The post-natal heart contains a myocardial stem cell population

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Abstract The recent identification of stem cell pools in a variety of unexpected tissue sources has raised the possibility that a pluripotent stem cell population may reside in the myocardium and contribute to the post-natal growth of this tissue. Here, we demonstrate that the post-natal myocardium contains a resident verapamil-sensitive side population (SP), with stem cell-like activity. When growth of the post-natal heart was attenuated through over-expression of a dominant negative cardiac transcription factor (MEF2C), the resident SP cell population was subject to activation, followed by a consequent depletion. In addition, cardiac SP cells are capable of fusion with other cell types, but do not adopt the corresponding gene expression profile. These observations suggest that a responsive stem cell pool resides in the adult myocardium, and may influence adaptation of the post-natal heart.

Key words: Cardiac; Stem cell; Myocyte-enhancing factor 2C; Fusion

1. Introduction

The adult heart retains a remarkable ability to adapt intrinsic cellular properties to alterations in the exterior milieu. A most critical aspect of this phenomenon is the synchronous pacing which matches myocardial enlargement to the growth of the individual in either pre- or post-natal life. During fetal development, myocardial growth is derived from proliferation of the existing cardiomyocyte population. This phenomenon is characterized by an elevated expression/activity of proteins that accelerate the cardiomyocyte cell cycle, concurrent to a repression of cell cycle inhibitors [1]. The gestational growth of the myocardium is followed by an equally dramatic expansion in heart size, which accompanies the transition from early post-natal life to maturity. Irrespective of the phenotypic similarity, the cellular adaptations that mediate fetal and post-natal growth of the myocardium are clearly divergent. Specifically, the proliferative capacity of cardiomyocytes is profoundly diminished at later stages of pre-natal life [2–5]. At this juncture, ambient growth of the myocardium is believed to be primarily derived from hypertrophy of individual cardiac myocytes [6–7]. This cellular adaptation is characterized by a number of features common to cells of a muscle lineage (cardiac and skeletal), including sarcomeric reorganization/myofibril assembly and enhanced contractile activity [8].

Despite the documented hypertrophy of the cardiomyocyte population, the potential for additional growth mechanisms in the post-natal myocardium cannot be excluded. For example, pluripotent bone marrow-derived stem cells have been shown to retain the capacity to form cardiomyocytes when directly injected into injured myocardial tissue [9,10]. These studies raise a number of interesting questions, paramount of which is the possibility that the post-natal heart may contain an endogenous population of stem cells programmed to contribute to myocardial growth. Indeed, many terminally differentiated tissues including the nervous system and skeletal muscle have been shown to harbor a population of cells with stem cell-like activity, which also maintain site-specific functions [11–14]. In the present study we report the novel observation that the adult myocardium retains an endogenous stem cell-like population, which is activated during growth challenge.

2. Materials and methods

2.1. Animal models

The transgenic models utilized were previously characterized: the Z/AP [15] and EGFP [16] mice as marker strains and the MEF2Cdn as a myocardial hypoplastic model [17]. Unless otherwise indicated, all subsequent experiments and primary cell derivations were performed with age-matched animals ~2 months.

2.2. Isolation of cardiac cell suspensions and cell culture

Primary cell cultures were prepared from ventricles and skeletal muscle as described previously ([18,19], respectively). Purification of primary cardiomyocytes was achieved with serial pre-plating in serum-depleted media. For co-culturing experiments, approximately 10 000 cells from the Z/AP or EGFP cardiac cell suspensions were plated with the indicated primary cultures (at 75% confluence) or in 3 ml of methocult medium (Stem Cell Technologies) for 14 days to assess hematopoietic potential [20]. To induce differentiation in primary derived and established muscle cell lines, cultures were maintained in 2% horse serum for the indicated time periods.

2.3. FACS sorting/profiling

Cardiac cell suspensions were isolated as described previously [19] and subject to Hoechst staining, verapamil treatment and fluorescence-activated cell sorter (FACS) analysis as previously described [20]. FACS was performed using a Becton-Dickinson FacStar flow cytometer equipped with dual lasers. Fluorescence was measured at two wavelengths using a 424BP44 filter for blue emissions and a 650 LP filter for red emissions. A 640 DMSP mirror was used to separate wavelengths.

2.4. Immunohisto- and immunocytochemistry

Hearts were fixed in 4% paraformaldehyde, sectioned midsagitally (6 μm) and counterstained with 0.05% toluidine blue [21]. Primary colonies isolated from methocult medium were fixed and stained as

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described in [25] using anti-mouse GR-1/Ly-6G (Pharmingen), and anti-mouse integrin α_M (M1-70)(Mac-1) (Pharmingen). Cardiac and skeletal muscle cells were fixed in 4% paraformaldehyde and stained for the following epitopes: connexin 43 (1:200, Chemicon); myogenin (1:5, Developmental Studies Hybridoma Bank) both with Cy3-conjugated goat anti-mouse IgG secondary (Chemicon); β-galactosidase (1:250, Abcam) with Cy3-conjugated goat anti-rabbit secondary (Jackson). Enzymatic β-galactosidase staining was performed as described in [21]. Immunofluorescence was visualized using a Zeiss Axioscope microscope.

3. Results and discussion

Prior studies using a variety of tissue sources have determined that Hoechst dye-stained cell suspensions reveal a Hoechst effluxing sub-population of cells (side population or SP cells). These cells possess stem cell-like activity, reduction or absence of differentiation markers, and are also characterized by a sensitivity to the presence of verapamil, an inhibitor of multi-drug resistance-like proteins [22–24]. Therefore, to evaluate the presence of a resident myocardial stem cell population, hearts of mature C57Bl6 mice (~2 months) were enzymatically dissociated into a single cell suspension and subjected to FACS analyses. FACS analyses of the myocardial cell suspensions revealed a robust Hoechst dye-excluding SP population, which represented ~1% of the total cell number in the adult heart (Fig. 1A). To test for lineage specificity, we purified myocardial SP cells from the EGFP strain of transgenic marker mice [16] and performed co-culture experiments with primary cardiomyocytes derived from non-marker mice of a similar genetic background (C57Bl6). These purified myocardial SP cells from EGFP mice were capable of forming cardiomyocytes under these co-culture conditions (as evidenced by co-localization of GFP and connexin 43 staining, Fig. 1C–E). We also monitored the activity of the cardiac cell suspensions (SPs and primary cardiomyocytes) using media preparations that promote the expansion of stem cells and stem cell progeny, i.e. methylcellulose stem cell medium [20]. Cells cultured in this manner gave rise to a number of presumptive stem cell colonies, at a low frequency of ~1 colony per 50 000 cells plated. Of interest, a portion of these suspended colonies became attached to surface adhered primary cardiomyocytes. Subsequently, the size of these colonies declined over time as the underlying cardiomyocyte colonies expanded and began rhythmic contractions (Fig. 1F–H).

Subsequently, we investigated the physiological response of this population to a myocardial growth challenge, i.e. do myocardial SP cell populations change with altered physiologic demands? Within this context, we compared the myocardial SP population from wild-type mice and MEF2C dominant negative (MEF2Cdn) mice. Previous studies from our laboratory have confirmed that cardiomyocyte-specific over-expression of a dominant negative MEF2C protein (MEF2Cdn) results in a substantial reduction in the size of individual cardiomyocytes leading to a post-natal hypoplastic myocardium (Fig. 2A,B; [17]). The MEF2Cdn strain is also unique in that there are no additional pathologic features such as fibrosis or immune cell infiltration, which are common to most models of cardiac insufficiency or damage. Quantification of the SP population from age-matched wild-type and MEF2Cdn hearts revealed a substantial reduction in the percentage of verapamil-sensitive SP cells in the MEF2Cdn genotype (~2.5-fold; Fig. 2C–G). The reduction was significant

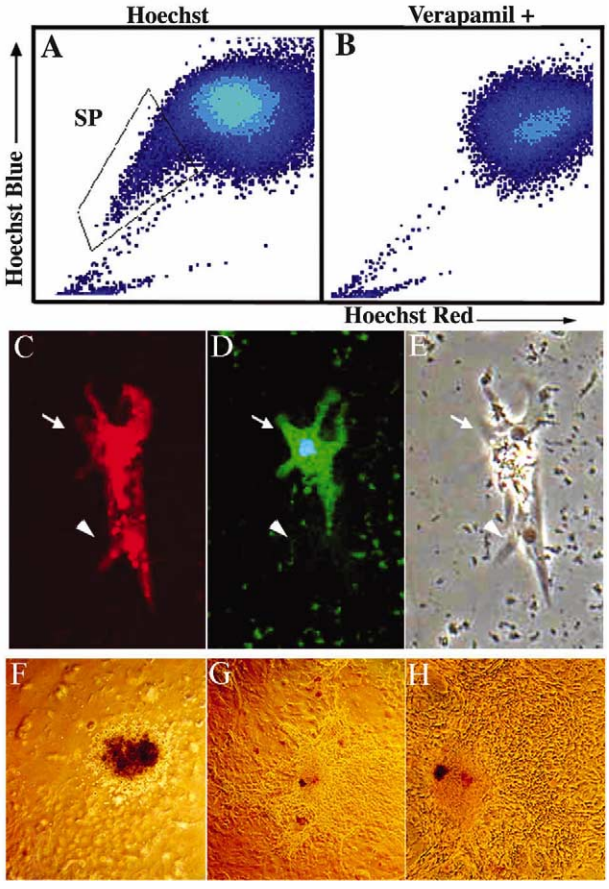

Fig. 1. FACS analysis of cardiac cell suspensions stained with Hoechst dye (A), or Hoechst dye plus verapamil (B). Cardiac-derived SP cells isolated from EGFP mice were co-cultured with wild-type 1° cardiomyocytes. Differentiated cardiomyocytes are marked by connexin 43 (C), while cardiac-derived SP cells are GFP-positive as indicated in panel D. The corresponding phase contrast image is shown in panel E. Arrows identify cardiac-derived SP cells expressing the cardiomyocyte differentiation marker, connexin 43. A wild-type cardiomyocyte cell is shown (arrowhead). Panels G–I illustrate the growth of an adhered stem cell-like colony contributing to the expansion of a cardiomyocyte colony at 8 days (50×; G) and 14 days (50×; H). Day 14 is shown at 200× magnification (I).

and reproducible ($P < 0.05$). These experiments did not specify the fate of the reduced SP cell population in the MEF2Cdn myocardium. Nevertheless, the concurrent increase in total cardiomyocyte number in this model suggests that the deleted SP cells were recruited and/or activated by the attenuation of post-natal cardiac growth. Indeed, cardiomyocyte counts in the MEF2Cdn are approximately 2.3-fold higher than in age-matched control myocardium, which is remarkably similar to the fold depletion in SP cells. Interestingly, the myocardial SP population also decreases with age, suggesting that these cells may also be physiologically responsive to growth demands in a non-disease myocardium (Fig. 2H).

The reduction in the MEF2Cdn SP cell population may reflect an activation/commitment process for this resident stem cell pool. Indeed, activated stem cell populations display evidence of lineage promiscuity. For example, stem cells derived from other tissue sources have been demonstrated to undergo lineage conversion when cultured with disparate cell types [11–13]. As an initial step, we assayed myocardial cell suspensions for the presence of stem cell progeny, i.e.

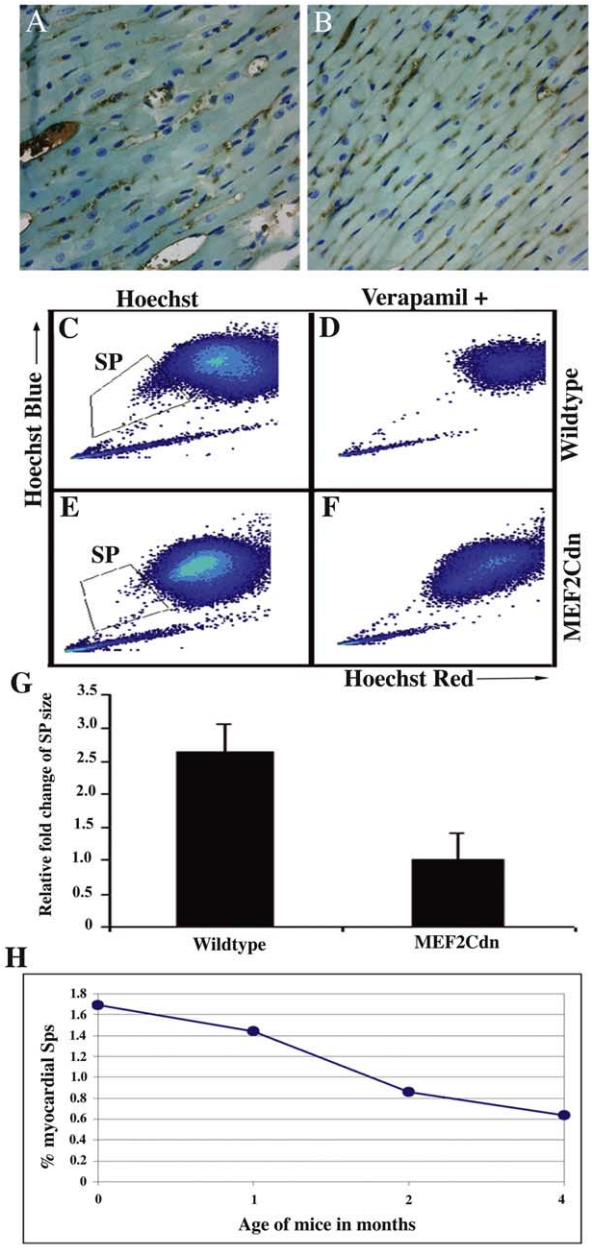

Fig. 2. Comparison of toluene blue-stained sections between wild-type (A) and MEF2Cdn (B) ventricles. MEF2Cdn sections show an increased density of nuclei compared to wild type. FACS analysis of cardiac cells from ventricles of wild-type (C, D) and MEF2Cdn (E, F) mice stained with Hoechst dye (C, E), or Hoechst dye plus verapamil (D, F). Comparison of the size of the SP fraction in three separate trials shows a >2.5-fold increase in wild-type vs. MEF2Cdn ventricles (G). Percentage of SP cells compared to total cells in the post-natal heart decreases with progressive aging, as illustrated in panel H.

colony formation using methylcellulose stem cell medium. Myocardial cell suspensions derived from wild-type hearts showed limited colony formation (Fig. 3A), yet MEF2Cdn myocardial cell suspensions (cultured under the same conditions) displayed robust hematopoietic colony formation (Fig. 3B–E). These colonies were comprised of both granulocytes and monocytes as determined by GR-1 and MAC-1 staining, respectively (Fig. 3F–H). The hematopoietic phenotypes of the myocardial cell suspensions were similar to the cell types derived from skeletal muscle-derived cell suspensions. However, the formation of hematopoietic colonies from myocardial cell suspensions is considerably lower than that observed for skeletal muscle (~10-fold difference), although a robust SP population is detectable from both sources [20]. In addition, FACS analyses using known stem cell markers for the hematopoietic and skeletal muscle lineage indicate that the cardiac SP population is not enriched for these proteins (CD34, c-kit, Sca-1, Flk-2, Thy1.1; data not shown). As

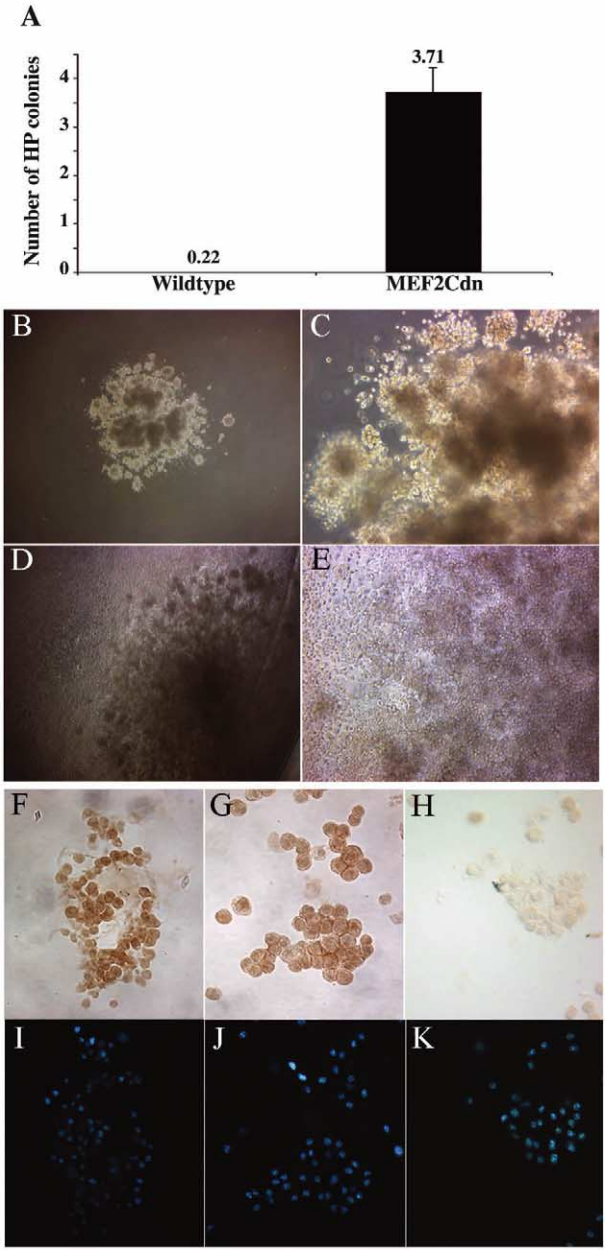

Fig. 3. Comparison of the stem cell-like activity of cardiac-derived SP cells between wild-type and MEF2Cdn mice, as determined by the formation of hematopoietic colonies on methylcellulose medium. Myocardial cell suspensions isolated from MEF2Cdn hearts show on average 3.7 colonies per 10 000 cells plated compared to on average less than one colony per plate from wild-type heart cell suspensions (A). Immunohistochemistry was performed on the colonies using hematopoietic markers GR-1 (F), and Mac-1 (G). Negative controls are shown in panel H. Nuclei were stained with Hoechst dye (I–G).

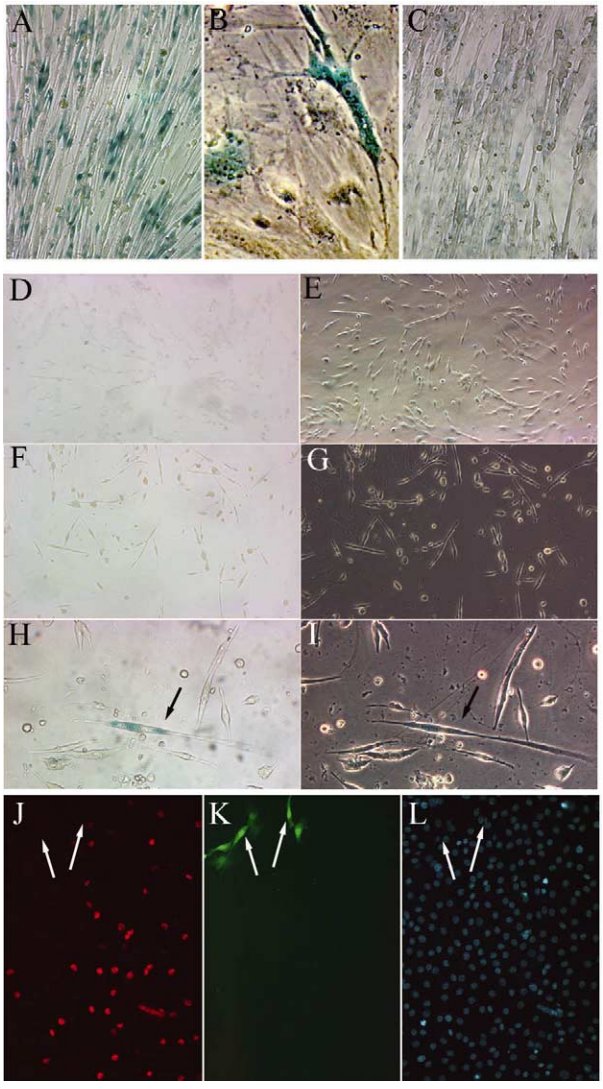

Fig. 4. Co-culture experiments demonstrate cardiac-derived SP cells are capable of fusion with skeletal muscle myoblasts, but not transdifferentiation in vitro. Cardiac-derived SP cells isolated from Z/AP mice cultured with wild-type 1° skeletal muscle cells (A) and H9C2 cardiomyocytes (B) show an ability for SPs to fuse with muscle cells as determined by β-galactosidase staining. Negative control is shown in panel C, in which no Z/AP cells were added. Cardiac-derived SPs from Myf5/LacZ transgenic mice were co-cultured with C2C12 cells (bright field (D) and phase contrast (E)), and 1° skeletal muscle cells (F–I) (bright field in panels F and H; phase contrast in panels G and I). Results were negative for β-galactosidase expression, with two rare exceptions depicted in panels H and I (arrow shown). Similar co-cultures were performed with SPs from GFP ventricles, plated with C2C12s (J–L). Panel J shows myogenin staining, while GFP-positive cells are seen in panel K. Nuclei were stained with DAPI (L). No double fluorescent-tagged cells were observed. Arrows show GFP-positive cells not seen in panel J.

such, we interpret this surge in colony formation to be the result of an in vivo stimulus to activate or commit a multipotent cell population, the stimulus in this instance being limited myocardial growth.

To directly test the ability for lineage conversion in the cardiac SP population, we purified myocardial SP cells from the Z/AP marker mice and performed co-culture experiments with non-cardiomyocyte cell types. The Z/AP strain utilizes a strong promoter–enhancer combination to yield systemic expression of a *lacZ* transgene [15]. The purified myocardial SP cells from Z/AP mice were capable of forming myotubes at a high frequency when co-cultured with primary-derived skeletal muscle myoblasts and H9C2 rat cardiomyocytes (β-galactosidase staining in the co-cultures ~25% of cell total; Fig. 4A,B). Although these results were suggestive of lineage conversion/transdifferentiation, the apparent incorporation of the SP cells into myotube structures may simply reflect cell fusion and not a true genetic reprogramming of the SP cell (a phenomenon observed with other stem cell types; [25,26]). Therefore, as an adjunct to the previous experiments we assayed the ability of myocardial SP cells to convert to a skeletal muscle genotype. The myocardial SP cells for these experiments were derived from the Myf5/lacZ mouse. This strain has a *lacZ* reporter gene targeted to the Myf5 locus, which ensures a skeletal muscle-specific expression pattern for the transgene [27]. Myf5/lacZ myocardial SP cells were co-cultured with both C2C12 skeletal muscle myoblasts and primary-derived skeletal muscle myoblasts, then subject to a low serum-induced differentiation time course. C2C12 co-cultures had a complete absence of β-gal staining (Fig. 4D,E) and primary skeletal muscle co-cultures displayed a very low frequency of 1 in 100 000 cells (Fig. 4F,I). This apparent lack of transdifferentiation was confirmed using a second transgenic marker strain with a ubiquitous expression pattern, the EGFP mouse [16]. As indicated in Fig. 4J–L, GFP-positive cardiac-derived SP cells were negative for the expression of the skeletal muscle transcription factor myogenin when co-cultured with C2C12 myoblasts.

In summary, while it has been previously shown that numerous tissue types possess stem cell populations, identification of an endogenous myocardial stem cell has not been forthcoming. The results of the current study provide the first evidence that the adult myocardium maintains a resident myocardial stem cell-like population. Cardiac SP cells retain characteristics of other stem cell populations, such as an effective fusion capacity with non-cardiac cell types. However, cardiac SP cells do not readily convert their genotype/gene expression profile, which suggests a myocardial-restricted function.

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