Cardiac Myocyte Progenitors from Adult Hearts for Myocardial Regenerative Therapy

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Background: The heart is a highly vascular organ and prolonged interruption of myocardial blood flow initiates events that culminate in cardiac myocyte death. Proposed experimental reparative strategies include harvesting potent cells followed by direct injection into ischemic myocardium to achieve myogenesis and angiogenesis.

Methods: Accordingly, we set out to isolate and expand a purified population of adult rat putative cardiomyocyte precursors, and to identify their characteristics *in vitro*. By using an acute myocardial infarction model and direct cell implantation, we further tested the hypothesis that these cells are an ideal cell source for myocardial regeneration and can enhance cardiac repair after implantation into the ischemic rat heart.

Results: We describe here the identification of a subpopulation of primitive cells from rat heart, processing stem cell marker, c-kit and myogenic transcriptional factors, GATA-4 and MEF 2C, and cardiac specific proteins, troponin-I, α-sarcomeric actinin and connexin-43. They exhibited a high *in vitro* proliferative potential. These findings strongly suggest that these cells are putative cardiomyocyte precursors. After transplantation, they were able to be retained and proliferate (13.63 ± 5.97% after 2 weeks) within the ischemic heart. Progeny of implanted cells migrated along the infarcted scar, reconstituted regenerated cardiomyocytes with incorporation into host myocardium, and inhibited cardiac remodeling with decreased scar formation.

Conclusion: Our findings suggest that putative cardiomyocyte precursors isolated from adult heart could potentially be an autologous cell source for myocardial regeneration cell therapy. [*J Chin Med Assoc* 2008;71(2):79–85]

Key Words: cells, myocytes, myocardial infarction

Introduction

The heart is a highly vascular organ, and prolonged interruption of myocardial blood flow initiates cardiac myocyte death.[1] Reparative strategies consider harvesting potent cells followed by their direct injection into ischemic myocardium to achieve myogenesis[2,3] and angiogenesis.[4] However, these strategies have been limited by the sources and fate of donor cells.[5,6]

We describe here the identification of primitive cells from rat heart cardiac myocyte progenitors (CMPs), expressing stem cell surface marker and myogenic transcriptional factors with the potential of cardiomyogenic differentiation. After transplantation, the cells could survive and proliferate within the ischemic heart. Progeny of implanted cells migrated toward the infarcted scar and reconstituted it with regenerated cardiomyocytes incorporating into host myocardium. Our findings suggest that CMPs isolated from adult heart could potentially serve as an autologous cell source for cell therapy to achieve myocardial regeneration.

Methods

Isolation and purification of CMPs

This study was approved by Taipei Veterans General Hospital's Animal Committee, and the *Principles of*

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Laboratory Animal Care (NIH publication no. 86-23, revised 1985) were followed. Hearts from 10 male Sprague-Dawley rats (~250 g each, from National Laboratory Animal Center, Taipei) were placed in cold, sterile calcium-free DMEM (Gibco). The atria and upper portion of the ventricles were removed by dissection, and the remaining tissue was cut into pieces (~2 mm cubes). After treatment with trypsin (0.25%; Gibco) and collagenase type II (0.1%; Gibco), tissue chunks were placed in calcium-free DMEM containing 1% bovine serum albumin (BSA; Sigma) and 100 μM $CaCl_2$, teased apart with forceps and further disrupted by repeated up-and-down pipetting. Tissue clumps were then allowed to settle repeatedly at 1*g*; centrifugation of the combined supernatants at 25*g* for 5 minutes yielded mature myocytes and non-myocyte cells that were suspended in calcium-free DMEM-1% BSA containing 500 μM $CaCl_2$ and layered over 5 mL of calcium-free DMEM containing 6% BSA. Centrifugation at 25*g* for 5 minutes separated a clear upper layer (1% BSA), a darker 6% BSA layer and the myocyte pellets.

The 6% BSA fraction from this gradient centrifugation, which contained cardiac primitive cells and endothelial cells, was placed in DMEM containing 10% fetal bovine serum (FBS; Sigma) on collagen-coated plates (10 μg/cm^2). After overnight incubation, most of the endothelial cells attached to the plates, while non-adherent cardiac primitive cells were collected and placed in DMEM and Ham's F12 (ratio 1:1) containing 10% FBS on regular plates. After 7–10 days, most cells became attached, and these were called *cardiac myocyte progenitors* (CMPs). They were continuously grown at subconfluent densities with serial passages. All media contained 100 μ/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B.

Cell immunocytochemistry

CMPs at passages 2 and 7 were grown on glass coverslips and permeabilized with ice acetone for 10 minutes. Immunocytochemical staining was performed with antibodies for stem cell surface marker c-kit (Santa Cruz Biotechnology) for myogenic transcriptional factor GATA-4 (Santa Cruz Biotechnology) and for heart-specific proteins troponin-I (cTn-I) (Serotec), α-sarcomeric actinin (α-SA) (Sigma) and connexin-43 (Sigma). Fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Chemicon) and propidium iodide (PI) for nuclear counterstaining were used.

Flow cytometry

The isolated and expanded CMPs were characterized at passage 2 by flow cytometric analysis of stem cell surface marker c-kit. The cells were harvested from the culture dishes and then stained for 30 minutes on ice with anti c-kit antibody (Santa Cruz Biotechnology) and then FITC-conjugated secondary antibody (Chemicon). The cell mixture was then washed twice with washing buffer (PBS/1% of FBS/0.1% of NaN_3) and fixed in 1% paraformaldehyde in PBS. Cells were analyzed using a fluorescence-activated cell sorter (FACS) (Vantage SE; Becton Dickinson) using a 525 nm bandpass filter for green FITC fluorescence.

Reverse transcriptase–polymerase chain reaction

Total RNA was extracted from CMPs and adult rat heart by Trizol Reagent (Life Technologies). Reverse transcriptase–polymerase chain reaction (PCR) of myogenic specific genes, including GATA-4, MEF-2C and Myf-5 was performed. cDNA was synthesized from total RNA (1 μg) using M-MuLV reverse transcriptase, and PCR was performed using cDNA as the template in a 30 μL reaction mixture containing a specific primer pair of each cDNA according to the published sequences (Table 1). The reaction mixture was incubated initially at 95°C for 1 minute, followed by 25–35 cycles of denaturation at 95°C for 30 seconds, annealing at 53–60°C for 1.5 minutes and 72°C for 1 minute with an additional 7-minute incubation at 72°C after completing the last cycle (number of PCRs optimized in each case to ensure that the intensity of each product fell within the linear phase of amplification). DNA product was separated by electrophoresis in 1% agarose gel, stained and photographed under UV light. β-actin was used as the internal control.

Cell labeling and preparation for implantation

Sterile CellTracker Orange stock solution (Molecular Probes) was added to the serum-free medium at a final concentration of 10 μM on the day of implantation.[7] The dye was allowed to remain in the culture dishes for 30 minutes and then replaced with complete medium, followed by incubating CMPs (passage 2) for another 30 minutes. CMPs were then rinsed 6 times in PBS to remove the dye outside the cells. CMPs were collected (approximately 1×10^6 cells for 1 implantation) and resuspended in minimal volume of serum-free medium and stored on ice (less than 1 hour) until implantation into the myocardium.

Surgical procedure

Ten male Sprague-Dawley rats (~250 g each) were subjected to ligation of the left anterior descending coronary artery to produce myocardial infarction, as

Table 1. PCR primers

GATA-4	
Sense, 5′-AGACATCGCACTGACTGAGAA-3′	
Antisense, 5′-GACGGGTCACTATCTGTGCAA-3′	
MEF-2C	
Sense, 5′-CCGATGCAGACGATTCAGTAG-3′	
Antisense, 5′-GTGTCACACCAGGAGACATAC-3′	
Myf-5	
Sense, 5′-GAGCCAAGAGTAGCAGCCTTCG-3′	
Antisense, 5′-GTTCTTTCGGGACCAGACAGGG-3′	
β-actin	
Sense, 5′-CAGCTTCTCTTTAATGTCACG-3′	
Antisense, 5′-GGGTCAGAAGGACTCCTACGT-3′	

previously described.[8] In brief, rats were anesthetized with enflurane (Ethrane), intubated, and ventilated with 1.5–2% of enflurane in oxygen. Left thoracotomy was performed at the fourth intercostal space, the pericardium was gently ruptured, and the heart was exteriorized. The left anterior descending coronary artery was ligated 2–3 mm from its origin at about the level of the tip of the left auricular appendage with a permanent 7-0 polypropylene suture. The heart was returned to the chest, the rib space and overlying muscles were closed, and the lungs were re-inflated by positive expiratory pressure. After recovery from anesthesia and extubation, the rats were returned to their cages. They were given water and standard rat chow and housed in a climate-controlled environment with a 12-hour light/12-hour dark cycle.

Two weeks after myocardial infarction, rats were anesthetized and underwent left thoracotomy again, during which the heart was carefully dissected free of adhesions to visualize the extent of left ventricular infarction. Then, 1×10^6 CMPs (passage 2) were injected at 3 or 4 points in the region bordering the infarct. Thereafter, the thoracotomy was closed, and the rats were allowed to recover.

Two weeks later, the rats were sacrificed. They were lightly anesthetized with enflurane and placed in a supine position. Median sternotomy was performed to expose the heart and great vessels. The RA was cut open, the ascending aorta was cannulated using a 22-G angiocatheter, and the distal aorta was ligated. High-potassium solution 50 mL (5 mL KCL in 500 mL normal saline) was administered at 100 cmH_2O via the catheter to arrest the heart in diastole. Later, 50 mL of 4% paraformaldehyde was infused through the coronary system to fix the heart at physiologic pressure. The hearts were excised and placed into ice-cold saline to allow the heart to remain in diastole and to remove the remaining blood. After removal of the atria and great vessels, the right ventricular free wall was excised. The left ventricle was transversely sectioned into 4 segments from apex to base and fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), paraffin-embedded, and sectioned.

Intraperitoneal 5-bromo-2-deoxyuridine (BrdU) (Sigma) was given (50 mg/kg) 12 hours before the sacrifice of rats to evaluate the proliferation of implanted CMPs.

Tissue immunohistochemistry

Immunohistochemical staining with antibodies against BrdU and the cardiac specific markers cTn-I, α-SA and connexin-43 were performed on the cryosections of individual blocks. FITC-conjugated second antibody was used. The proliferation rate of implanted CMPs was evaluated by examination of 5 randomly selected fields of sections from a series of blocks. Proliferative CMPs were recognized as cells co-localized with Cell-Tracker Orange in the cytoplasm and BrdU in the nucleus.

Results

Characteristics of CMPs in vitro

We separated small non-myocyte cells from myocytes by gradient centrifugation first. Small non-myocyte cells included cardiac primitive cells, fibroblasts, endothelial cells and hematopoietic cells. We further separated these cells by selective adhesion. Most of the fibroblasts and endothelial cells adhered quickly to the collagen-coated plates and were discarded. The non-adherent cells were transferred to medium with DMEM and Ham's F12 (ratio 1:1) containing 10% FBS on regular plates. CMPs then preferably attached and were separated from other cells by this selective culture media. Using these methods, we successfully isolated CMPs from 10 rats (~500 cells/rat). They were continuously grown at subconfluent densities with serial passages at intervals of around 7–10 days. After passage 7 (~6×10^6 CMPs/rat), they appeared to have reached growth arrest and senescence with morphologic changes from small nuclear-dominant cells to flat cytoplasm-rich cells (Figure 1A *vs.* 1D and 1E).

By immunocytochemistry and flow cytometry (data not shown), over 50% of CMPs expressed stem cell surface marker, c-kit (Figure 1A), at passage 2. These cells also demonstrated differentiation through myogenic lineage by activating genes of myogenic transcriptional factors including GATA-4, MEF-2C

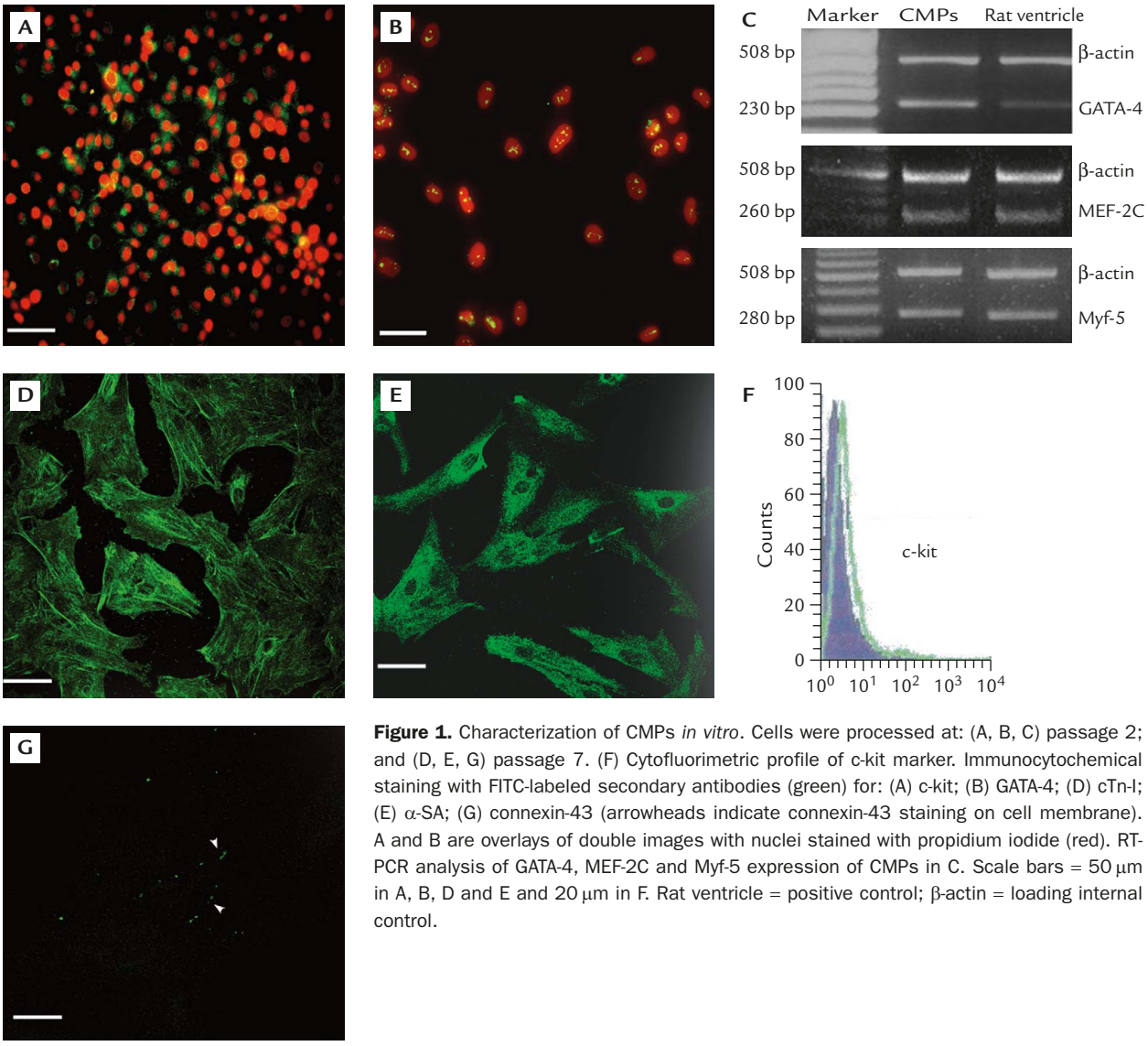

Figure 1. Characterization of CMPs *in vitro*. Cells were processed at: (A, B, C) passage 2; and (D, E, G) passage 7. (F) Cytofluorimetric profile of c-kit marker. Immunocytochemical staining with FITC-labeled secondary antibodies (green) for: (A) c-kit; (B) GATA-4; (D) cTn-I; (E) α-SA; (G) connexin-43 (arrowheads indicate connexin-43 staining on cell membrane). A and B are overlays of double images with nuclei stained with propidium iodide (red). RT-PCR analysis of GATA-4, MEF-2C and Myf-5 expression of CMPs in C. Scale bars = 50 μm in A, B, D and E and 20 μm in F. Rat ventricle = positive control; β-actin = loading internal control.

and Myf-5 (Figure 1C). Moreover, GATA-4 could be detected on almost all CMPs (Figure 1B). No cardiac specific proteins were demonstrated at this point (data not shown). However, these cells expressed cardiac specific markers, cTn-I (Figure 1D), α-SA (Figure 1E) and connexin-43 (Figure 1F) at passage 7 when they were no longer proliferating rapidly.

CMPs sustained proliferation ability after transplantation

We injected 1×10^6 CellTracker-labeled CMPs into the peri-infarcted area of the rat hearts. At another 2 weeks after injection, we treated the rats with BrdU and used BrdU immunoreactivity as a marker of cells undergoing division. By counting the number of CellTracker-labeled cells and the number of such cells co-localized with BrdU immunostain, we estimated that 13.63 ± 5.97% of CMPs expressed proliferative ability 2 weeks after implantation (Figures 2A–C).

CMPs migrated along infarcted scar

We observed clusters of CMPs oriented in the same direction as the native cardiomyocytes in the border zone and in the myocardial scar (Figure 2D). These findings demonstrated that CMPs could integrate with host myocardium and migrate specifically toward infarcted scar.

CMPs expressed markers of cardiomyocytes after transplantation

To address whether implanted CMPs contributed to myocardial regeneration, we examined their phenotype expression after transplantation. Immunostaining showed that labeling of CellTracker co-localized with

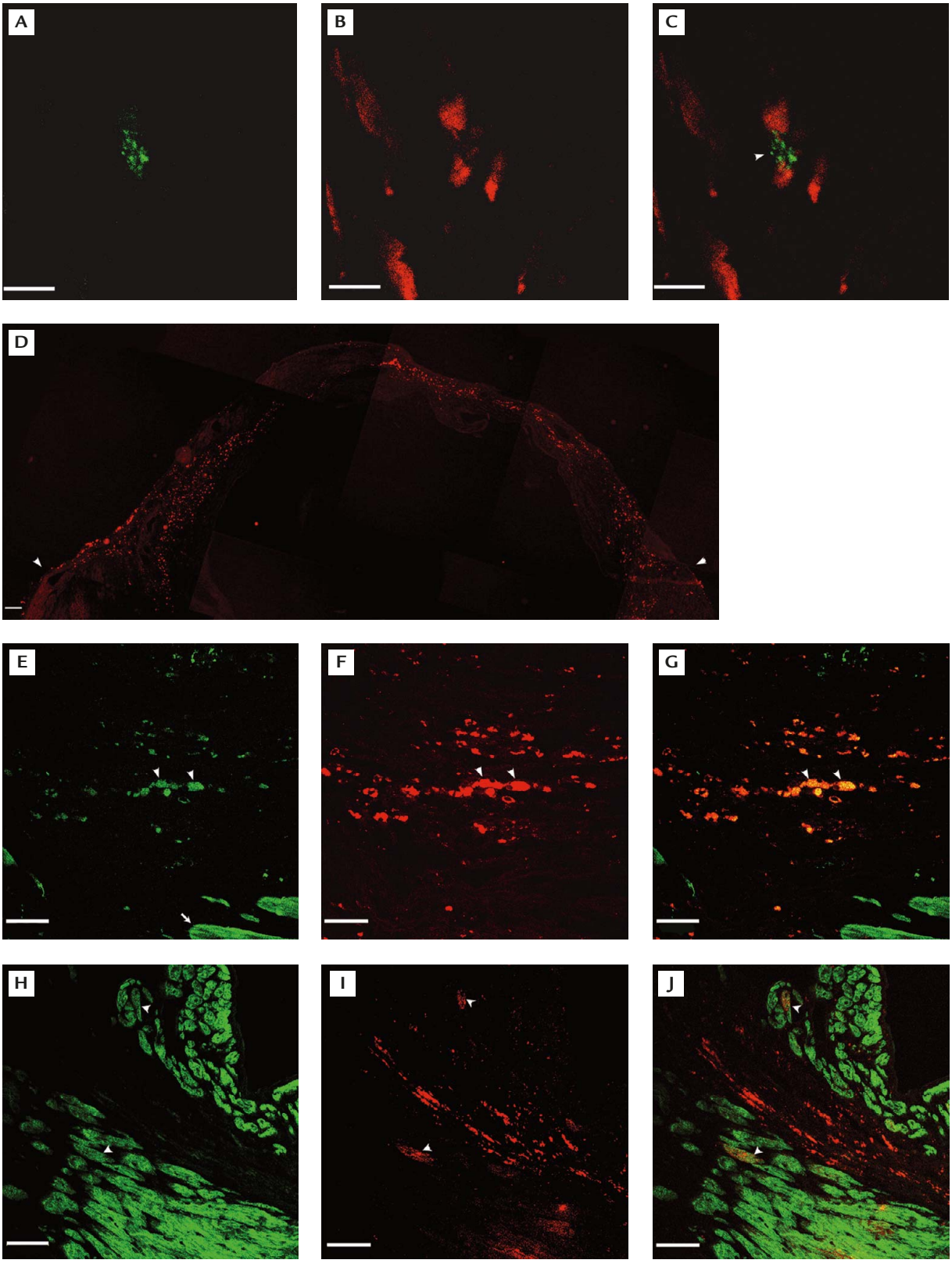

Figure 2. *(Continued)*

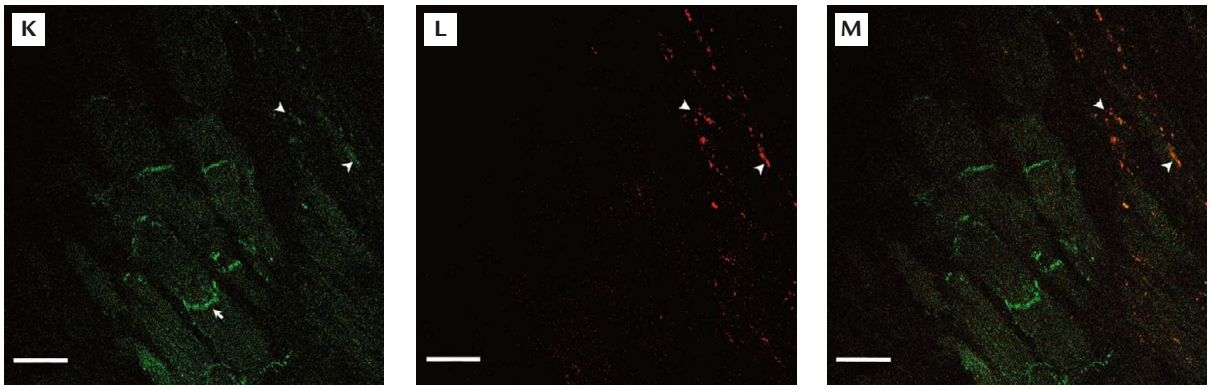

Figure 2. Proliferation, migration and cardiomyocyte phenotype expression of implanted CMPs. Sections were from hearts 2 weeks after implantation and stained for: (A) BrdU; (E) cTn-I; (H) α-SA; and (K) connexin-43 with FITC-labeled secondary antibodies (green). (B, D, F, I, L) CellTracker-labeled CMPs (orange). (C, G, J, M) Overlays of double images. Arrowheads in C and E–M indicate cells co-localized with immunostain markers and CellTracker. Arrowheads in D show the border zone of scar in infarcted hearts. Arrow in E indicates the host cardiomyocyte. Scale bars = 10 μm in A–C, 500 μm in D, 50 μm in E–G and K–M, and 100 μm in H–J.

cardiac specific cTn-I (Figures 2E–G) and α-SA (Figures 2H–J). Labeled CMPs also expressed connein-43 (Figures 2K–M) at contact points with each other and native cardiac myocytes. Together, these results established that implanted CMPs expressed cardiomyocytic phenotype and that they connect with each other by gap junctions.

Discussion

CMPs can be used as donor cells for myocardial regeneration

In this study, we used adult CMPs as cell therapy for the ischemic rat heart because we wanted to correlate with the situation of using autologous cells in aging humans. Autologous cardiac cells might be more effective than other cells in the setting of cell therapy for myocardial regeneration.[9] However, isolated adult cardiomyocytes have no ability to divide as they have been withdrawn from cell cycle.[10] Recent studies suggest that the heart may not be a post-mitotic organ, and that myocyte renewal occurs throughout life in the normal or diseased myocardium.[11,12] In patients with aortic stenosis, investigators found intense new myocardium formation from cardiac stem cells.[13] They further isolated these cells by antibody-based techniques to evaluate the feasibility of using these cells to repair the acute ischemic heart.[8]

Different from their results, the CMPs isolated here expressed a lower percentage (50% *vs.* 90%) of c-kit with an extremely high percentage (~100% *vs.* 7%) of myogenic transcriptional factor, GATA-4. After serial passages, they expressed cardiac specific proteins including cTnI, α-SA and connexin-43. Compared with the results from Anversa and Nadal-Ginard's study,[9] which showed that isolated cells are clonogenic and multipotent, our results demonstrated that CMPs are a heterogeneous population as to its stage of differentiation, and that most of them are already committed and restricted to the myogenic lineage. After implantation, CMPs still preserve their myogenic phenotypes, including contractile proteins (cTnI and α-SA) and gap junction protein (connexin-43). Furthermore they appear to integrate with host cardiac myofibers, which is essential for implanted cells to improve cardiac function. Further studies to compare their efficacy with other sources for myocardial regeneration would be highly desirable.

CMPs can proliferate in vivo

Previous experiments have shown that it is possible to generate new myocardium by cell therapy, but the amount of new myocardium is severely limited by cell death.[6,14] An alternative strategy to obtain an adequate quantity of graft tissue would be to introduce a smaller number of cells initially, which would, however, be capable of proliferation *in vivo*. CMPs isolated by our method demonstrated proliferating ability (about 14%) 2 weeks after being implanted into the ischemic heart. This characteristic may represent a superior feature compared to other potential donor cell sources to replace a substantial fraction of the myocardium lost due to infarction.

CMPs have many theoretical advantages as donor cells for myocardial regeneration. They are already lineage-committed to cardiomyocytes, thus may not be affected in their differentiation by an adverse microenvironment, such as that of scar tissue. They can be autologous, so there is no need for immunosuppression. They

can be vastly multiplied in number *in vitro*, and are capable of *in vivo* replication, thus addressing the issues of cell loss after implantation as well as the quantitative requirement of cell therapy to replace a great loss of myocardial tissue as often encountered clinically. Harvesting and expanding CMPs from a biopsy obtained from the healthy portion of the patient's heart and implanting them back into the infarcted area, for example, could be a strategy worthy of further exploration.

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