

## Characterization of Cardiac-derived Myelo-monocytoid Progenitor Cells

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### Abstract

Cells migrating from heart tissue biopsies during ex vivo culture have been associated with cardiomyogenic and angiogenic potential. We have characterized cells shed by murine cardiac explants cultured in MesenCult MSC medium. A relatively uniform population with a distinguishable morphology emerged from the early cellular outgrowth. This population expressed monocyte/macrophage and hematopoietic markers (CD11b<sup>+</sup>, CD14<sup>+</sup>, CD45<sup>+</sup>) but neither mesenchymal (CD90<sup>-</sup>, CD105<sup>-</sup>) nor endothelial (CD31<sup>-</sup>) markers. It was maintained in culture with a stable phenotype for more than 10 months. When cultured in differentiation medium, cells expressed cardiac sarcomeric  $\alpha$ -actinin. They spontaneously formed sphere clusters ("cardiospheres"). This process was enhanced by TNF $\alpha$ . Spheres from neonatal mice exhibited spontaneous beating. To assess whether "contaminating" myocardial tissue fragments would explain CS beating, neonatal Z/EG transgenic mice were injected with an adeno-associated virus (AAV) vector expressing Cre-recombinase from a cardiac-specific promoter (Ncx1), resulting in the excision of a *lacZ* gene and activation of the expression of the second reporter gene, enhanced green fluorescent protein (EGFP). Cardiac explants from Z/EG mice receiving AAV-9.Ncx1-CRE expressed EGFP, whereas the cellular outgrowth did not, demonstrating that the presence of cardiomyocytes in the outgrowth was not required for the generation of cardiac progenitor cells. Whether the myelo-monocytoid population originated from a retained hematologic component of explants or cardiac-resident leukocytes remains unclear. These results are in line with recent data on monocyte-derived multipotent cells (MOMC) with cardiomyogenic potential. They suggest that the cardiac-derived myelo-monocytoid cell, although not considered a classical adult progenitor cell, may have regenerative potential.

**Keywords:** Stem cell; Progenitor; Heart; Mouse; Monocyte

### Introduction

Over the past decade, accumulating evidence has suggested that the adult mammalian heart may harbor one or more populations of progenitor cells endowed with regenerative potential [1]. Whether these cells represent adult cardiac-resident progenitor cells (CPCs) or bone marrow (BM)-derived cells homing to the heart is incompletely understood. We and others have shown that BM-derived cells can acquire stem cell properties in the damaged heart [2,3]. At minimum, 5 seemingly different populations of cardiac stem cells have been described [4]. Whether these populations represent distinct entities of cardiac stem cells or different developmental and/or physiological stages of a single entity of stem cell remains unclear.

For the prospective isolation of CPCs, many studies have relied upon specific antibodies that recognize cell-surface antigen markers expressed by stem cells in other tissues, and particularly by hematopoietic stem cells, such as c-kit (CD117; stem cell factor receptor) [5] and stem cell antigen-1 (Sca-1) [6]. However, no single cell surface marker definitely identifies a single entity of CPC.

Alternatively, progenitor cells have been obtained from heart tissue biopsies maintained in primary culture [7-14]. Cells shed by cardiac explants in primary culture initially form a layer of fibroblast-like cells on which numerous small round "phase-bright" cells appear with a delay of 1 to 2 weeks [11]. The cellular outgrowth from murine neonatal cardiac explants is heterogeneous and includes both hematopoietic (CD45<sup>+</sup>) and non-hematopoietic (CD45<sup>-</sup>) cells. The hematopoietic component of the cellular outgrowth can be minimized by perfusing the heart with heparinized saline before placing the tissue explant in culture [12]. This observation suggests that CD45<sup>+</sup> cells in the cellular outgrowth may originate from a retained hematologic component. The cellular outgrowth from saline-perfused hearts displayed a heterogeneous immunophenotype consistent with a mixed population

of CPCs (differentiation lineage-negative [Lin<sup>-</sup>, c-kit<sup>+</sup>], endothelial cells (CD31<sup>+</sup>, CD34<sup>+</sup>), and mesenchymal/stromal cells (CD90<sup>+</sup>, CD105<sup>+</sup>), but not hematopoietic cells (CD45<sup>-</sup>).

Cells shed by cultured cardiac explants from rodents and humans can form multicellular sphere clusters, or "cardiospheres" (CS). CS formation is associated with an increase in progenitor cells, endothelial cells and mesenchymal cells but a decrease in hematopoietic cells [1,12,13]. It has been suggested that human CS may result from stem cells endowed with cardiomyogenic and angiogenic potential [12,13]. In animal models of myocardial infarction, CS as well as CS-derived cells injected intramyocardially enhanced blood vessel formation and functional recovery. However, a recent study challenged the view that CS from rodents are a source of stem cells with cardiomyogenic potential [14]. This study claimed that beating CS were explained by "contaminating" myocardial tissue fragments that detached from the explants as these became less cohesive after extended periods of time in culture. Removing cell aggregates with filters minimized CS beating. This issue remains controversial [14]. Here, we have isolated, expanded

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and characterized cells shed by cardiac explants from neonatal or adult mice in primary culture. We consistently obtained a relatively uniform cell population with a distinguishable morphology, which expressed monocyte/macrophage and hematopoietic markers, but neither endothelial nor MSC markers (with the exception of CD44). These myelo-monocytoid cells grew slowly but could be maintained in culture with a stable phenotype for more than 10 months. Cells from neonatal mice, but not those from adult mice, formed spontaneously beating CS. To assess whether “contaminating” myocardial tissue fragments could explain CS beating, we used the Z/EG double-reporter system. Cardiac-specific expression of Cre-recombinase resulted in the excision of a *lacZ* gene and activation of expression of the second reporter gene (enhanced green fluorescent protein; EGFP) in the heart of Z/EG transgenic mice. After Cre-recombinase gene transfer, EGFP expression was observed in Z/EG heart tissue explants but not in the cellular outgrowth, demonstrating that the presence of cardiomyocytes in the outgrowth was not required for the generation of CPC. These findings suggest that myelo-monocytoid cells isolated from cardiac explant outgrowths may be of potential interest as a cell source for cardiac repair.

## Materials and Methods

### Cell isolation and culture

Hearts were collected from neonatal (1-2 days of age) or adult (8-10 weeks of age) male C57Bl/6 mice (Charles River Labs, France). The neonatal heart was rinsed abundantly with ice-cold heparinized saline and ventricles were cut into small pieces that were placed in Corning Costar 6-well plates (Sigma) with no extracellular matrix (ECM) protein coating. The adult heart was retrogradely perfused with ice-cold heparinized saline and ventricles were cut into small pieces that were incubated with 5 mL RPMI supplemented with 12.5  $\mu$ L Liberase II and 25  $\mu$ L DNase I (both from Roche) for 30 min at 37°C (this treatment was performed because of the higher ECM content of the adult heart compared with the neonatal). Tissue pieces were placed in 5.5-cm (Greiner) or 10 cm plastic dishes (Sarstedt) with no extracellular matrix (ECM) protein coating. Cardiac explants were cultured in MesenCult medium consisting of MesenCult MSC Basal Medium supplemented with serum-containing MesenCult MSC Stimulatory Supplements (both from Stem Cell Technologies). After 2 weeks, tissue explants were removed from the plates or dishes, and the cellular outgrowth was allowed to grow as a plastic-adherent monolayer. When cells reached near-confluence, they were detached from plastic with PBS-EDTA, collected, and seeded onto new plates.

### Flow cytometric analyses

For flow cytometric analysis, cells were gently detached from plastic with PBS-EDTA, filtered through a 70- $\mu$ m filter, centrifuged, and resuspended in reagent A Leucoperm B4FO9B (AbD Serotec) for 15 min at RT. Then, they were washed in PBS, centrifuged, resuspended in reagent B, incubated with primary antibody (Supplementary Table 1) for 30 min at RT, washed with PBS and, when necessary, labeled with Alexa 488 (Invitrogen; 1:25 dilution) for 30 min at RT. Flow cytometric analysis was performed using a FACSCalibur system (BD Bioscience) and the CellQuest software. Gates used to resolve antigen-expressing cells were set using appropriate isotype-specific control antibodies.

### Differentiation media

To induce cardiac differentiation, adherent cells were grown in MesenCult medium for 2-3 months and then transferred

to differentiation medium consisting of MesenCult medium supplemented with 1.3  $\mu$ M dexamethasone, 50  $\mu$ g/mL ascorbic acid, and 10 mM glycerol-2 phosphate for 14 days [15]. To induce adipogenic differentiation, cells were transferred to MesenCult medium supplemented with 1.3  $\mu$ M dexamethasone.

### Immunocytochemistry

Immunohistochemistry was performed as previously described [15]. Briefly, cells were seeded on Lab-Tek Chamber-Slides (Nunc) and fixed with 4% paraformaldehyde (PFA). For immunostaining of sarcomeric  $\alpha$ -actinin, a monoclonal anti- $\alpha$ -actinin sarcomeric antibody (Sigma; 1:750 dilution) labeled with Alexa 488 (Invitrogen; 1:400 dilution) was used. For immunostaining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), a rabbit polyclonal anti- $\alpha$ -SMA antibody (Abcam; 1:750 dilution) labeled with Alexa 594 (Invitrogen; 1:400 dilution) was used. Nuclei were stained with DAPI. CS were stained with anti-tubulin A antibody labeled with Alexa 488, and nuclei were stained with Hoechst dye (1:1000 dilution).

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA was extracted using the RNeasy Mini kit (Qiagen), as described [15]. The different cDNAs were generated using the Quantitect reverse transcription kit (Qiagen) on 500 ng total RNA, with a genomic DNA digestion step. The cDNA was then used for quantification using the Quantitect SYBR Green PCR Kit (Qiagen) and the Rotor-Gene 2000 (Qiagen). Quantitative PCRs were set up in 10 mL reaction volume with 5 mL of Quantimix, 0.5 mL of 10 mM forward primer, 0.5 mL of 10 mM reverse primer, 3 mL cDNA, and 1 mL water. The polymerase was heat-activated for 15min at 95°C. The reactions were then cycled 50 times (94°C, 15 s; 50°C, 30 s; 72°C, 30s) followed by a melting step. The following primers were used for SYBR Green real-time PCR: GATA4, fw: 50-ccccaatctcgatattgttga-30, rev: 50-atggccccaattgaca-30; cardiac  $\alpha$ -actinin, fw: 50-gctgctccaactgacctgtcc-30, rev: 50-cg-gccaccgatggacgggaa-30; GAPDH, fw: 50-tcaagaaggtggtgaagcag-30, rev: 50-aagtgaagagtgggagtt-30. Relative expression was calculated using the comparative DDCT method. GAPDH was used as a reference gene.

### CS formation

To study CS formation, the explant outgrowth from neonatal or adult mice was cultured in MesenCult medium for 2 to 5 months. In a subset of experiments, MesenCult medium was changed to Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum (DMEM-10% FCS) to compare the two media in terms of CS formation. Moreover, the effect of cytokines and growth factors on CS formation was addressed by supplementing MesenCult medium with 5 ng/mL of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), bone morphogenetic proteins (BMP) 2 and 4, or transforming growth factor- $\beta$ 1 (TGF $\beta$ 1), or with 10 ng/mL of vascular endothelial growth factor-165 (VEGF<sub>165</sub>) or basic fibroblast growth factor (bFGF). In separate experiments, CS formed in MesenCult were transferred to low-serum medium consisting of 2 volumes of B27-based medium (100 mL DMEM supplemented with Glutamax, 2 mL B27 50x [Gibco], and 1 mL penicillin/streptomycin/fungizone 100x) and 1 volume of MesenCult medium. Cell cultures were monitored for the appearance of beating CS under the microscope. Epinephrine was used to test the responsiveness of CS beating rates to an adrenergic stimulus.

### Experiments using Z/EG transgenic mice

To determine whether the cellular outgrowth from cardiac explants contained “contaminating” myocardial tissue fragments, we

used the Z/EG double-reporter system. In Z/EG transgenic mice, Cre-recombinase (CRE) activity results in the excision of a *lacZ* reporter gene and the concomitant activation of the expression of enhanced green fluorescent protein (EGFP) as the second reporter gene [16]. Cardiac-specific expression of CRE was mediated by a serotype-9 adeno-associated virus vector containing the Ncx1 promoter (AAV-9. Ncx1-CRE). A vector containing a cytomegalovirus immediate-early promoter (AAV-9.CMV-CRE) was used as a control. AAV vectors were generated and characterized as described [17]. Vector stock titers ranged from  $1 \times 10^{12}$  to  $1 \times 10^{13}$  viral genome copies per milliliter. AAV vectors were injected intraperitoneally to neonatal mice anesthetized with 4% isoflurane. Green fluorescence in cardiac explants and cellular outgrowths was assessed by fluorescence microscopy.

### Statistical analysis

The non-parametric Mann-Whitney-U test was used for comparisons between two groups.  $P < 0.05$  was considered to be statistically significant.

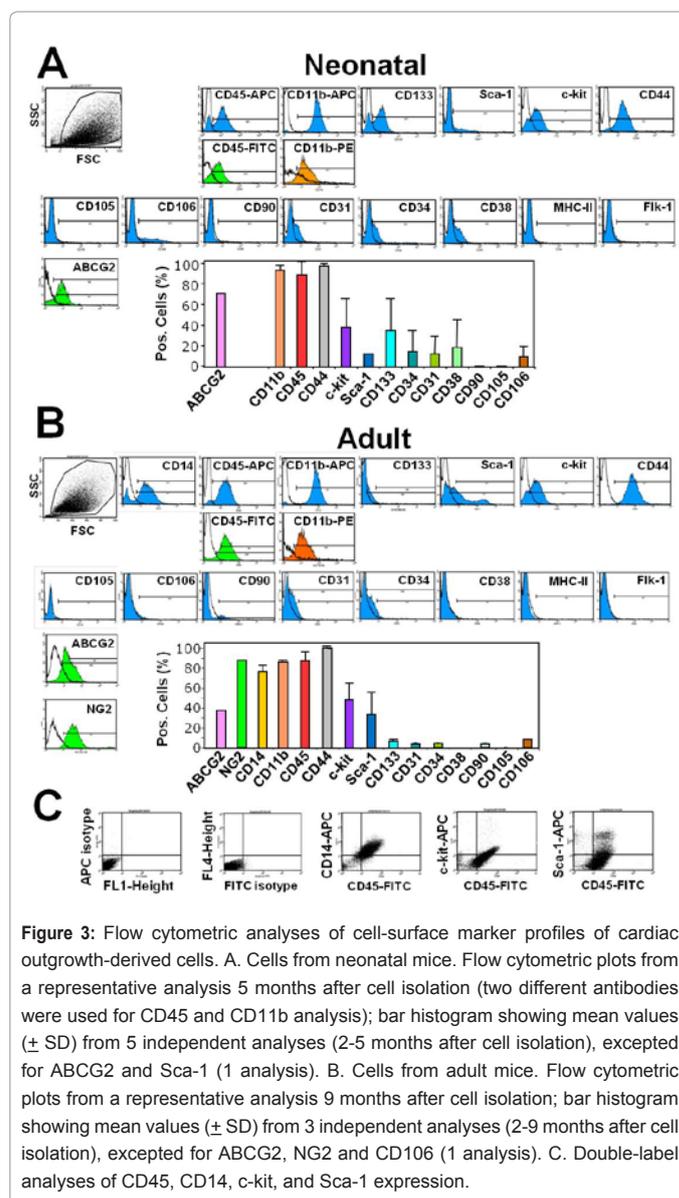
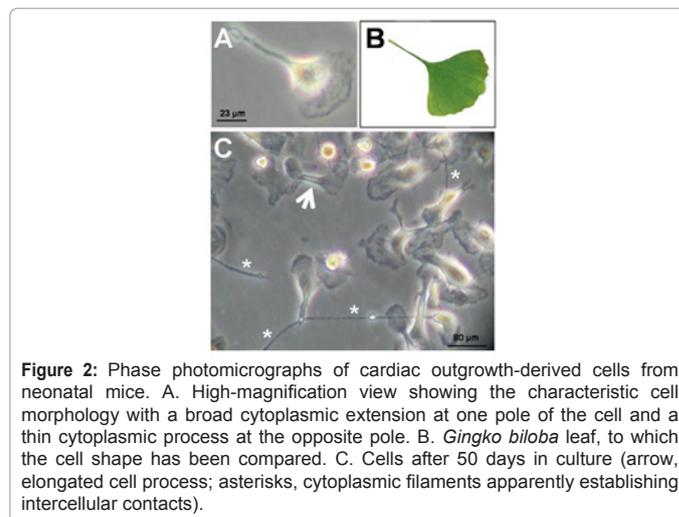
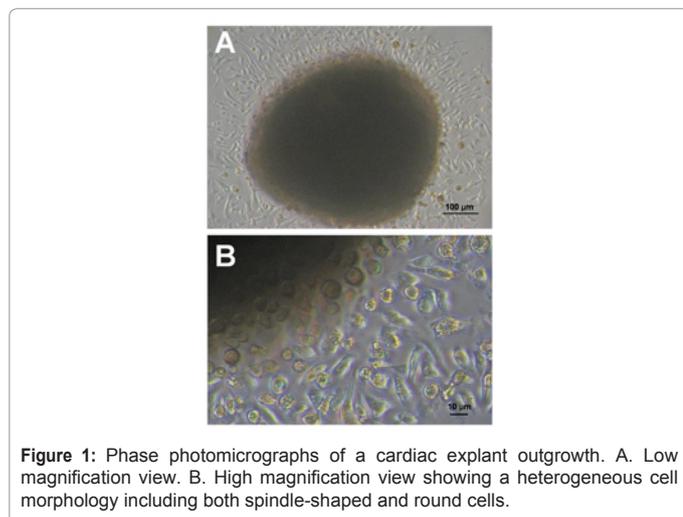
## Results

### Cell isolation and culture

Cultured cardiac explants from neonatal or adult mice gave rise to cellular outgrowths becoming visible after 3-4 days. The early outgrowth was comprised of spindle-shaped cells and round, highly refringent (phase-bright) cells (Figure 1). Tissue explants were removed from culture after 2 weeks. After 4-6 weeks, cells from neonatal or adult mice acquired a relatively uniform, distinguishable morphology characterized by one or multiple, thin cytoplasmic processes (the compared the shape of cells with a single process to that of *gingko biloba* leaves; Figure 2). Cells grew slowly but could be maintained in culture for more than 10 months with a stable morphology and immunophenotype (see below).

### Flow cytometric analyses

Flow cytometric analyses of cell surface antigens were performed on cardiac outgrowth-derived cells from neonatal mice ( $n = 5$ ) after 2 to 5 months in culture, as well as from adult mice ( $n = 3$ ) after 2 to 9 months in culture. Cells from neonatal or adult mice displayed similar cell-surface marker profiles (Figure 3). These were characterized by expression of monocyte/macrophage and hematopoietic markers



(CD11b, CD14, CD45), CD44 (considered a MSC and endothelial progenitor cell [EPC] marker), and NG2 proteoglycan (a marker of pericytes/perivascular cells and mesoangioblasts). Regarding stem cell-associated markers, ATP-binding cassette G2 (ABCG2) was expressed by a significant subpopulation, CD133 was expressed by a subpopulation from neonatal but not adult mice, and Sca-1 was expressed by a larger subpopulation from adult compared with neonatal mice. The specificity of Sca-1 detection was tested using cells from Sca-1-deficient mice (data not shown). CD90 and CD105 (MSC markers), CD31 (an endothelial marker), CD34 (a hematopoietic and EPC marker), CD3e (T-cell receptor complex subunit), CD19, CD38 and CD45R (B-cell markers), CD49a, CD106 (vascular cell adhesion molecule-1; VCAM-1), Flk-1 and major histocompatibility complex (MHC) class II molecules were essentially undetectable. Double-label analyses showed that c-kit<sup>+</sup> cells and a majority of Sca-1<sup>+</sup> cells were CD45<sup>+</sup>. To assess changes of the cell-surface marker profile over time, the same cell population from a neonatal heart was analyzed by flow cytometry after 2 and 5 months (data are shown in Figure 4C and Figure 3A, respectively). Occasionally, cells lost their original morphology, enlarged, and ceased growth. To assess changes in cell-surface markers associated with morphological changes, we analyzed two subpopulations derived from the same population after 2 months in culture, the first one having partially lost the original morphology, and the second one having maintained it (Figure 4A and 4B, respectively). The subpopulation with altered morphology showed decreased percentages of CD11b<sup>+</sup> and CD45<sup>+</sup> cells but increased percentages of CD106<sup>+</sup> cells compared with the subpopulation with unchanged morphology. These changes were paralleled by increased mRNA expression of GATA-4 and sarcomeric  $\alpha$ -actinin. These data suggest a shift from a hematopoietic to a non-hematopoietic phenotype.

### Cell differentiation potential

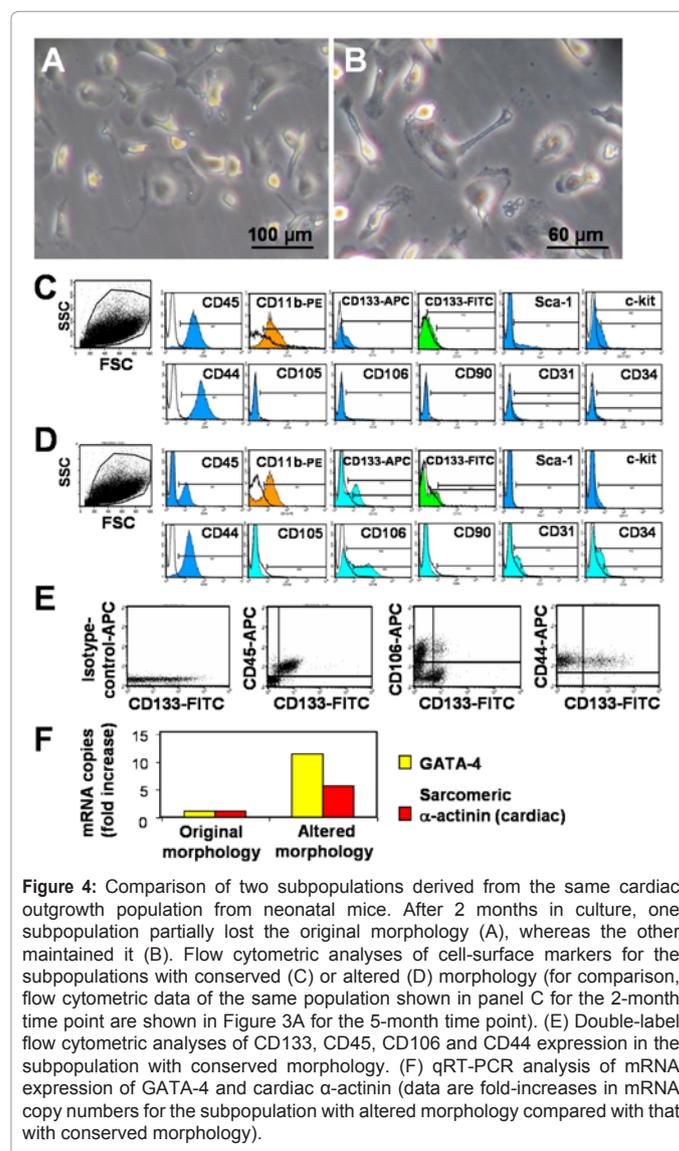
Cardiac outgrowth-derived cells cultured in cardiac differentiation medium stained positive for sarcomeric  $\alpha$ -actinin (Figure 5), which appeared to be organized in microfibrils. A minority of cells stained positive for  $\alpha$ -SMA. Cells cultured in Mesencult supplemented with 1.3  $\mu$ M dexamethasone enlarged and acquired intracellular droplets indicative of adipogenic differentiation.

### CS formation

Plastic-adherent outgrowth-derived cells from neonatal or adult mice spontaneously formed CS after 2 to 5 months in culture (Figure 6). Neither low-adherence plates nor specific CS growth media were needed for CS formation. Mesencult medium was more efficient than DMEM-10% FCS in this regard. The effect of cytokines or growth factors, supplemented to Mesencult medium, on CS formation was evaluated. TNF- $\alpha$  significantly increased the number of CS. CS from neonatal, but not adult, mice occasionally exhibited spontaneous beating activity in MesenCult medium. Virtually all CS from neonatal mice exhibited spontaneous beating in low-serum B27-based medium (see: Supplementary electronic file S1). Beating activity lasted for several days to a few weeks. Epinephrine increased beating rates up to  $\approx$ 180 bpm.

### Cell tracing using the Z/EG reporter system

To assess whether “contaminating” myocardial tissue fragments were responsible for CS beating, we used the double-reporter Z/EG system. Neonatal Z/EG cardiomyocytes transduced with AAV-9.Ncx1-CRE or AAV-9.CMV-CRE in vitro were irreversibly labeled with EGFP (Figure 7). Neonatal cardiac fibroblasts transduced with



**Figure 4:** Comparison of two subpopulations derived from the same cardiac outgrowth population from neonatal mice. After 2 months in culture, one subpopulation partially lost the original morphology (A), whereas the other maintained it (B). Flow cytometric analyses of cell-surface markers for the subpopulations with conserved (C) or altered (D) morphology (for comparison, flow cytometric data of the same population shown in panel C for the 2-month time point are shown in Figure 3A for the 5-month time point). (E) Double-label flow cytometric analyses of CD133, CD45, CD106 and CD44 expression in the subpopulation with conserved morphology. (F) qRT-PCR analysis of mRNA expression of GATA-4 and cardiac  $\alpha$ -actinin (data are fold-increases in mRNA copy numbers for the subpopulation with altered morphology compared with that with conserved morphology).

AAV-9.CMV-CRE, but not those transduced with AAV-9.Ncx1-CRE, were similarly labeled. These data demonstrated cardiac-specific CRE expression using the Ncx1 promoter-containing vector. After 2 weeks in culture, heart tissue explants from neonatal Z/EG mice previously injected with AAV-9.Ncx1-CRE exhibited green fluorescence, whereas the cellular outgrowth did not, demonstrating lack of cardiomyocyte in the outgrowth.

### Discussion

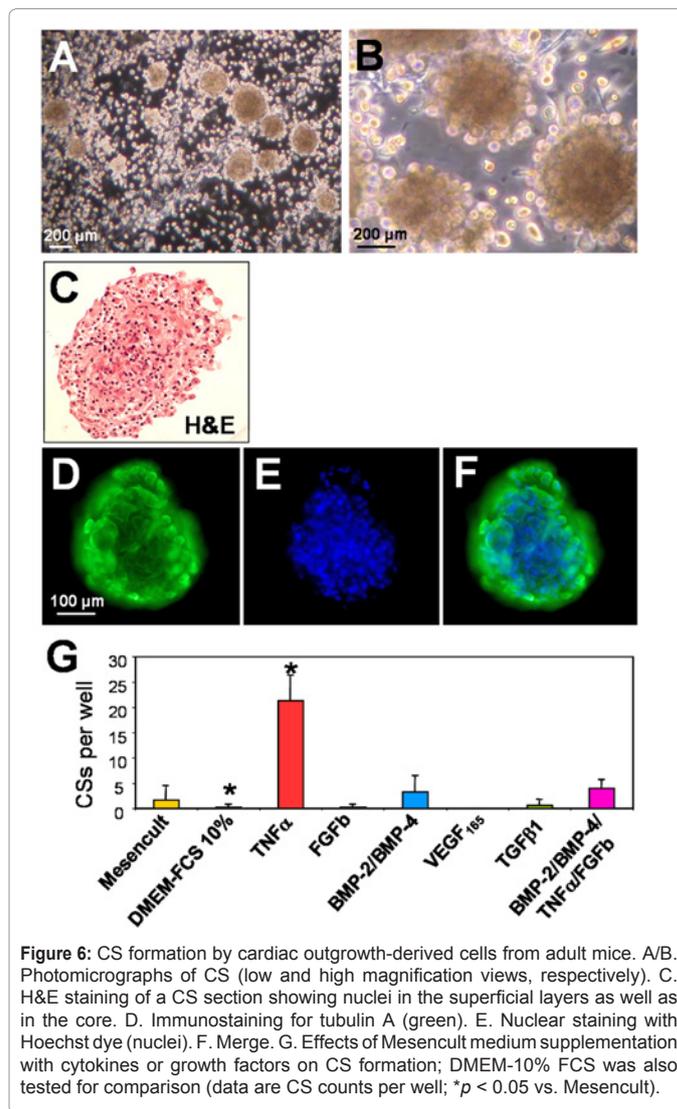
We have obtained a myelo-monocytoid cell population by outgrowth from murine cardiac explants maintained in primary tissue culture. This population exhibited a distinguishable morphology and could be maintained in culture for more than 10 months. The vast majority ( $\approx$  80-90%) of cells expressed the leukocyte common antigen CD45, the monocyte/macrophage markers CD11b and CD14 [18-20], CD44 (generally considered a mesenchymal and EPC marker, but not expressed by primary BM MSC and progenitor cells [21]), or the pericyte/perivascular cell marker NG2 [9,22,23]. Subpopulations expressed the stem cell-associated markers ABCG2, c-kit, and CD133

(the latter in cells from neonatal mice only). A small minority of cells expressed the endothelial marker CD31. Expression of the MSC markers CD90 and CD105 [24] was barely detectable. This cell-surface marker profile pointed to a myelo-monocytoid phenotype.

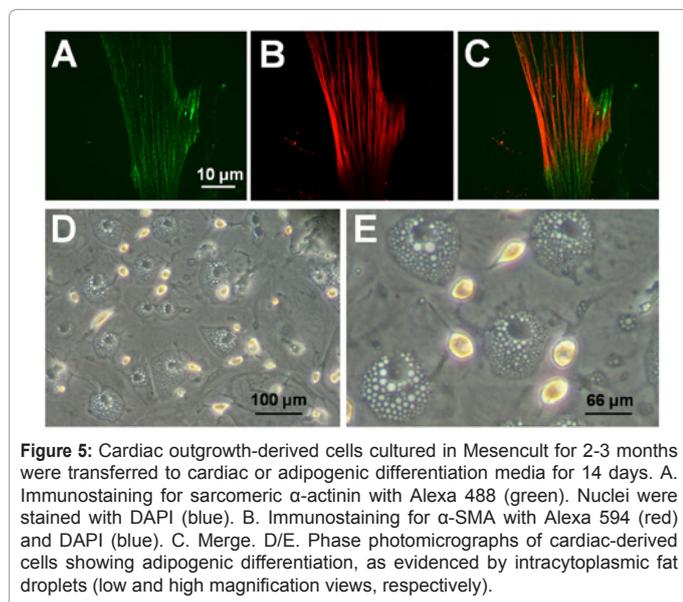
Monocyte-derived cells include macrophages, fibrocytes, dendritic cells, osteoclasts, and adipocytes [18-20]. Lack of expression of CD105 by the isolated population suggested a monocytic phenotype, rather than a macrophage or fibrocytic [19]. Expression of NG2 proteoglycan has been observed on perivascular cells including pericytes and cardiac mesoangioblasts. The latter displayed cardiomyogenic potential [9]. Moreover, human MSC in multiple organs were shown to express NG2 in vivo, thereby suggesting a perivascular origin of these cells [23]. In analogy to cardiac mesoangioblasts [9], the population isolated in the present study expressed NG2, CD44 and, in part, c-kit. On the other hand, it clearly differed from mesoangioblasts with respect to CD45, CD31, and CD34 expression.

Expression of CD44 by the isolated population may seem at odds with that of hematopoietic and monocytic markers. It should be noted, however, that blood monocyte-derived mesenchymal progenitors (MOMP) with mixed phenotypic features of monocytes, MSC, and endothelial cells have been described [25]. Moreover, MSC from BM and other tissues have been shown to express CD45 under certain conditions [26-29]. In some cases, CD45 expression by MSC was dramatically downregulated in culture [26,27]. Collectively, these data demonstrate the existence of CD45<sup>+</sup> cells expressing mesenchymal markers, while also suggesting that certain CD45<sup>-</sup> populations expressing such markers may have originally been CD45<sup>+</sup>.

The myelo-monocytoid phenotype of the isolated population conflicted with published data. It should be emphasized that we used an unconventional protocol based on Mesencult medium, whereas Messina *et al.* [7] used an Iscove's Modified Dulbecco's Medium [IMDM]-based medium supplemented with 10% FCS. It has been shown that the composition of the culture medium alter the phenotype of cardiac outgrowth-derived cells [12]. Several groups independently reported that human cardiac outgrowths expressed MSC markers (CD90<sup>+</sup>, CD105<sup>+</sup>) but not hematopoietic markers (CD45<sup>-</sup>, CD34<sup>-</sup>,

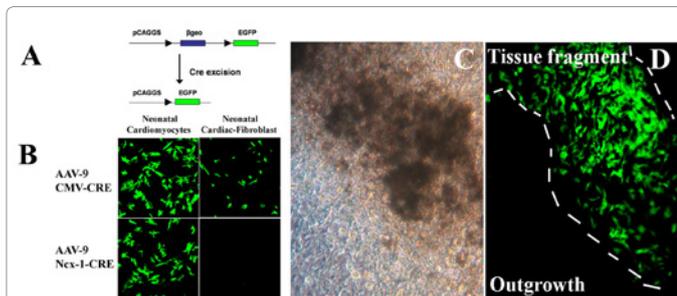


**Figure 6:** CS formation by cardiac outgrowth-derived cells from adult mice. A/B. Photomicrographs of CS (low and high magnification views, respectively). C. H&E staining of a CS section showing nuclei in the superficial layers as well as in the core. D. Immunostaining for tubulin A (green). E. Nuclear staining with Hoechst dye (nuclei). F. Merge. G. Effects of Mesencult medium supplementation with cytokines or growth factors on CS formation; DMEM-10% FCS was also tested for comparison (data are CS counts per well; \**p* < 0.05 vs. Mesencult).



**Figure 5:** Cardiac outgrowth-derived cells cultured in Mesencult for 2-3 months were transferred to cardiac or adipogenic differentiation media for 14 days. A. Immunostaining for sarcomeric  $\alpha$ -actinin with Alexa 488 (green). Nuclei were stained with DAPI (blue). B. Immunostaining for  $\alpha$ -SMA with Alexa 594 (red) and DAPI (blue). C. Merge. D/E. Phase photomicrographs of cardiac-derived cells showing adipogenic differentiation, as evidenced by intracytoplasmic fat droplets (low and high magnification views, respectively).

CD11b<sup>-</sup>, CD14<sup>-</sup>, CD19<sup>-</sup>) [10,13,30,31]. In rodents, cardiac outgrowths displayed a more heterogeneous phenotype. Andersen *et al.* [14] reported that rat cardiac outgrowths included fibroblasts, endothelial cells, EPCs, CD45<sup>-</sup> CS-forming cells, and CD45<sup>+</sup> hematopoietic cells. Davis *et al.* [12] showed that retrograde perfusion of rat hearts with heparinized saline minimized the number of CD45<sup>+</sup> cells in the cellular outgrowth. This was comprised of putative CPCs (c-kit<sup>+</sup>), endothelial cells and EPCs (CD31<sup>+</sup>, CD34<sup>+</sup>), and mesenchymal cells (CD90<sup>+</sup>). In the present study, perfusion of adult hearts with heparinized saline did not prevent the isolation of CD45<sup>+</sup> cells. However, this observation does not rule out an origin of these cells from the retained hematologic component of cardiac explants. Incidentally, it should be mentioned that we were not able to isolate a similar cell population from mouse BM using the same culture conditions (data not shown). An alternate hypothesis is that the isolated population originated from cardiac-resident leukocytes. In this regard, it has recently been shown that the unstressed mouse heart harbors  $2.3 \times 10^3$  resident leukocytes/mg of tissue (as measured after heart perfusion according to Langendorff with an oxygenated medium for 5 minutes), the most prominent fraction being antigen-presenting mononuclear cells (CD11b<sup>+</sup>, CD11c<sup>+</sup>, F4/80<sup>+</sup> and MHC-II<sup>+</sup>) followed by B-cells, monocytes, and T-cells [32].



**Figure 7:** Primary tissue culture from a neonatal Z/EG mouse heart. Upper left panel: Schematics of the Z/EG double reporter (lacZ, EGFP) system. Lower left panel: Tissue specificity of EGFP expression using AAV-9.Ncx1-CRE as compared with AAV-9.CMV-CRE. EGFP expression using the first vector was restricted to cardiomyocytes. Middle panel: Photomicrograph of Z/EG heart tissue explant and cell outgrowth. Right panel: Corresponding fluorescence photomicrograph (the boundaries of the tissue explant are indicated by a dashed line). Green fluorescence is detectable within the explant but not in the outgrowth.

Regardless of whether the isolated population originated from blood cells trapped in the intravascular space of the explant or from cardiac-resident leukocytes, the high proportion of CD45<sup>+</sup> and CD11b<sup>+</sup> cells in the cultured population suggested an advantage for these cells in terms of survival or growth compared with other cells in the early outgrowth. Alternatively, these myelo-monocytoid cells might have resulted from the differentiation of progenitor cells. In either case, this outcome was unanticipated since MesenCult has been optimized for growth of MSC. Indeed, we previously showed that mouse cardiac MSC (Lin<sup>-</sup>, Sca-1<sup>+</sup>, CD90<sup>+</sup>, CD105<sup>+</sup>, CD45<sup>-</sup>, CD31<sup>-</sup>) could be expanded for more than 25 passages in MesenCult medium with a preserved immunophenotype [15].

Cardiac-derived myelo-monocytoid cells appeared to have cardiomyogenic potential. When cultured in differentiation medium, they stained positive for sarcomeric  $\alpha$ -actinin. When cultured in MesenCult medium, they occasionally gave rise to free-floating multicellular sphere clusters. This process was enhanced by TNF  $\alpha$ . Sphere clusters were first described with neural stem cells (“neurospheres”) [33]. Spheres have been considered – or named, at least – a feature of stemness. It should be noted, however, that spheres may result from either cell proliferation or aggregation. Non-clonal spheres increase with increasing cell density in the culture plate, and therefore clonal sphere-formation assays need to be performed at very low cell densities, and ideally at the single-cell level [34]. In the present study, sphere formation was observed at relatively high cell densities, a possible hint to non-clonality. Previous studies indicated that human clonal CS resulted from stem cells with cardiomyogenic and angiogenic potential [12,13]. However, recent data in rodents questioned this notion [14]. Again, it is important to note methodological differences between our study and the original report by Messina *et al.* [7]. To induce CS formation, these investigators placed the cells in suspension culture in poly-D-lysine-coated plates in a base medium supplemented with bFGF, EGF, cardiotrophin-1, thrombin, and B27 as a serum substitute. In contrast, we observed CS formation from plastic-adherent cells (as opposed to suspension cultures) in MesenCult medium (as opposed to CS-forming medium).

The most obvious evidence of cardiomyogenic differentiation was the demonstration of beating. We occasionally observed spontaneous CS beating from neonatal mice in MesenCult medium. Virtually all spheres placed in a low-serum, B27-based medium showed

spontaneous beating. Beating rates were responsive to an adrenergic stimulus, a feature of electrophysiologically mature cardiomyocytes.

Andersen *et al.* [14] recently suggested that CS beating from neonatal rodents might be explained by “contaminating” myocardial tissue fragments in the cellular outgrowth, especially when cardiac explants were not removed from culture plates. In the present study, tissue explants were removed from cultures after 2 weeks. In MesenCult medium, beating activity appeared 3 to 6 months after cell isolation. It seems unlikely that “contaminating” myocardial fragments would resume beating after such a long interval. To directly address this question, we used the Z/EG double-reporter system. Cardiac explants from neonatal Z/EG mice injected with the AAV-9.Ncx1-CRE vector *in vivo* exhibited green fluorescence, whereas the cellular outgrowth did not. This observation demonstrated that the presence of cardiomyocytes in the cellular outgrowth was not required for the generation of CPC.

The theoretical possibility that cardiac-derived myelo-monocytoid cells originated from cardiac-resident MSC seems unlikely. Freisinger *et al.* [35] previously showed that clonally isolated, adipose tissue-resident MSC (CD90<sup>+</sup>, CD105<sup>+</sup>, CD44<sup>+</sup>, CD4<sup>-</sup>, CD11b<sup>-</sup>, CD34<sup>-</sup>, CD45<sup>-</sup>, CD68<sup>-</sup>) cultured in various differentiation media (IL-1 $\beta$ , IL-3, M-CSF and MTG) differentiated into cells expressing early hematopoietic (c-kit) and monocyte/macrophage markers (CD11b). However, we did not use these differentiation media in the present study. Zhao *et al.* [36] described a subpopulation of adult pluripotent stem cells (PSC) from human peripheral blood monocytes, which in appearance resembled fibroblasts, expanded in the presence of M-CSF, expressed monocytic and hematopoietic markers (CD45, CD34, CD14), and could be induced to differentiate into various cell types in the presence of appropriate growth factors. Kuwana *et al.* [25,37] described human blood monocyte-derived mesenchymal progenitors (MOMP) as well as monocyte-derived multipotent cells (MOMC) expressing CD45, CD34, CD14 and type-I collagen. These cells showed a fibroblast-like morphology and mixed phenotypic features of monocytes, endothelial cells, and MSC. They contained progenitors with the capacity to differentiate into bone, cartilage, fat, skeletal and cardiac muscle, neuron, and endothelium [25,37-39]. Romagnani *et al.* [40] described clonogenic, multipotent, circulating CD14<sup>+</sup>CD34<sup>low</sup> cells that proliferated in response to stem cell growth factors. Our data on myelo-monocytoid cells obtained by outgrowth from cardiac explants are in line with these reports on multipotent monocytes. These findings suggest that the monocyte, traditionally viewed as a committed precursor for macrophages and dendritic cells, may have a larger differentiation potential. Further studies are warranted to compare the regenerative potential of cardiac-derived myelo-monocytoid cells with that of other CPC populations in animal models of heart injury.

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