, hCPCs were immunoselected for their expression of c-kit, put in culture or used to prepare cell sheets on thermo-responsive surfaces. Cell pellets were frozen at −80°C for subsequent analyses. The bioinformatic analysis was performed on datasets obtained from competitive hybridization of sample RNAs on topic-defined PIQOR Stem Cell Microarrays after T7 RNA amplification. Human cell sheet samples were labeled with Cy5 and hCPCs from conventional monolayer cultures, used as hybridization reference, were labeled with Cy3. The kit is designed to analyze 942 genes involved in stem cell status maintenance, proliferation, and lineage commitment. Genes having a significant variation in the same direction in all the four tested samples were filtered. Differences in gene expression were selected using a "one-class" t test with very permissive settings [t test against zero; $n = 4$, Welch approximation for unequal variance, p-values determined based on distribution, no p-value adjustment for multiple testing, p-value cut-off of 0.05 (uncorrected)]. To discriminate questionable results from relevant results, the cells of spots/genes that did not pass the quality filtering were blanked because they were either flagged or had very low signal intensities (<2-fold above average signal intensities of the background in both Cy3 and Cy5 channels).

hCPC Sheet Transplantation and Light Microscope Examination

C57Bl/6 mice ($n = 6$) were treated with cyclosporine (25 μg/g) and antibiotics 2 days before the implant and then every second day until 6 days after the implant. Animals were anesthetized with Avertin (0.2 ml/g) and mechanically ventilated by Minivent (Hugo Sacks, March-Hugstetten, DE). Thoracotomy was performed via the forth left-intercostal space, the parietal pericardium was partially removed and the left ventricle exposed. Cell sheets (3 × 3 mm areas containing approximately 1 million Vybrant Red-labeled hCPCs) were placed on a plastic sheet and leaned face down onto the visceral pericardium corresponding to the left ventricle without suture; after 20-30 minutes, the plastic sheet was removed and chest closed. Thereafter, mice were allowed to recover. Control sham-operated mice were treated with the same procedure except no cell sheet was stuck on the heart surface. After 1-2 weeks, mice were killed, their hearts excised, perfused with PBS containing cadmium chloride, embedded in OCT solution (Bio-Optica), snap-frozen, and cut in 5-μm sections. Animal studies were performed according to the guidelines of the Animal Care and Use Committee of the University of Rome "Tor Vergata."

Hemodynamic Measurements of Cardiac Function

Cardiac function was assessed in cell sheet-treated versus control mice 2 weeks after the engineered tissue implantation in the heart. After adequate anesthesia was attained with ketamine and fentanyl, the right carotid artery was isolated and cannulated with a 1.4 French Millar SPR 671 catheter (Millar Instruments, TX, USA). The catheter was passed down the aorta into the left ventricle and the heart rate, left ventricular end-diastolic and end-systolic pressure and the maximum rate of increase (dP/dt_{max}) and decline (dP/dt_{min}) in left ventricular pressure were measured.

Statistical Analysis

The results are shown as mean ± standard deviation (SD) as derived by unpaired t test. The values are considered significantly different when $p < .05$. The number of replicated experiments performed is given as n.

RESULTS

Human Right Auricle as Source of Undifferentiated Multipotent Sca-1^{pos} Progenitor Cells

After 4-7 days of culture in complete medium, fibroblast-like cells migrated from the myocardial fragments (Fig. 1A) obtained from the right auricle samples of cardiosurgery patients ($n = 13$). When confluent, the cells were detached and, after cytofluorimetric analysis for c-kit expression (Fig. 1B), the positive fraction was immunomagnetically enriched and subjected to further analyses and experiments. The preparations obtained showed patient-to-patient variability in c-kit expression and a preparation dubbed Torino patient 2 (TO2) (cell preparation 2 in Fig. 1B) was chosen for further evaluation. After three passages, further cytofluorimetric characterization showed that the selected cells expressed other mesenchymal stemness markers, namely Sca-1 (99%), CD90 (95%), CD105/endoglin (70%), with only a small proportion being ABCG2^{pos} (5%) and CD34^{pos} (<1%). With time (from passage 3 to passage 10), c-kit, CD90 and CD105 positive cells were reduced, while Sca-1 expression remained rather stable (Fig. 1C). Indeed, 76.6% of the freshly isolated hCPCs coexpressed c-kit and Sca-1 markers, with 14.7% expressing only Sca-1 and 7.6% expressing only c-kit. At passage 13, c-kit was coexpressed on 15.5% of Sca-1^{pos} cells, while 84% of the population was exclusively Sca-1^{pos} (Fig. 1D). Interestingly, any time the c-kit selection was repeated, the percentage of c-kit^{pos} cells dropped constantly below 20% within few passages (Fig. 1E).

Immunofluorescence analysis of c-kit-enriched hCPCs was performed at passage 10 confirming previous cytofluorimetric data and showing that a small number of cells expressed FLK-1, GATA-4, and Nkx-2.5 (Fig. 2A, 2C). Cells were negative for CD31 and markers characterizing fully differentiated cardiomyocytes [α-actinin, caveolin 3, and cardiac troponin I (cTnI)], while, as expected, they were positive for the control mesenchymal marker αSMA. Moreover, freshly isolated

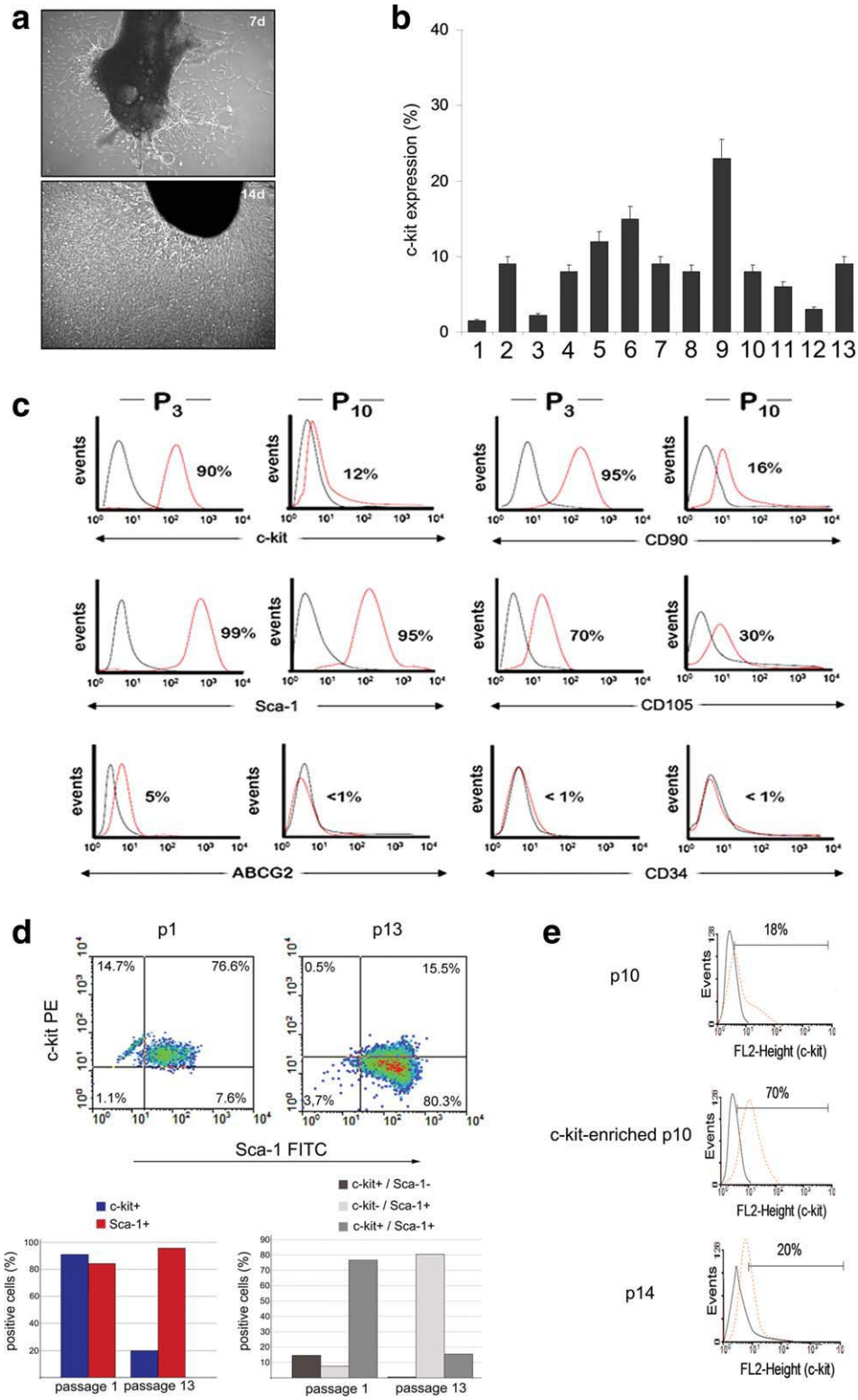
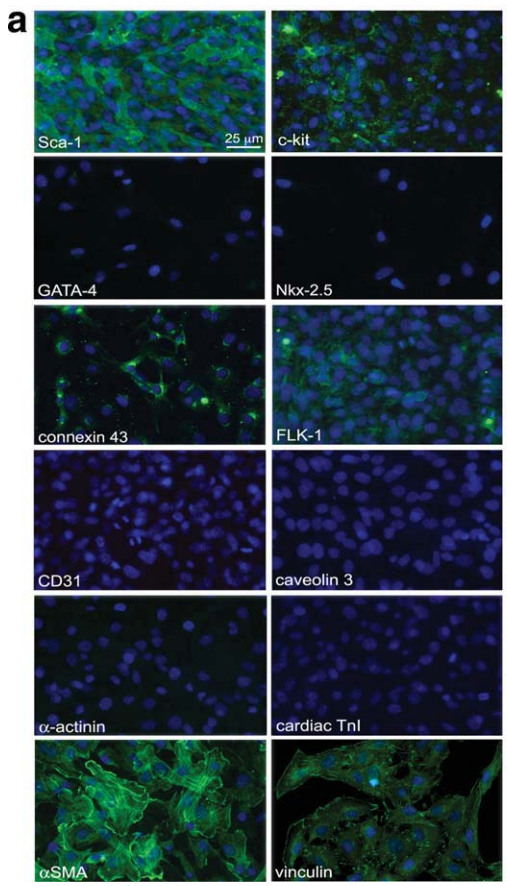
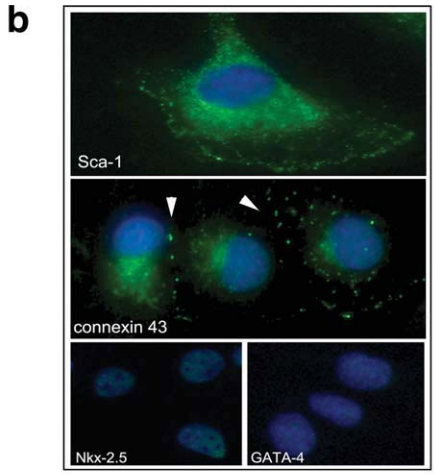


Figure 1. Sca-1 is a stable and reliable marker of human cardiac progenitor cells isolated from right auricles. Fibroblast-like cells migrated from right auricular biopsy fragments excised from 13 patients and cultured for 7-14 days (**A**). Among them, a mean of 9.7% cells were positive for c-kit expression ($n = 13$, **B**). When confluent, human auricular cells expressing c-kit were selected by immunosorting and analyzed in cytofluorimetry. The expression of c-kit, CD90, and CD105 in c-kit-enriched cells was downregulated within the first 10 passages in culture, while the Sca-1 antigen was stably expressed over the culture. ABCG2 and CD34 were hardly detected in human cells (**C**). The shift in c-kit, Sca-1 expression was confirmed in coexpression experiments (passage 1: p1 and passage 13: p13, **D**). Passage 10 c-kit-enriched cells were selected again for c-kit expression; nonetheless, within few passages (p14), the percentage of c-kit-positive cells was reduced again to 20% (**E**). Representative cytofluorimetric profiles obtained in one out of three experiments performed for each preparation are reported. Abbreviations: FITC, fluorescein isothiocyanate; FL-2, fluorescence 2; PE, phycoerythrin; and Sca-1, stem cell antigen-1.





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	Passage 10	Passage 35	Passage 50
Sca-1	93.5±4.5%	92.0±1.5%	93.0±2.0%
c-kit	12.3±2.5%	9.2±1.5%	8.2±1.7%
GATA-4	5.3±2.6%	5.0±1.2%	4.7±1.5%
Nkx-2.5	6.4±1.8%(#)	13.5±1.5%	21.2±2.3%(##)
connexin 43*	6.0±1.1%	5.2±0.9%	13.4±1.7%(¶)
FLK 1	8.5±1.6%	6.5±1.6%	5.3±1.7%
CD31	n.d.	n.d.	n.d.
caveolin 3	n.d.	n.d.	n.d.
α-actinin	n.d.	n.d.	n.d.
cardiac TnI	n.d.	n.d.	n.d.

Figure 2. Human Sca-1^{pos} cardiac cells retain undifferentiated phenotype in culture. Fresh human cardiac progenitor cells in standard culture conditions were fixed and stained for indirect immunofluorescence. Most of the cells were Sca-1 and αSMA positive, only few were c-kit, GATA-4, Nkx-2.5, and FLK-1 positive, and no staining for CD31, caveolin 3, α-actinin, or cardiac TnI was detected. Instead, proper vinculin expression and perinuclear connexin 43 distribution were observed in most of the cells (**A**). In long-term culture (passages 35 and 50), Sca-1 expression was maintained in a vast number of cells, while the number of Nkx-2.5 and connexin 43 positive cells was increased, with most of these cells displaying functional membrane localization of connexin 43 (arrowheads in **B** and * in **C**). No staining for structural cardiomyocyte proteins was detected at any stage of the culture. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole. The data in (C) are reported as mean ± SD. $^{\#}p < .05$ when compared with passages 35 and 50; $^{\#\#}p < .05$ when compared with passage 35. $^{\P}p < .05$ when compared with passages 10 and 35; $n = 6$. Abbreviations: FLK-1, fetal liver kinase 1; GATA-4, GATA binding protein-4; Nkx-2.5, NK2 transcription factor related, locus 5; Sca-1, stem cell antigen-1; αSMA, α-smooth muscle actin; and TnI, troponin I.

hCPCs exhibited a perinuclear localization of connexin 43 that translocated to the membrane in a number of cells in long-term culture (13.4% ± 1.7% at passage 50; $p < .05$). Conversely, the expression of Sca-1 was preserved (>90% at passages 35 and 50) and Nkx-2.5 was upregulated (21.2% ± 2.3% cells at passage 50; $p < .05$), while GATA-4 was not significantly affected throughout the culture ($p > .05$ in Fig. 2B, 2C). Such evidences suggested that hCPCs, although maintaining stemness phenotypic features (Sca-1 expression), displayed some characters of the early cardiac phenotype.

Sca-1^{pos} hCPC multilineage potential was positively assessed by stimulation with specific adipogenic, osteogenic, and chondrogenic media (Fig. 3A). More importantly, the cardiomyogenic potential was assessed in hCPCs cocultured with murine nCMs (ratio 1:5) for 1 week. In these conditions, Vybrant-labeled hCPCs displayed a marked upregulation of GATA-4 expression (45.9% ± 4.8% when compared with 5.3% ± 2.6%; $p < .01$), translocation of connexin 43 to the membrane (59.5% ± 5.1% when compared with 6.0% ± 1.1%; $p < .01$) and de novo expression of cTnI (31.3% ± 3.4%) and α-sarcomeric actinin (α-actinin; 25.4% ± 3.0%) (Fig. 3B, 3C). These data eventually demonstrated that hCPCs endowed with the acknowledged properties (stemness markers, ability to be propagated in tissue culture and multipotentiality, including the cardiogenicity) can actually be isolated from human auricles by means of anti-murine Sca-1 antibodies.

Fabrication of Cell Sheets of Undifferentiated Human CPC

Sca-1^{pos} hCPCs were cultured on PNIPAAm thermo-responsive dishes to obtain human cell sheets, which were then detached by lowering the temperature below 20°C (Fig. 4A). The possibility that hCPC sheets could undergo apoptosis was ruled out in preparations assessed 1, 3, and 7 days after the detachment, when compared with hCPCs control cultures grown in conventional tissue culture polystyrene (TCPS) plates or hCPCs treated with 5 μM H_2O_2 (Fig. 4B). After detachment, each cell sheet was four- to five-cell thick and displayed a surface of approximately 3.5 cm^2, contained an average of 3.5×10^6 hCPCs expressing high levels of Sca-1, CD105, and αSMA proteins, and being negative for multidrug resistance 1 (MDR-1), c-kit, Nkx-2.5, GATA-4, and cardiac structural proteins (α-myosin heavy chain and α-sarcomeric actinin). Vinculin and connexin 43 expression was also detected within the cell sheets (Fig. 4C).

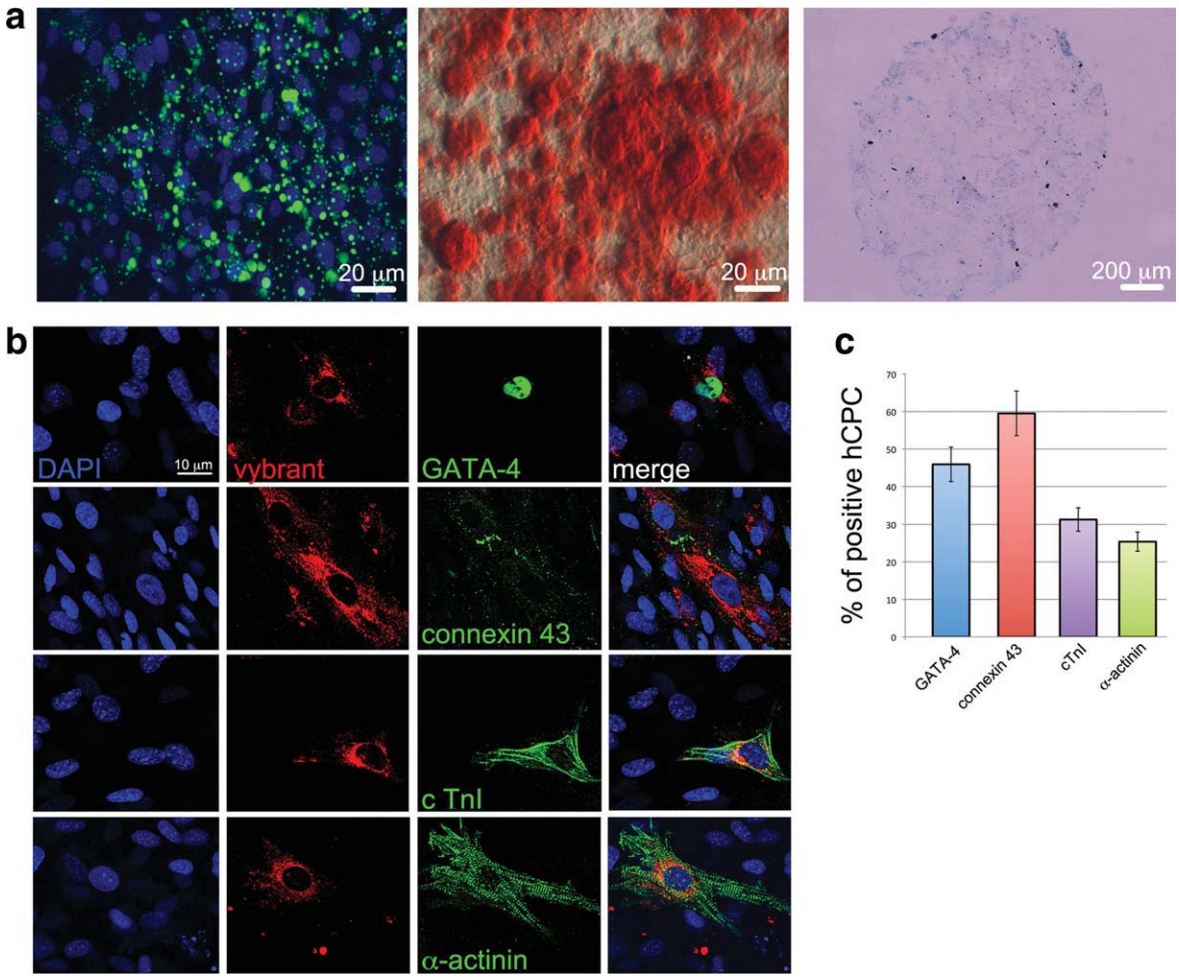

Figure 3. Human stem cell antigen-1 positive (Sca-1^{pos}) cardiac cells are multipotent in vitro. Specific treatments induced Sca-1^{pos} human cardiac progenitor cells (hCPCs) toward adipogenic (Adipored staining in **A**, left panel), osteogenic (Alizarin Red S staining in A, central panel) and chondrogenic (Alcian Blue staining in A, right panel) differentiation. Vybrant Dil-labeled hCPC (red) cocultured with neonatal murine cardiomyocyte (ratio 1:5) for 7 days were induced to express GATA-4, membrane connexin 43, and sarcomeric proteins like cTnI and α sarcomeric actinin (α-actinin, **B**). The quantification of human differentiated cells after 7-day coculture with murine neonatal cardiomyocytes is reported in (**C**). The results are shown as mean ± SD and are obtained counting 12 independent fields at ×10 magnification ($n = 3$). Abbreviations: cTnI, cardiac troponin I; DAPI, 4′,6′-diamidino-2-phenylindole; and GATA-4, GATA binding protein-4.

hCPCs within cell sheets were compared to freshly isolated Sca-1^{pos} hCPCs in standard culture conditions by PIQOR DNA microarray for a panel of 942 genes. Genes-encoding proteins involved in the angiogenetic pathways (TEK, VGR1, ANGPT1, and FGF11), extracellular matrix (COL1A1, COL1A2, COL6A1, COL6A2, COL11A2, ITGA3, ITGAB8, LAMA 1, NIDOGEN, and FN1), and cardiac progenitor maturation (GATA-4, ISLET 1, TGF βI, Myocardin, and BMPR 2) were upregulated in hCPC cell sheets. By contrast, genes playing a role in Notch signaling (Wnt family and Jag 1), cell cycle progression (TERT, MDM2, HDAC 2, HRAD17, CDC25B, CYCLIN E, CYCLIN B2, and P53), and the maintenance of the undifferentiated phenotype (KIT, ID1, THY 1, TGF βRI, and TGF βRII) were downregulated (Table 1).

Cell Sheets as hCPC Delivery Systems

To investigate the possibility that cell sheet technology could be used as delivery system for hCPCs, a cell sheet fragment (3 × 3 mm) was stuck onto the visceral pericardium of the left ventricular wall of healthy immunosuppressed mice. One week after the implantation, cell sheet fragments stained positive for human collagens I and XVIII and laminin, but not for collagen IV (Fig. 5A). At this time point, the cell sheet fragments could be still found on the ventricular wall, as demonstrated by hematoxylin/eosin staining (Fig. 5B and Supporting Information Fig. 1). However, Vybrant Red-prelabeled Sca^{pos} hCPCs crossed the visceral pericardium and migrated into the thickness of the ventricular wall, while still expressing Sca-1 antigen (Fig. 5B, bottom panel). Few Vybrant-positive cells could be found in contact with host cardiomyocytes and starting expressing α-sarcomeric actinin (Fig. 5B). Two weeks after sheet implantation, hCPCs could be detected inside the host tissue, as demonstrated by anti-human nuclei antibody staining (in red). Such cells displayed cardiomyocyte features, namely α-sarcomeric actinin organized in typical bands (Fig. 5C, left panel). Moreover, few human cells acquired a vascular phenotype (anti-αSMA in red and anti-human nuclei in green), being fully integrated within the host coronary walls (Fig. 5C, right panel). Interestingly, when hCPC-rich areas were investigated for connexin 43 expression, homotypic as well as heterotypic junctions were found within the infiltrated tissue (Fig. 5D), thus testifying the formation of electromechanical coupling between human and host cells. No CD45 positive cells were detectable in the implant area (data not shown), suggesting the absence of

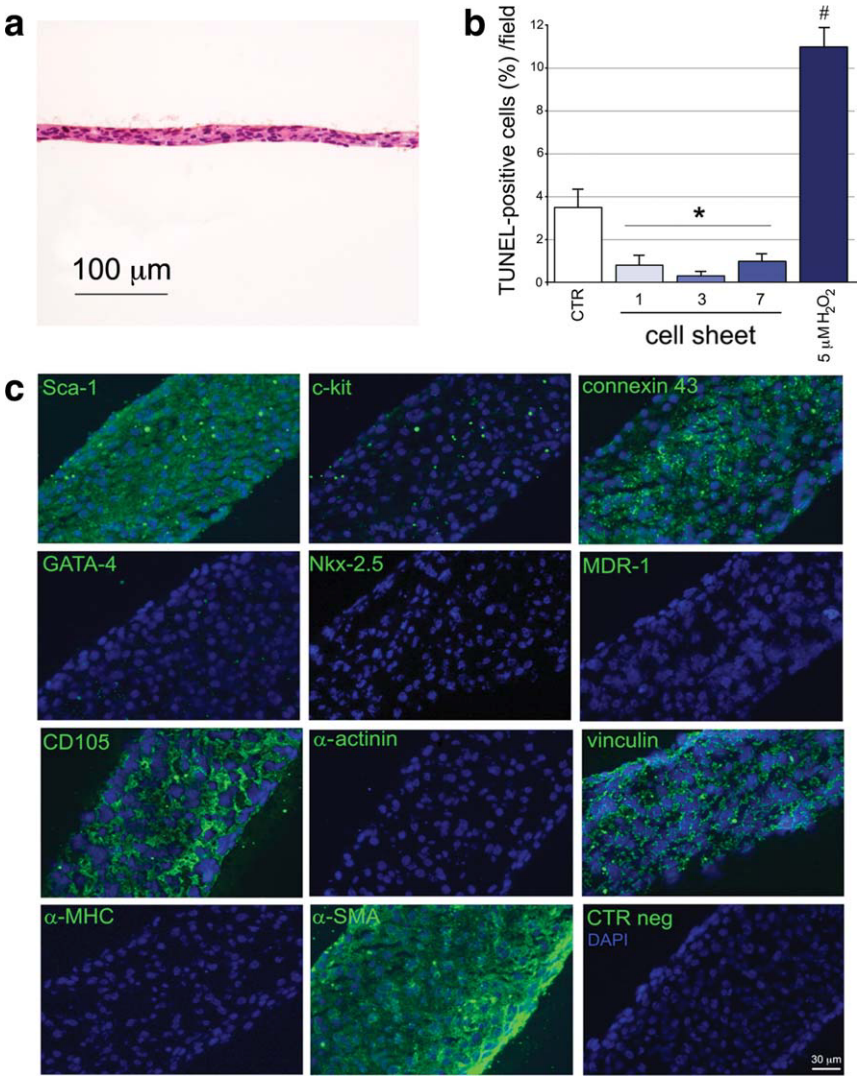

Figure 4. Generation and characterization of human Sca-1^{pos} cardiac progenitor cell sheets using thermo-responsive technology. Sca-1^{pos} human cardiac progenitor cells (hCPCs) grown to confluence onto thermo-responsive dishes (35 mm) at 37°C were exposed to 20°C to allow cell layer spontaneous detachment. The detached sheets were four- to five-cell layers thick (**A**, hematoxylin-eosin staining) and the number of apoptotic cells 1, 3, and 7 days after sheet detachment was compared to those of Sca-1^{pos} hCPCs either in CTRs or treated with H_2O_2 (5 μM) (**B**, TUNEL assay). The data represent the mean ± SD of three independent experiments carried out at least in triplicate. The immunostaining of the cross-sections **(C)** showed that hCPC sheets expressed high levels of αSMA and of the stemness markers Sca-1 and CD105, while no expression of c-kit and MDR-1 was detectable. Also, cardiac markers like GATA-4, Nkx-2.5, α-MHC, or α sarcomeric actinin (α-actinin) were not detectable. Abbreviations: CTR, control culture plate; GATA-4, GATA binding protein-4; MDR-1, multidrug resistance 1; α-MHC, α-myosin heavy chain; Nkx-2.5, NK2 transcription factor related, locus 5; Sca-1, stem cell antigen-1; α-SMA, α-smooth muscle actin; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

significant inflammatory processes. Furthermore, no significant alterations in cardiac function parameters were detectable 2 weeks after the engineered tissue implantation in the heart (Supporting Information table).

Discussion

Cardiac cell therapy applied to the injured myocardium remains an unkept promise owing to the low viability of the transplanted cells (<1% of the cells are detectable in the target area after 1 week) [21] and the insufficient mechanical support to the heart function [22] attainable by present technologies. In fact, among others, current protocols for cardiac cell therapy do not allow preserving an optimal cell viability/function and maximizing differentiation yields, since progenitor cells are cultured under conventional conditions that have been established decades ago for differentiated cells and neglect the stem cell peculiar environment (niches) in vivo. The subsequent enzyme manipulation damages cell membrane and destroys the self-produced extracellular matrix required for progenitor cell homing and differentiation [19, 23]. Finally, cells deprived of their natural adhesion structures are delivered as single cell suspension while exposed to distressing conditions (such as syringe and intraneedle pressure, and host critical ischemic microenvironment), to which they, usually embedded in the less-stressed atrial or apical myocardium [24], are not structurally and functionally adapted. As a result, most of the progenitor cells injected into the myocardium are removed by the blood stream and eventually entrapped in the lungs and the liver, or induced to apoptosis by unfavorable environmental conditions [25].

Table 1. The table reports the data obtained by PIQOR Stem Cell microarray performed on stem cell antigen-1 positive (Sca-1^{pos}) human cardiac progenitor cell (hCPC) sheets vs. Sca-1^{pos} hCPC in standard culture conditions

Gene	Fold increase	Ref. seq.
KRT14	6.6	NM_000526
COL1A1	4.4	NM_000088
FN1_REPEAT-B	4.3	NM_002026
BUB1	3.7	NM_004336
NRG3	3.6	NM_001010848
TC10-PIGF	3.4	NM_002643
FN1_EIIIA	3.3	NM_002026
PTN	3.3	NM_002825
ISL1	3.2	NM_002202
COL1A2	3.0	NM_000089
ITGA3	3.0	NM_002204
NIDOGEN	2.8	NM_002508
GATA-4	2.6	NM_002052
ITGB8	2.4	NM_002214
ANXA2	2.4	NM_001002857
VIM	2.2	NM_003380
RARB2_1	2.1	NM_000965
ACTG2	2.1	NM_001615
ALPL	2.1	NM_000478
COL6A2_1	2.1	NM_001849
CD44_EX7-9_HUMAN	2.0	NM_000610
COL6A1	2.0	NM_001848
MGST1	1.9	NM_020300
COL11A2	2.0	NM_080679
LAMA1	2.1	NM_005559
TGFB1	4.0	NM_000660
BMPR2	2.4	NM_001204
ANGPT1	2.3	NM_001146
TEK	2.1	NM_000459
MYOCD	2.0	NM_153604
PAX5	1.9	NM_016734
TGFB3	1.9	NM_003239
VGR1	1.9	NM_002019
FGF11	1.8	NM_004112
RPL24	−2.0	NM_000986
DDX21	−2.0	NM_004728
NRG1	−2.1	NM_013956
GNL3	−2.1	NM_014366
P53	−2.1	NM_000546
ATF4	−2.2	NM_001675
TCF4	−2.2	NM_003199
COL8A1	−2.3	NM_001850
HSPA9	−2.3	NM_004134
MTHFD2	−2.4	NM_006636
ITGB7	−2.4	NM_000889
NPM1	−2.4	NM_001004419
NOP5	−2.5	NM_015934
TGFBR1	−2.6	NM_004612
CLCN3	−2.7	NM_001829
BRCA1	−2.8	NM_007294
HDAC2	−2.9	NM_001527
ID1	−3.0	NM_002165
SNRPF	−3.1	NM_003095
IL6	−3.6	NM_00060
COL18A1_1	−3.5	NM_030582
HSPA4_1	−3.5	NM_002154
TNFSF11	−5.8	NM_003701
CCNE1	−11.6	NM_001238
BMP5	−2.0	NM_021073
GATA-2	−2.0	NM_032638
FGF7	−2.1	NM_002009
BMP8A-BMP8B_HUMAN	−2.5	NM_001720
AKT1	−2.6	NM_001014431
CDKN2A_1	−2.8	NM_000077
WNT10B	−2.8	NM_003394
WNT3	−3.0	NM_030753
TGFBR2	−3.9	NM_001024847
KIT	−4.0	NM_000222
JAG1	−4.8	NM_000214
WNT11	−5.2	NM_004626
TERT	−5.7	NM_198253
MDM2	−6.2	NM_002392
WNT4	−8.2	NM_030761
THY1	−9.2	NM_006288

The experiment was repeated in quadruplicate and only genes displaying consistent deviation in all the four tested samples were considered.

The present investigation demonstrated that, applying a different less-stressful strategy, a large number of viable human progenitor cells can be fairly delivered into the myocardium, where they differentiated and definitively integrated into the pristine tissue architecture.

Human progenitor cells were isolated from surgically excised auricular fragments of patients aged 45-83 years and potentially candidated to receive a cell implant into their injured hearts. Initially, cells migrating from auricle fragments were immunosorted for the c-kit surface marker expression and cultured in conventional conditions to expand the cell colony. Consistently with previous reports [26], the expression of c-kit revealed to be unstable and declined rapidly, stabilizing in no more than 15%-20% of cell population, while most of the isolated cells retained the expression of the stemness marker Sca-1. However, few passages after enriching the c-kit^{pos} cell fraction from 15%-20%-90%, the original c-kit^{pos}/Sca-1^{pos} ratio was restored. This could imply that c-kit^{pos} and Sca-1^{pos} progenitor cells, so far considered rather different cell population with overlapping figures [27] could represent two phenotypic stages of the same original population, in which c-kit expression could mark the more immature, slowly dividing progenitor cell pool, identified also in niches in vivo [20], while a more mature, actively growing and potentially cardiogenic progenitor cell population (a transit-amplifying population?) can be identified by the Sca-1 expression, as already demonstrated both in vitro and in vivo [13, 14, 25, 28]. The hCPC phenotype was homogeneous in all preparations from the 13 donor cardiac patients and stable also in long-term conventional cultures (Sca-1^{pos} cells are able to uphold their phenotype for more than 50 passages).

After initial passages (p7-p10) in conventional conditions, the Sca-1^{pos} hCPC culture was continued in a three-dimensional scaffoldless system represented by four/five-cell-thick sheets, in which cells, embedded in self-produced extracellular matrix, could establish more physiological spatial relationships. In the present investigation, the scaffoldless cell sheet technology, originally developed to fabricate rat mesenchymal and murine cardiac progenitor cell sheets [4, 7], was adopted for the first time to fabricate human cardiac engineered tissues to emulate the natural hCPC environmental architecture and to minimize the stress caused by conventional manipulations. Indeed, engineered hCPCs retained their Sca-1^{pos} undifferentiated phenotype, but dramatically reduced their growth rate; in fact, genes involved in cell cycling were downregulated and very few apoptotic events occurred within 7 days after sheet fabrication. In addition, progenitor cells embedded in the engineered tissue upregulated the transcription of the genes involved in early cardiac commitment (GATA-4 and Myocardin), but in the absence of any detectable expression of the corresponding proteins or other cardiomyocyte structural

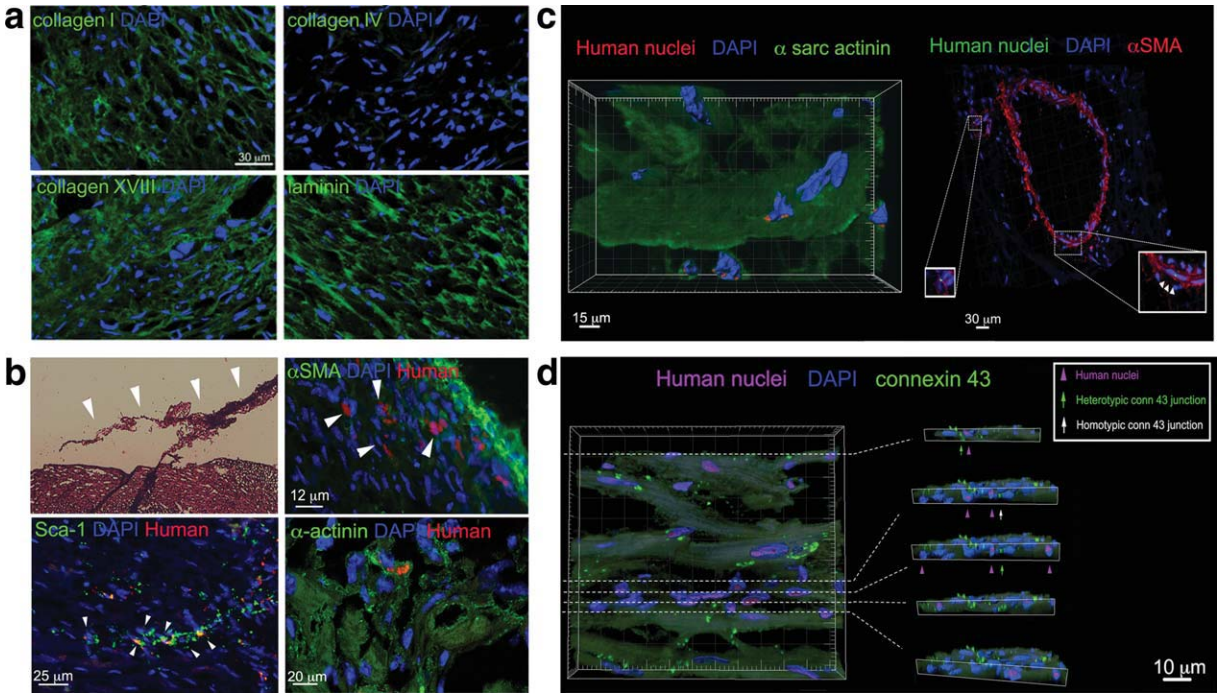

Figure 5. Implant of human Sca-1^{pos} cardiac progenitor cells on murine hearts. Human cardiac progenitor cell (hCPC) sheets were leant on the left ventricular wall of murine hearts. One week after the implant, animals were sacrificed and fragments of the Sca-1^{pos} hCPC sheets leant on the ventricular wall were recovered and analyzed in immunofluorescence for the expression of the extracellular matrix components **(A)**. The expression of collagens I and XVIII and laminin, but not collagen IV, was detected. At the same time point, cell sheets could be found on the left ventricle (**B**, top, arrowheads, hematoxylin-eosin) and human Vybrant Red-labeled cells (B, second panel, red, arrowheads) migrated across the visceral pericardium (αSMA positive in green). These migrating cells still expressed Sca-1 (B, third panel, green, arrowheads) or started to express α-sarcomeric actinin (B, fourth panel, green). Two weeks after the implant, human cells identified by an anti-human nuclei antibody **(C)** were found to express α-sarcomeric actinin (α-actinin, in green) within the host myocardium or integrated within the wall of the host coronary arteries (αSMA in red). Finally, when connexin 43 expression was investigated in human cell-infiltrated areas, appropriate connections between human and murine cells (heterotypic junctions, in purple) were detected **(D)**. Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole; Sca-1, stem cell antigen-1; and αSMA, α-smooth muscle actin.

proteins. Sheet-embedded cells also expressed genes encoding for components of the embryonic cardiac extracellular matrix (laminin, collagens I, XI, and XVIII) [29–31]. These findings indicate that, in some respect, a three-dimensional engineered tissue could mimic the natural environment allowing progenitor cells embedded in a proper extracellular matrix to stably display a very low-rate division and apoptosis [23]. Noteworthily, each cell sheet spanned approximately 3.5 cm^2 (although larger sizes are feasible), with a density of cells (mainly the single cell type Sca-1^{pos} hCPC) per cm^2 of 0.7-1 million, which is very close to that usually administered by direct injection into the myocardium [32]. In the mammalian heart and in other regions of the body, there are no examples of so large clusters of progenitor cells, but only few progenitors and some other supporting cells are located into niches. The mechanisms presiding over the extended intercellular stability inside the engineered tissue remains to be elucidated; however, the downregulated expression of the Wnt and Notch systems occurring in the sheet-embedded cells could play a relevant role considering their stage-dependent positive and negative effects on early mesoderm development and cardiac specification and differentiation [33–35].

The current model of physiological cell turnover in organs characterized by terminally differentiated cells (like the myocardium) asserts that a self-limited number of numerary myocardial cells is contained in healthy hearts. Numerary cardiomyocytes are homeostatically regulated by a fractional low-rate cardiomyocyte dismission [36] that, through feeble biological signals spread throughout the entire myocardial microenvironment by single suffering or dying cells, implausibly induces progenitor cells located in the auricles or in the apex to generate substitute cells in a one-to-one ratio. This mechanism, being tuned by the sluggish and limited demand from the differentiated healthy myocardium, does not require a multiplicity of resident progenitor cells to substitute few unsuitable cardiomyocytes, but is unfit to match the enhanced demand elicited by increased amounts of inflammatory cytokines released in association with extensive tissue damage. Therefore, it has been proposed to inject additional progenitor cells into the myocardium to repair the damage and/or high doses of growth factors/cytokines to overstimulate the native progenitor cells. Unfortunately, both strategies failed to allow an efficient myocardial repair, very likely, because of the inappropriateness of the progenitor cell culturing and expansion protocols as well as of the delivery system so far adopted. Instead, in the present study, a significant number of Sca-1^{pos} CPCs migrated from cell sheets implanted onto the ventricular wall surface into the inner myocardial layers of immunosuppressed mice, where they not only adopted the phenotype of either differentiated cardiomyocytes or smooth muscle cells but also fully integrated into the texture of the contractile tissue, as shown by the multiple connexin 43 junctions, or coronary wall, respectively, without modifying cardiac function. In this context, a pivotal relevance had the strategy adopted to culture and deliver progenitor cells. In fact, the Sca-1^{pos} hCPC culture into cell sheets allowed preserving their native potential much better than in cells cultured in conventional systems. In addition, the preservation of the self-produced fetal extracellular matrix was part of this

potential, since it could sustain progenitor cell migration, differentiation, and integration into the myocardium [22, 37]. The progenitor cell migration into the myocardium occurred within 14 days from implantation, was self-regulated and did not require injections, external administration of growth factors/cytokines or direct surgical maneuvers on the myocardium, which could distress cells and forcefully induce them to locate into the ventricular wall. In fact, progenitor cells were delivered by leaning fragments (~0.5 million cells per each) of Sca-1^{pos} hCPC sheets on the visceral leaflet of the pericardial sac. Inside this cavity, Sca-1^{pos} hCPCs were supplied with an optimal array of biochemical signals (growth factors and cytokines released by the myocardium) and mechanostructural cues (e.g., stiffness and tessellation of the visceral pericardium [19, 38]) able to trigger their migration and differentiation into the host myocardium [2, 21]. Intriguingly, the host organ was represented by healthy hearts, in which, on the basis of the current vision, supernumerary terminally differentiated cardiomyocytes should not be accepted. Conversely, the present findings demonstrated that the healthy myocardium is characterized by a constitutive cell cupidity, very likely, directed to collect as many fit cardiomyocytes as possible to guarantee the best myocardial function in all conditions. This continued demand is not self-restricted, but is independently regulated by the limited release of new cardiomyocytes from the two marginally located (atria and apex) sites hosting quiescent cardiac progenitor cells [20]. The conceivable finality of this mechanism could be the preservation of an optimal cardiac structure and function avoiding a disproportionate thickness of the ventricular walls. In fact, in ventricular overloaded hearts, since the excess of demand cannot be matched by the limited augmentation in progenitor cell-derived contractile cells [27], the myocardium adopts a different strategy based on the release of a specific array of signals increasing the number of sarcomeres in each cardiomyocyte to enhance the contractile strength. In this context, when an alien unrestricted source of efficient hCPCs was made available by cell sheets stuck on the heart surface, cell release was no more restrained and a large number of progenitor cells migrated to the healthy myocardium unveiling, for the first time, the real relationship between cardiomyocytes and progenitor cells presiding over cardiac cell homeostasis.

Collectively, the present study introduces a novel concept about myocardial cell homeostasis with many implications in designing efficient cardiac cell therapy protocols. In fact, the meager number of progenitor cells homing and integrating into the myocardial architecture after conventional procedures is not determined by inherent myocardial impedimenta [39], but by the lack of at least two irremissible factors: proper culture of optimal progenitor cells and most favorable host tissue environment. Consistently, the present study was designed to verify the capability of properly cultured progenitor cells to home in a favorable host tissue environment (healthy myocardium). The final aim was to define a benchmark to prospectively assess the relative efficiency of the different protocols proposed for repairing the damaged myocardium. So far, comparable investigations have never been carried out, very likely, supposing that healthy hearts contain a fixed number of terminally differentiated cardiomyocytes and supernumerary contractile cells could not be allowed [2, 3, 40, 41]. Previous investigations on heart repair were exclusively focused on restoring the integrity of the cardiomyocyte number in the injured myocardium, neglecting the cell type multiplicity and the architectural complexity of the heart. Indeed, cell types suitable for heart regeneration in human remain to be defined [42] and generating new cardiomyocytes may be not sufficient to efficiently repair the texture of the myocardial tissue, which also includes fibroblasts, smooth muscle cells, endothelial cells, and adipocytes, among others. In this context, only 10%-20% of the cell complex constituting the whole healthy human heart are cardiomyocytes [15, 43]. Present findings, disclosing that supernumerary cells can be integrated in different myocardial structures (muscle and vessels) definitively clarify the limited relevance of current protocols to engraft suboptimal stem cells in an unfavorable ischemic tissue. The consequent modest heart structural and functional improvement so far observed can only be ascribed to paracrine factors rather than to the mechanical support of newly implanted cells implying that current cell therapy protocols are not adequate to restore heart integrity. Furthermore, the observation that patients with limited residual necrotic regions after heart infarction and adequate ejection fraction (>40%) can survive without major restrictions to their daily activities suggest that the preservation of the functional efficiency in the injured hearts is not necessarily related to the re-establishment of the original cardiomyocyte number. The migration of progenitor cells from an external source and their differentiation to cardiomyocytes and other cell types should maintain the myocardial cell number and the organ function above a critical threshold without necessarily substituting all the damaged cells. The implanted cells, even if scattered and integrated in different cardiac structures (muscle and vessels), will contribute to attain an adequate heart function not only when localized damages are present within the myocardium (infarction) but also when injured cells are widespread throughout the entire cardiac tissue (cardiomyopathy). Therefore, based on the knowledge accumulated in the present study, specific investigations must be designed to adapt the implantation procedure here described to the peculiar microenvironmental conditions of differently damaged myocardia.

Conclusion

In conclusion, present data demonstrate that progenitor cells can fairly migrate and properly home into the myocardium provided that, after isolation, they are cultured in environmental conditions mimicking the native ones. Furthermore, the study introduces a novel concept postulating that the myocardium displays a continued cell cupidity of new cells regulated by the limited capability of the progenitor cells sources. The exploitation of these novel findings could eventually allow an efficient cell therapy of cardiac injuries circumventing the hurdles hampering the current approaches.

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Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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