Stimulation of endogenous cardioblasts by exogenous cell therapy after myocardial infarction

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Abstract

Controversy surrounds the identity, origin, and physiologic role of endogenous cardiomyocyte progenitors in adult mammals. Using an inducible genetic labeling approach to identify small non-myocyte cells expressing cardiac markers, we find that activated endogenous cardioblasts are rarely evident in the normal adult mouse heart. However, myocardial infarction results in significant cardioblast activation at the site of injury. Genetically labeled isolated cardioblasts express cardiac transcription factors and sarcomeric proteins, exhibit spontaneous contractions, and form mature cardiomyocytes in vivo after injection into unlabeled recipient hearts. The activated cardioblasts do not arise from hematogenous seeding, cardiomyocyte dedifferentiation, or mere expansion of a preformed progenitor pool. Cell therapy with cardiosphere-derived cells amplifies innate cardioblast-mediated tissue regeneration, in part through the secretion of stromal cell-derived factor 1 by transplanted cells. Thus, stimulation of endogenous cardioblasts by exogenous cells mediates therapeutic regeneration of injured myocardium.

Keywords  cardiac stem cells; cardioblasts; cardiomyocyte progenitor cells; cardiosphere-derived cells; heart regeneration

Introduction

The adult mammalian heart is now viewed as a dynamic organ, capable of endogenous regeneration. While the rate of cardiomyocyte renewal in adults is low (Bergmann et al., 2009), exploitation of innate regenerative processes may open up novel therapeutic options favoring cardiomyocyte repopulation in the diseased heart. Improved insights into the cellular and molecular mechanisms of endogenous heart regeneration are needed, as the field is rife with controversy (Laflamme & Murry, 2011; Steinhauser & Lee, 2011; Garbern & Lee, 2013). While adult cardiomyocytes retain a detectable (albeit small) ability to proliferate (Soonpaa & Field, 1997; Malliaras et al., 2013a; Senyo et al., 2013), the role of endogenous progenitor cells in adult cardiomyogenesis is unclear (Laflamme & Murry, 2011; Steinhauser & Lee, 2011; Garbern & Lee, 2013; Roudstaal et al., 2013). During the past decade, numerous populations of putative adult endogenous cardiomyocyte progenitors have been proposed, mainly on the basis of expression of surface markers and functional properties that have been used to mark progenitors in other organs, and expression of cardiac proteins in vitro or after delivery into recipient hearts following ex vivo expansion (Beltrami et al., 2003; Oh et al., 2003; Martin et al., 2004; Messina et al., 2004; Ott et al., 2007; Chong et al., 2011; Tamura et al., 2011). However, the physiologic importance of these various progenitor populations and their contribution to cardiomyocyte replenishment in the normal or injured adult heart remains topics of intense debate.

Genetic fate mapping offers the unique ability to study organ regeneration in vivo (Kretzschmar & Watt, 2012). Using an inducible fate mapping approach [where Cre recombinase activity, driven by the cardiac α-myosin heavy chain (αMHC) promoter, is induced prior to myocardial infarction (MI) to genetically label pre-existing cardiomyocytes], multiple groups have detected a dilution of the labeled myocyte pool post-injury (Hsieh et al., 2007; Loffredo et al., 2011; Qian et al., 2012; Song et al., 2012), consistent with the generation of new cardiomyocytes by unlabeled myocyte progenitors. By combining the aforementioned experimental approach (which retrospectively captures the net result of endogenous progenitor cell activation and differentiation, but fails to reveal the identity and properties of said progenitors) with long-term BrdU pulsing, we have previously provided indirect evidence that cell therapy with exogenous cardiosphere-derived cells (CDCs) upregulates endogenous progenitor cell-mediated heart regeneration (Malliaras et al., 2013a). Here, we took a different approach: induction of Cre recombinase activity following (and not prior to) myocardial injury. We sought to achieve prospective genetic labeling of endogenous cardioblasts, as they turn on the αMHC promoter post-injury on their way to cardiomyogenic differentiation. Using this approach, we directly investigated the existence, identity, and origin of cardioblasts in the injured adult mouse heart, and how these progenitors are influenced by exogenous cell therapy.
Results

Activation of endogenous cardioblasts post-MI

In bitransgenic Myh6-MerCreMer/ZEG mice, transient exposure to tamoxifen induces an irreversible genetic switch from β-galactosidase expression to green fluorescent protein (GFP) expression in cells with zMHC promoter activity, resulting in permanent labeling of zMHC+ cells by GFP (Hsieh et al., 2007). Bitransgenic mice were randomized to undergo sham surgery or MI by permanent ligation of the left anterior coronary artery (LAD), followed by daily pulsing with 4OH-tamoxifen (Fig 1A). Ten days later, harsh enzymatic digestion of the explanted hearts (followed by multiple filtering steps) resulted in efficient depletion of adult cardiomyocytes (Supplementary Fig S1). Examination of the isolated cardiomyocyte-depleted cell fraction revealed small (11.5 ± 3.7 μm) round GFP+, lacZ+ cells which we go on to implicate as cardioblasts (Fig 1D, E and I). Quantification by flow cytometry and epifluorescence microscopy revealed that GFP+ cardioblasts were rare in sham-operated hearts (0.12 ± 0.06% of non-myocyte cells) (Fig 1B and C, Supplementary Fig S2), but their number increased >10-fold post-MI (1.34 ± 0.48% of non-myocyte cells in the risk area) (Fig 1B and C). GFP+ cardioblasts were highly enriched in the infarct and peri-infarct area (Fig 1F, Supplementary Fig S3), compared to the remote myocardium (Fig 1G and H). These results demonstrate that MI results in cardioblast activation (which includes ‘turning on’ the zMHC promoter) within the risk area.

Properties of endogenous cardioblasts

We investigated expression of cardiac transcription and structural factors at the protein level in fluorescence-activated cell sorting (FACS)-sorted GFP+ cardioblasts by fluorescent immunocytochemistry (without any intermediate culture step) and tissue immunohistochemistry. The majority of GFP+ cardioblasts expressed NKKX2-5 (69%) and GATA4 (74%), while 16% were positive for MEF2C (Fig 2B and D, Supplementary Fig S4). We could not detect expression of TBX5 or Isil in GFP+ cardioblasts. With regard to sarcomeric proteins, 38% of GFP+ cardioblasts expressed α-sarcomeric actinin and 39% expressed zMHC (Fig 2C and D). The discrepancy between zMHC promoter activity and protein expression (zMHC at the protein level was expressed in only a subset of GFP+ cardioblasts) can be rationalized by the fact that promoter activity is several steps upstream of protein synthesis, one of several factors underlying the poor correlation between mRNA and corresponding cellular protein abundance (Vogel & Marcotte, 2012). No GFP+ cardioblasts expressed endothelial (CD31) or smooth muscle cell (α-smooth muscle actin) markers. Profiling of proteins associated with cell cycle progression revealed that 17% of GFP+ cardioblasts were positive for Ki67 (a marker of late G1, S, G2, and M phases of cell cycle) and 2% were positive for phosphorylated histone H3 (H3P, a marker of karyokinesis) (Fig 2E and G, Supplementary Fig SSA). Immunohistochemistry identified GFP+ cardioblasts appearing to undergo mitosis in the border zone (Fig 2F and Supplementary Fig SSB).

To investigate whether GFP+ cardioblasts exhibit contractile activity, enzymatically digested cardiomyocyte-depleted heart cells were plated onto fibronectin-coated dishes (without FACS for GFP, to avoid traumatic cell injury). While few GFP+ cardioblasts survived in culture, approximately 11% (7/66) exhibited weak spontaneous contractions in vitro over the course of the first 2 days post-plating, without exposure to cardiac differentiation medium (Fig 2A, Supplementary Movies S1 and S2). Modifications in the culture conditions (including plating isolated cardioid cell preparations onto a feeder layer, or culturing cells in embryonic stem cell medium) failed to improve survival of GFP+ cardioblasts in vitro. While rare examples of dividing GFP+ cardioblasts could be detected (Supplementary Fig S6), no GFP+ cardioblasts survived more than 1 week in culture.

To investigate expression of cardiogenic molecules and cardiac structural factors at the transcript level, we performed RT-qPCR in RNA extracted from FACS-sorted GFP+ and GFP-myocyte-depleted cardiac cells (obtained from the same hearts, n = 5 hearts) and from adult cardiomyocytes (n = 3) for genes that are upregulated during cardiomyogenic differentiation of embryonic stem cells (Paige et al., 2012). With regard to cardiac transcription factors and cardiogenic molecules, we found upregulation of NKKX2-5 (7.8-fold), MEF2C (3.8-fold), TBX3 (2.6-fold), FOXC2 (5.1-fold), and BMP2 (5.4-fold) expression in GFP+ cardioblasts compared to GFP− cardiocytes. A non-significant increase in GATA4 expression (1.8-fold, P = 0.151 versus GFP− cells) was observed [a finding that may also reflect GATA4 expression in GFP− cardiac fibroblasts (Zaglia et al., 2009)], while no major differences in expression of Isl1, TBX2, TBX5, TBX20, MESP1, MESP2, GATA6, WNT2, WNT5a, WNT11, DKK1, HAND1, HAND2, FOXC1, and SMARCD3 could be detected. Compared to adult cardiomyocytes, GFP+ cardioblasts exhibited lower expression of NKKX2-5, similar expression of MEF2C, TBX3, and GATA4, and increased expression of BMP2 and FOXC2 (Fig 3). With regard to cardiac structural factors, GFP+ cardioblasts exhibited an ‘intermediate’ phenotype between GFP− non-myocyte cardiac cells and adult cardiomyocytes. Specifically, we observed upregulation of MYH6 (13.5-fold), MYH7 (7.7-fold), MYL2 (15.9-fold), MYL3 (13.7-fold), TNNI3 (5.5-fold), TNN2 (6.7-fold), MYBPC3 (16.4-fold), PLN (2.4-fold), DES (4.0-fold), NPPA (4.0-fold), CKM (11.0-fold), and TTN (3.8-fold) in GFP+ cardioblasts compared to GFP− cardiac cells. Compared to adult cardiomyocytes, GFP+ cardioblasts exhibited decreased expression of all investigated cardiac structural factors, with the exception of MYH7 (which was expressed at similar levels in both cell populations). Importantly, expression of fibroblast (VIM)-, endothelial (VWF)-, and smooth muscle cell (ACTA2)-related genes was lower in GFP+ cardioblasts compared to GFP− non-myocyte cardiac cells, providing additional evidence that FACS for GFP+ myocyte-depleted cardiac cells enriches a committed cardioblast population (Fig 3).

To examine whether GFP+ cardioblasts expressed surface markers or exhibited functional properties previously attributed to various putative endogenous cardiomyocyte progenitor populations, we looked for expression of Sca-1 (Oh et al., 2003), c-kit (Beltrami et al., 2003), nestin (Tamura et al., 2011), PDGFrα (Chong et al., 2011), and stage-specific embryonic antigen 1 (SSEA1) (Ott et al., 2007) in GFP+ cardioblasts, as well as the ability to actively efflux Hoechst dye (side population cells) (Martin et al., 2004) by immunocytochemistry and flow cytometry. We found that 54.5 ± 15.3% of GFP+ cardioblasts (as determined by immunocytochemistry) were positive for Sca-1, but Sca-1 was not specific for cardioblasts (only ~3% of Sca1+ cells expressed GFP; Fig 4A and D). A minority of
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Figure 1.

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GFP+ cardioblasts (10.8 ± 3.2%, as determined by immunocytochemistry) expressed nestin (Fig 4C and D), while < 1% of GFP+ cardioblasts actively effluxed Hoechst dye (Fig 4B and D). Immunocytochemistry demonstrated that GFP+ cardioblasts were negative for c-kit, SSEA1, PDGFRα, and CD34 (Fig 4D). To exclude the possibility that enzymatic tissue digestion altered the expression of surface markers via cleavage of stem cell-related antigens, we performed RT-qPCR in RNA extracted from FACS-sorted GFP+ surface markers via cleavage of stem cell-related antigens, we performed RT-qPCR in RNA extracted from FACS-sorted GFP+ myocyte-depleted cardiac cells (obtained from five hearts) for expression of KIT, FUT4 (SSEA1), PDGFRα, and CD34. KIT and FUT4 (SSEA1) were not detected in any samples. Very low expression of PDGFRα and CD34 was detected in 3/5 samples [on average > 1,000-fold less compared to expression of MYH6 (α-MHC) in the same samples] [Supplementary Fig S7]. However, we cannot exclude the possibility that expression of stem cell-related antigens may be lost as progenitors begin to differentiate.

To investigate whether GFP+ cardioblasts can differentiate into mature cardiomyocytes in vivo, small cells isolated from infarcted mice 10 days following MI and daily tamoxifen pulsing were FACS-sorted for GFP positivity and lack of 7-actinomyocin D incorporation (to select for live cells). We then injected these cardioblasts into normal (n = 3) or infarcted (n = 3) recipient hearts of background non-transgenic mice (10,000 cardioblasts/heart, Fig 5A). Employing a model of cardioblast isolation and transplantation into non-transgenic recipients was necessary, as our fate mapping model also labels pre-existing cardiomyocytes, complicating the investigation of cardiomyogenic differentiation of GFP+ cardioblasts in situ. Three months post-injection, recipient hearts were examined for GFP+ cells, either by immunohistochemistry or by epifluorescent microscopy of dissociated cardiomyocytes. Rare events of cardiomyogenic differentiation could be detected in four of six cardioblast-injected hearts [2/3 normal (4.2 ± 2.2 GFP+ cardiomyocytes/heart section) and 2/3 infarcted (3.4 ± 3.6 GFP+ cardiomyocytes/heart section) hearts; Fig 5B–D]. GFP+ cardiomyocytes appeared fully mature and structurally integrated with the surrounding myocardium, as they shared gap junctions with neighboring GFP+ myocytes (Fig 5B). While we cannot exclude cell fusion (between injected GFP+ cells and host myocytes) as a source of GFP+ cardiomyocytes, such fusion events are exceedingly rare in the myocardium (Alvarez-Dolado et al, 2003), occurring at a frequency 10- to 100-fold smaller compared to the frequency of GFP+ cardiomyocytes observed post-injection of GFP+ cardioblasts here. No instances of GFP+ endothelial (von Willebrand factor+) or smooth muscle cells (α-smooth muscle actin+) cells could be detected. Even though we did not perform a controlled study to investigate whether transplantation of GFP+ cardioblasts confers therapeutic benefit to infarcted mice, comparison of cardiac function by echocardiography between baseline and 5 weeks post-MI revealed a significant decline in ejection fraction (30 ± 5% versus 22 ± 7%, P = 0.036). The lack of major functional benefit can be rationalized by the paucity of long-term engrafted GFP+ cardiomyocytes. The latter may reflect the low dose of injected GFP+ cardioblasts, likely compounded by limited survival of GFP+ cardioblasts following traumatic FACS purification and transplantation into recipient hearts; however, a low efficiency of mature cardiomyogenic differentiation by GFP+ cardioblasts in vivo cannot be excluded.

Origin of endogenous cardioblasts

Since contribution of bone marrow-derived cells to cardiomyogenesis is controversial in the adult mammalian heart (Laflamme et al, 2002; Quaini et al, 2002; Deb et al, 2003; Balsam et al, 2004; Murry et al, 2004; Nygren et al, 2008), we sought to investigate whether activated GFP+ cardioblasts arise from hematogenous seeding. Immunohistochemistry of infarcted hearts demonstrated that GFP+ cardioblasts were negative for the pan-leukocyte marker CD45 (Supplementary Fig S8). However, since bone marrow also contains CD45+ cells that have been reported to express cardiac proteins (Kucia et al, 2006; Dawn et al, 2008), we performed bone marrow transplantation experiments in order to investigate definitively a potential bone marrow origin of GFP+ cardioblasts. Bone marrow was isolated from bitransgenic Myh6-MerCreMer mice and transplanted into lethally irradiated non-transgenic background mice (n = 9) (Wang et al, 2006). Four weeks later (a time period sufficient to ensure bone marrow reconstitution by donor cells), transplanted mice were randomized to undergo sham surgery (n = 4) or MI (n = 5), followed by daily pulsing with 4OH-tamoxifen (Fig 6A). Ten days later, lacZ+ cells were readily detectable in the infarct region (Fig 6B), indicating successful reconstitution of the bone marrow by transplanted cells obtained from bitransgenic donors.
Figure 2. Endogenous cardioblasts exhibit spontaneous contractions and express cardiac transcription factors, sarcomeric and cycling proteins.

A Still images of GFP+ cardioblasts exhibiting spontaneous contractile activity in vitro after 1 day in culture (see Supplementary Movies S1 and S2 for the corresponding Movies) (scale bars, 10 μm) [green: GFP, bright field (BF)].

B Fluorescent immunocytochemistry of FACS-sorted GFP+ cardioblasts for nuclear expression of cardiac transcription factors NKX2-5, GATA4, and MEF2C. Note that GFP− cells (middle & right panels, resulting from imperfect FACS for GFP) are negative for cardiac transcription factors (blue: DAPI, green: GFP, red: NKX2-5, GATA4, MEF2C).

C Fluorescent immunocytochemistry of FACS-sorted GFP+ cardioblasts for cytoplasmic expression of α-sarcomeric actinin (αSA) and α-myosin heavy chain (αMHC). Image on the right shows a GFP+ cardioblast expressing αMHC in the cytoplasm and NKX2-5 in the nucleus. Note that GFP− cells (left panel) do not express sarcomeric proteins (blue: DAPI, green: GFP, red: αSA/αMHC).

D Quantification of expression of cardiac transcription factors and sarcomeric proteins in GFP+ cardioblasts by fluorescent immunocytochemistry (n = 4 mice). No expression of TBX5 or ISL1 could be detected in GFP+ cells.

E Fluorescent immunocytochemistry of FACS-sorted GFP+ cardioblasts for Ki67 and phosphorylated histone H3 (H3P). Note that the GFP+ cell is Ki67− (left panel) and the GFP+ dividing cardioblast is expressing H3P in both nuclei (right panel) (blue: DAPI, green: GFP, red: Ki67/αSA, white: H3P).

F Confocal microscopy revealed the presence of GFP+ cardioblasts appearing to undergo mitosis in the border zone. Image on the right is a magnified image of highlighted area on left. Image of confocal scanning across the XZ plane is also provided (blue: DAPI, green: GFP, red: αSA).

G Quantification of expression of cycling proteins in GFP+ cardioblasts by fluorescent immunocytochemistry (n = 4 mice).
animals. Not a single GFP+ cell could be detected by either tissue immunohistochemistry or epifluorescence microscopy and immunocytochemistry of enzymatically digested myocyte-depleted cell preparations isolated from sham-operated and infarcted hearts (Fig 6B and D). Flow cytometry revealed a percentage of GFP+ cells similar to that measured in background non-transgenic (non-GFP-expressing) non-transplanted mice (0.04% of cells were detected as GFP+), which did not increase after MI (Fig 6C and D). These results exclude the possibility that GFP+ cardioblasts arise from hematogenous seeding.

To investigate whether the increase in GFP+ cardioblasts observed post-MI originates from dedifferentiation of resident myocytes or from expansion of a pre-existing pool of already committed (αMHC+*) progenitors, on the basis of conventional retrospective analysis (Malliaras et al, 2010). We previously concluded that CDCs upregulate endogenous progenitors, on the basis of conventional retrospective analysis (Malliaras et al, 2013a). Using the prospective genetic labeling approach described here, we directly tested whether transplantation of exogenous CDCs upregulates endogenous cardioblast activation in injured hearts. Bitransgenic mice were randomly assigned to 1 of 3 groups: (a) sham surgery, (b) MI and (c) MI followed by intramyocardial injection of mouse CDCs (2 × 10⁵, grown from background non-transgenic animals) into the infarct border zone. Mice were subsequently pulsed daily with 4OH-tamoxifen. Ten days later, flow

Cardiosphere-derived cells (CDCs) (Smith et al, 2007) have been shown to exert regenerative effects in animal models of ischemic cardiomyopathy (Johnston et al, 2009; Malliaras et al, 2012, 2013b) and in patients with convalescent MI (Makkar et al, 2012; Malliaras et al, 2014). We previously concluded that CDCs upregulate endogenous progenitors, on the basis of conventional retrospective analysis (Malliaras et al, 2013a). Using the prospective genetic labeling approach described here, we directly tested whether transplantation of exogenous CDCs upregulates endogenous cardioblast activation in injured hearts. Bitransgenic mice were randomly assigned to 1 of 3 groups: (a) sham surgery, (b) MI and (c) MI followed by intramyocardial injection of mouse CDCs (2 × 10⁵, grown from background non-transgenic animals) into the infarct border zone. Mice were subsequently pulsed daily with 4OH-tamoxifen. Ten days later, flow
cytometry and epifluorescent microscopy revealed a significant upregulation of GFP+ cardioblasts in infarcted hearts that had been injected with CDCs \( 4.78/0.88\% \) of non-myocyte cells in the risk area expressed GFP (Fig 7A).

The indirect effects of CDCs to activate cardioblasts motivate a search for the relevant paracrine signals. Three sets of findings point to stromal cell-derived factor 1 (SDF1) and vascular endothelial growth factor (VEGF) as potentially important secreted factors in CDC-mediated stimulation of GFP+ cardioblasts post-MI: (1) SDF1 (Segers et al., 2007; Unzek et al., 2007) and VEGF (Lin et al., 2012) can upregulate putative endogenous cardiomyocyte progenitors; (2) CDCs are potent secretors of both SDF1 and VEGF (Li et al., 2012); and (3) immunocytochemistry revealed that a subset of GFP+ cardioblasts expressed CXCR4 (the receptor for SDF1, Fig 7B) and VEGFR1 (but not VEGFR2 or VEGFR3, Fig 7C). We therefore suppressed SDF1 or VEGF expression in CDCs by transduction with short hairpin (sh)RNA-expressing lentiviral vectors. Knockdown of SDF1 and VEGF in CDCs decreased in vitro production of SDF1 \([\text{to levels } \sim 18\% \text{ of those produced by sh-control-transduced CDCs (Fig 7D)}]\) and VEGF \([\text{to } \sim 23\% \text{ of the levels produced by sh-control-transduced CDCs (Fig 7E)}]\) and decreased myocardial abundance of SDF1 (Fig 7F) and VEGF (Fig 7G) in vivo, 4 days after MI and injection of shRNA-transduced CDCs. Subsequently, bitransgenic mice underwent MI and were randomly assigned to receive intramyocardial injections of sh-control-transduced CDCs, sh-SDF1-transduced CDCs, or sh-VEGF-transduced CDCs, followed by daily pulsing with 4OH-tamoxifen. Ten days later, flow cytometry and epifluorescent microscopy revealed that knockdown of SDF1 (but not VEGF) in CDCs resulted in significant attenuation of CDC-mediated GFP+ cardioblast upregulation in the infarcted area (Fig 7H).

We next sought to examine whether inhibition of SDF1-mediated stimulation of endogenous cardioblasts resulted in decreased...
Figure 5. Endogenous cardioblasts differentiate into mature myocytes after transplantation into recipient hearts.

A Study schematic. FACS-sorted GFP⁺ cardioblasts activated post-MI were injected into non-injured (n = 3) or infarcted (n = 3) hearts of non-transgenic background mice.

B Confocal microscopy in tissue sections from uninjured hearts revealed rare instances of GFP⁺ cardiomyocytes 3 months post-injection. GFP⁺ cardiomyocytes appeared fully mature and were connected by gap junctions [connexin 43 (Cx 43)] to neighboring GFP⁻ myocytes. Images on the right are high-power images of highlighted areas on left [blue: DAPI, green: GFP, red: α-sarcomeric actin (αSA), purple: Cx 43].

C Fluorescence micrograph of a GFP⁺ cardiomyocyte after enzymatic dissociation of an uninjured heart on a Langendorff apparatus. Image on the right is a high-power image of inset on left (scale bars, 50 µm) [green: GFP, bright field (BF)].

D Confocal microscopy of tissue sections from infarcted hearts revealed rare instances of GFP⁺ cardiomyocytes in the infarct border zone 3 months post-injection (blue: DAPI, green: GFP, red: αSA).
structural and functional benefits in the infarcted heart. Pre-pulsed bitransgenic mice were randomly assigned to undergo (a) MI, (b) MI followed by intramyocardial injection of sh-control-transduced CDCs, or (c) MI followed by intramyocardial injection of sh-SDF1-transduced CDCs. Mice pre-pulsed with 4OH-tamoxifen were used for this set of experiments in order to allow for comparison of the dilution of the pool of GFP+ cardiomyocytes by endogenous progenitors (see below). Five weeks post-MI, both sh-control CDC- and sh-SDF1 CDC-treated hearts exhibited smaller scar mass and increased viable myocardium compared to untreated infarcted controls (Fig 8A–C). While no significant differences in total scar mass or total viable mass could be observed between sh-control CDC- and sh-SDF1 CDC-treated hearts, knockdown of SDF in transplanted CDCs increased scar transmurality and decreased viability in the infarcted area (Fig 8A, D and E). Sh-control CDC-treated animals displayed superior global function, attenuation of adverse remodeling, and increased capillary density in the peri-infarct area by immunohistochemistry (left) and immunocytochemistry (right), indicating successful reconstitution of the bone marrow (blue: DAPI, green: GFP, red: αSMA, white: lacZ).

Figure 6. Origins of endogenous cardioblasts.
A Study schematic. Bone marrow from Myh6-MerCreMer was transplanted into lethally irradiated non-transgenic background mice. Bone marrow-reconstituted mice were randomized to undergo sham surgery or MI, followed by 4OH-tamoxifen pulsing.
B LacZ cells were readily detectable in the infarct region by immunohistochemistry (left) and immunocytochemistry (right), indicating successful reconstitution of the bone marrow (blue: DAPI, green: GFP, red: αSMA, white: lacZ).
C Representative flow cytometry plots of enzymatically digested myocyte-depleted cardiac cell preparations for side scatter (SSC) and GFP expression (color gating has been applied to the images). Numbers indicate average % GFP positivity in each group.
D Quantification of GFP+ cardioblasts by flow cytometry (FC) and epifluorescence microscopy (epi) in sham-operated (sham) and infarcted (MI) hearts from bone marrow-reconstituted mice. Not a single GFP+ cell was detected by epifluorescence microscopy (n = 4–5 mice/group).
E Study schematic. Bitransgenic mice, pre-pulsed with 4OH-tamoxifen, were randomized to undergo sham surgery or MI.
F Representative flow cytometry plots of enzymatically digested myocyte-depleted cardiac cell preparations for side scatter (SSC) and GFP expression (color gating has been applied to the images). Numbers indicate average % GFP positivity in each group.
G Quantification of GFP+ cardioblasts by flow cytometry (FC) and epifluorescence microscopy (epi) in sham-operated (sham) and infarcted (MI) hearts from pre-pulsed bitransgenic mice (*P < 0.05 compared to sham, n = 3–5 mice/group).
Figure 7. SDF1-dependent cardiosphere-derived cell-mediated upregulation of endogenous cardioblasts post-MI.

A Representative flow cytometry plots of enzymatically digested myocyte-depleted cardiac cell preparations for side scatter (SSC) and GFP expression (color gating has been applied to the images). Numbers indicate average % GFP positivity in each group (left). Quantification of GFP+ cardioblasts by flow cytometry (FC) and epifluorescence microscopy (epi) in sham-operated (sham), infarcted (MI), and cell-treated (CDCs) mouse hearts (*P < 0.05 compared to sham, #P < 0.05 to MI, n = 3–5 mice/group).

B, C Fluorescent immunocytochemistry and quantification of CXCR4 (B) and VEGFR1 (C) receptors in GFP+ cardioblasts (blue: DAPI, green: GFP, red: CXCR4/VEGFR1) (n = 3 mice).

D-G Knockdown of SDF1 and VEGF resulted in decreased in vitro production of SDF1 (D) and VEGF (E) (*P < 0.05 compared to sh-control-transduced CDCs, n = 3) and in decreased myocardial levels of SDF1 (F) and VEGF (G) in vivo, 4 days after MI and injection of shRNA-transduced CDCs (*P < 0.05 compared to MI, #P < 0.05 compared to shSDF1- or shVEGF-transduced CDCs, n = 3 mice/group).

H Representative flow cytometry plots of enzymatically digested myocyte-depleted cardiac cell preparations for side scatter (SSC) and GFP expression (color gating has been applied to the images). Numbers indicate average % GFP positivity in each group (left). Quantification of GFP+ cardioblasts by flow cytometry (FC) and epifluorescence microscopy (epi) in infarcted hearts injected with sh-control-, sh-SDF1- and sh-VEGF-transduced CDCs (*P < 0.05 compared to shSDF1-transduced CDCs, n = 5 mice/group).
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Discussion

Elucidation of the cellular sources of innate adult cardiomyogenesis is a crucial step toward the development of therapeutic strategies to amplify regeneration of infarcted myocardium. While adult cardiomyocytes can re-enter the cell cycle and proliferate post-MI (Soonpaa & Field, 1997; Malliaras et al, 2013a; Senyo et al, 2013), controversy surrounds the existence and identity of endogenous cardiomyocyte progenitors as well as their role in the generation of new myocytes in the injured adult mammalian heart. Previous studies using an inducible fate mapping approach (where Cre recombinase activity, driven by the αMHC promoter, is induced prior to myocardial injury) have provided indirect evidence that endogenous progenitors replenish lost cardiomyocytes post-MI (Hsieh et al, 2007; Laffredos et al, 2011; Qian et al, 2012; Song et al, 2012; Malliaras et al, 2013a). This approach cannot directly label said endogenous progenitors and thus cannot discern their molecular identity, origin, and functional properties. Expanding upon these studies, we used the same fate mapping model and hypothesized that induction of Cre recombinase activity after MI would enable prospective genetic labeling of endogenous progenitors, as they would presumably activate the αMHC promoter post-injury on their way to cardiomyogenic differentiation.

By employing the prospective labeling approach in combination with cardiac cell isolation protocols designed to achieve efficient myocyte depletion, we demonstrate the existence of rare non-myocyte cells exhibiting αMHC promoter activity in the adult uninjured heart. MI results in activation of the αMHC promoter in a substantial subset of non-myocyte cells in the risk area. These cells (which express cardiac transcription factors, cardiac structural proteins and proteins associated with cell cycle progression) can be isolated in a manner consistent with αMHC reporter activity (MHC promoter post-injury on their way to cardiomyocyte specification). This approach also labels resident cardiomyocytes. Since the presence of differentiated myocyte progenitors, rather than uncommitted, undifferentiated parent stem cells. With regard to their anatomic origin, we demonstrate that the identified endogenous cardioblasts do not arise from hematogenous seeding. While we show that the majority of cardioblasts are activated (i.e., turn on the αMHC promoter) post-MI, a small minority may originate from a pre-existing, already committed cardioblast pool or from dedifferentiation of resident myocytes.

Perhaps most importantly from a therapeutic and translational perspective, we find that cell therapy with CDCs stimulates endogenous cardioblast-mediated myocardial regeneration. CDCs have been shown to confer therapeutic benefit in animal models of acute MI and ischemic cardiomyopathy (Smith et al, 2007; Johnston et al, 2009; Malliaras et al, 2012, 2013b), and recently in humans (Makkar et al, 2012; Malliaras et al, 2014); in the prospective randomized CADUCEUS trial, intracoronary infusion of autologous CDCs in patients with convalescent MI induced an increase in viable myocardium of approximately 22 g (while no significant changes were observed in the control group) and resulted in improved regional contractility of the infarcted area (Makkar et al, 2012; Malliaras et al, 2014); these results are consistent with therapeutic heart regeneration. Multiple lines of evidence from preclinical studies suggest that the mechanism of benefit is indirect: CDCs stimulate endogenous reparative and regenerative pathways, resulting in durable benefits despite evanescent cell survival (Chimenti et al, 2010; Malliaras et al, 2013a). The indirect mechanism of action rationalizes the efficacy of allogeneic CDCs in animal models of MI (Malliaras et al, 2012, 2013b); indeed, allogeneic human CDCs have recently entered the clinical arena in the ongoing ALLSTAR trial (NCT01458405). The present study offers mechanistic insights into therapeutic regeneration: CDCs upregulate endogenous cardioblasts in part through the secretion of SDF1. Overexpression of SDF1 in mesenchymal stem cell lines has been shown to increase their therapeutic efficacy (Zhang et al, 2007), while physical (Assmus et al, 2013)-, gene transfer (Penn et al, 2013), and protein (Seger et al, 2007)-based approaches that increase SDF1 myocardial levels have yielded promising results in animals and human subjects.

Limitations

Our study has several limitations. First, our genetic labeling approach also labels resident cardiomyocytes. Since the presence of
Figure 8.
labeled whole cardiomyocytes or cardiomyocyte remnants in the isolated cell preparations could confound our results, we took extensive care to ensure efficient cardiomyocyte depletion, by combining a harsh enzymatic digestion protocol with multiple filtering steps. We found that our approach effectively eliminated labeled resident cardiomyocytes from myocyte-depleted cardiac cell preparations (Supplementary Fig S1). In addition, in our analysis of cardiac cell preparations by epifluorescence microscopy and fluorescence immunocytochemistry, we only examined small cells [cell area in isolated GFP+ cardioblasts was ~110 μm², much smaller compared to that of isolated neonatal (~330–870 μm²) (Tamamori et al., 1998; Finn et al., 1999) or adult myocytes (~2500 μm²) (Shioi et al., 2000)] with intact morphology and a central nucleus (to rule out myocyte fragments). In flow cytometry experiments, forward and side scatter gates were set to exclude cellular debris and cells with exceedingly high forward and side scatter [which are known characteristics of myocytes (Diez & Simm, 1998; Malliaras et al., 2013a)]. In tissue immunohistochemistry experiments, only cells positive for GFP but negative for sarcomeric proteins were considered as cardioblasts. In any case, if our cell isolation approach resulted in a significant contamination of cell preparations by labeled whole cardiomyocytes or cardiomyocyte remnants, one would not expect to detect differences in the number of labeled cells in sham-operated, infarcted, and cell-treated hearts (as well as in pre-pulsed hearts), as those conditions would not alter the level of contamination by labeled resident cardiomyocytes in our cell preparations. Finally, it should be noted that the majority of the detected labeled cells did not express sarcomeric proteins (Fig 2D), which by default excludes the possibility that they were cardiomyocyte remnants. Nevertheless, we acknowledge labeling of resident cardiomyocytes as a limitation of our study. Second, our genetic labeling approach cannot identify the identity of the parent cell (prior to activation of the αMHC promoter) that gives rise to endogenous committed cardioblasts. The investigation of surface receptor expression in cardioblasts identified Sca-1 as a potential (albeit non-specific) marker that may be expressed in parent cells. It should be noted, though, that Sca-1 (Oh et al., 2003) is expressed in several putative adult cardiac progenitors [including c-kit+ (Bailey et al., 2012) and PDGFRα+ (Chong et al., 2011) cells]. Ultimately, forward-fate mapping experiments for putative stem cell markers will be required to reveal the identity of the parent cell that gives rise to cardioblasts. To that end, a recent study employing forward fate mapping for Sca-1 demonstrated a contribution of Sca-1-derived cells to the myocyte pool in the normal heart, which increases post-myocardial injury (Uchida et al., 2013). Third, even though we show that endogenous cardioblasts can differentiate into mature cardiomyocytes after injection into recipient non-transgenic hearts, our fate mapping model (which also labels cardiomyocytes) does not allow for the investigation of cardiomyogenic differentiation of cardioblasts in situ or for direct quantification of the number of cardiomyocytes that arise from this cell population. Our previous study using the same fate mapping model (in which administration of 4OH-tamoxifen prior to injury was combined with long-term BrdU pulsing post-injury) provided indirect evidence that endogenous progenitors replenish approximately 1% of total myocytes post-MI and approximately 2.5% of total myocytes post-cell therapy with CDCs, during the first 5 weeks post-injury (Malliaras et al., 2013a). Fourth, even though we show that cell therapy with CDCs upregulates endogenous cardioblasts partially through SDF1 secretion, our model cannot discern whether this is a result of increased survival, activation, proliferation, or recruitment of endogenous cardioblasts. SDF1 has been shown to affect cell homing to the injured myocardium (Abbott et al., 2004) [including recruitment of putative endogenous cardiomyocyte progenitors (Segers et al., 2007; Unzek et al., 2007)] and to exert cytoprotective (Zhang et al., 2007), pro-proliferative (Lataillade et al., 2000), and angiogenic (Segers et al., 2007; Zhang et al., 2007; Saxena et al., 2008; Kanki et al., 2011) effects (the latter were also observed in our study, Supplementary Fig S8), all of which could directly or indirectly contribute to the detection of increased numbers of endogenous cardioblasts in the infarcted area post-cell therapy. Finally, while we show a correlation between CDC-induced functional benefits and a SDF-1-dependent increase in the number of endogenous cardioblasts, our study does not unequivocally demonstrate that cardioblasts play a direct role in the benefits afforded by CDC therapy, since: (a) our fate mapping model does not allow for direct quantification of the number of cardiomyocytes that arise from GFP+ cardioblasts and (b) SDF1 has pleiotropic effects (Frangogiannis, 2011; Penn et al., 2012).

Conclusions

In summary, our study provides evidence for the activation of endogenous cardioblasts in the injured adult mammalian heart. We provide insight into the molecular identity, functional properties, and anatomic origin of said cardioblasts and identify treatment strategies and paracrine signaling pathways that could be used for therapeutic stimulation of innate progenitor cell-mediated cardiomyocyte replenishment.

Materials and Methods

Generation of bitransgenic MerCreMer/ZEG Mice and 4-OH-Tamoxifen pulse

All animal procedures were approved by the Cedars-Sinai Medical Center Animal Care and Use Committee (IACUC2557). Bitransgenic mice were generated by crossbreeding female transgenic B6129-Tg (Myh6-cre/Esr1)1Jmk/J (Myh6-MerCreMer) mice with male B6.Cg-Tg(ActB-Bgeo/GFP)21Lbe/J (ZEG) reporter mice (Jackson Laboratory). Myh6-MerCreMer mice carry a fusion transgene of Cre recombinase flanked by mutated estrogen receptor ligand-binding domains, driven by the cardiac αMHC promoter (encoded by Myh6). ZEG reporter mice carry a lacZ transgene flanked by LoxP sites, followed by stop codons and then the GFP gene; therefore, upon excision of the LoxP sites and stop codons mediated by Cre recombinase activity, the reporter switches to GFP (driven by the β-actin promoter), permanently and specifically marking αMHC+ cells and their progeny as GFP+. Animal genotype was verified by RT–PCR on tail genomic DNA (Zhang et al., 2010; Malliaras et al., 2013a). 4-OH-tamoxifen (Sigma), dissolved in peanut oil (Sigma) at a concentration of 5 mg/ml, was intraperitoneally injected into double heterozygous bitransgenic MerCreMer/ZEG mice daily at a dose of 0.5 mg/d to induce Cre recombination.
Mouse CDCs were expanded from explanted hearts obtained from 8-week-old male B6129SF1/J mice as described (Malliaras et al., 2013a). In brief, myocardial specimens were cut into fragments less than 1 mm³ and cultured as cardiac explants on fibronectin-coated dishes. After a variable period of growth, a layer of stromal-like cells emerged from the cardiac explant over which phase bright cells proliferated. The cells surrounding the explant were harvested using enzymatic digestion with 0.05% trypsin and were seeded at 50,000 cells/ml on poly-D-lysine-coated dishes, generating cardiospheres. Three days later, free-floating cardiospheres were harvested and plated on fibronectin-coated flasks to generate CDCs.


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Quantitative analysis was performed using Summit software (Beckman Coulter, Inc.).

ShRNA knockdown of SDF1 and VEGF in CDCs

Knockdown of SDF1 and VEGF in mouse CDCs was performed by commercially available lentiviral vectors expressing shRNA against mouse SDF1 (sequence CGGGGATCCAAGGTACCTGGAGAACTCTGATTTCCTCAGTACTTCTGGATCTTTTTTG, clone number TRCN 0000184347) and VEGF (sequence CGGGGATCCAAGGTACCTGGAGAACTCTGATTTCCTCAGTACTTCTGGATCTTTTTTG, clone number TRCN0000066822) (MISSION lentiviral transduction particles, Sigma-Aldrich), according to the manufacturer’s instructions. A non-targeting shRNA that activates RISC and the RNAi pathway, but does not target any mouse genes served as a negative control (sh-control).

Mouse CDCs (plated 24 h prior to transduction) were transduced at 50–80% confluency, after the addition of hexadimethrine bromide (8 µg/ml) (MOI: 20). The next day, the medium was replaced with fresh culture medium. The next day, the medium was replaced with puromycin-containing culture medium (5 µg/ml). Puromycin-resistant CDCs were subsequently expanded (in puromycin-containing medium) until the time of the experiments. To confirm successful knockdown of SDF1 and VEGF in CDCs, shRNA-transduced CDCs were cultured in FBS-free IMDM medium for 2 days. The supernatants (conditioned media) were collected and the concentrations of SDF1 and VEGF were measured with mouse-specific ELISA kits (R&D Systems Inc., according to the manufacturer’s instructions. To investigate whether transplantation of shRNA-transduced CDCs resulted in decreased myocardial levels of SDF1 and VEGF in vivo, mice were sacrificed 4 days after MI and injection of shRNA-transduced CDCs. Myocardial protein was isolated from the peri-infarct area and Western blots were performed as described (Malliaras et al., 2012). Quantitative analysis was performed by ImageJ software, and expression levels were normalized to β-actin.

Bone marrow transplantation

Bone marrow transplantation was performed as described (Wang et al., 2006). In brief, bone marrow was isolated from the femurs and tibias of donor 8-week-old MerCreMer/ZEG mice. Recipient B6129SF1/J mice (8-week-old) were lethally irradiated with 1,100 rads, given in two doses 4 h apart. Subsequently, 5 million bone marrow cells were injected into the tail vein of the recently lethally irradiated recipient mice. Two mice did not receive bone marrow transplantation and served as controls to ensure lethal irradiation of irradiated recipient mice. Two mice did not receive bone marrow transplantation and served as controls to ensure lethal irradiation (both mice died within 2 weeks post-irradiation).

RT–qPCR

RNA was extracted from FACS-sorted GFP+ and GFP– myocyte-depleted cardiac cells (20,000 cells per condition/heart, obtained from the same hearts) and from isolated cardiomyocytes using RNeasy Micro Kit (Qiagen). Two-step RT–qPCR (with 14 cycles of cDNA preamplification) was performed in 7900HT Fast real-time PCR System, using commercially available primers and probes (Taqman assays, Applied Biosystems) according to the manufacturer’s instructions. The expression of the following genes was investigated: NKK2-5, MEF2C, GATA4, TBX3, FOXC2, BMP2, ISL1, TBX2, TBX5, TBX20, MESP1, MESP2, GATA6, WNT2, WNT5a, WNT11, DKK1, HAND1, HAND2, FOXC1, SMARC3, MYH6, MYH7, MYL2, MYL3, TNNT2, MEF2C, PLN, DES, NPPA, CKM, TTN, VIM, WVF, ACTA2, PDGFRα, KIT, FUT4, and CD34. GAPDH was used as the internal control. Data were analyzed using the 7900HT Sequence Detection System Software (Applied Biosystems), and fold change was calculated with the comparative CT method.

Epifluorescence microscopy, immunocytochemistry, and histology

Enzymatically digested myocyte-depleted cardiac cell preparations resuspended in HBSS (supplemented with 2% FBS and 10 mM HEPES) were plated onto laminin-coated slides and incubated with Hoechst 33342 (H1399, Molecular probes). Thirty minutes later, the percentage of GFP+ cells was quantified using epifluorescence microscopy (Nikon, ECLIPSE TE2000). Enzymatically digested myocyte-depleted cardiac cell preparations (either freshly isolated or after FACS for the purification of GFP+ cells) were cytopsonted onto laminin-coated slides to undergo fluorescent immunocytochemistry. Fluorescent immunocytochemistry was performed with antibodies against against GFP (ab13970, Abcam), α-sarcromeric actin (A7811, Sigma), α-myosin heavy chain (ab15, Abcam), NKK2-5 (ab35842, Abcam), GATA4 (ab84593, Abcam), MEF2C (ab64644, Abcam), TRX5 (ab101227, Abcam), Isl1 (ab20670, Abcam), Ki67 (RM-9106, Thermo Scientific), H3P (ab5176, Abcam), lacZ (ab9361, Abcam), Sca-1 (ab25195, Abcam), Nestin (ab6142, Abcam), c-kit (ab5506, Abcam), SSEA1 (ab16285, Abcam), CD34 (ab8158, Abcam), PDGFRα (ab61219, Abcam), CD31 (ab28364, Abcam), CD45 (ab10558, Abcam), CXC4 (ab2074, Abcam), VEGFR1 (ab32152, Abcam), VEGFR2 (ab2349, Abcam), and VEGFR3 (ab27278, Abcam).

For histology, hearts were arrested with KCl solution, explanted, frozen in OCT compound, and sectioned in 6-µm sections on a cryostat. Cryosections were subsequently fixed with 4% paraformaldehyde. Quantitative morphometric analysis with Masson’s trichrome staining was performed on 6-µm sections from cryostat. Cryosections were subsequently fixed with 4% paraformaldehyde.
The paper explained

Problem
The identity, properties, and physiologic role of endogenous cardiomyocyte progenitors in the adult mammalian heart are unknown.

Results
We demonstrate that the adult mouse heart contains endogenous cardiomyocyte progenitors (cardioblasts); these cells express cardiac transcription factors and sarcomeric proteins, exhibit spontaneous contractions, and form mature cardiomyocytes in vitro. Endogenous cardioblasts are rarely evident in the normal adult mouse heart. However, myocardial infarction results in significant cardioblast activation in the site of injury. The activated progenitors do not arise from hematopoietic seeding, cardiomyocyte dedifferentiation, or mere expansion of a preformed progenitor pool. Cell therapy with cardioblasts derived cells amplifies innate cardioblast-mediated tissue regeneration, in part through the secretion of stromal cell-derived factor 1.

Impact
We provide insight into endogenous cardiomyocyte progenitors in the adult mammalian heart and identify treatment strategies and paracrine signaling pathways that could be used for therapeutic stimulation of innate progenitor cell-mediated cardiac regeneration.

Echocardiography
Mice underwent echocardiography at 24 h (baseline) and 5 weeks after surgery using Vevo 770 TM Imaging System (VISUALSONICS) as described (Malliaras et al., 2013a). LVEF, LV end-diastolic volume, and LV end-systolic volume were measured with VisualSonics V1.3.8 software from 2D long-axis views taken through the infarcted area at the level of the greatest LV diameter.

Statistics
Results both in text and in figures are presented as means ± SD. Statistical significance was determined by independent samples t-test, non-parametric Mann–Whitney U-test, and one-way ANOVA followed by LSD post hoc test (SPSS 16.0). Differences were considered significant when P < 0.05. The exact P-values and the specific statistical test performed for each experiment are provided in Supplementary Table S1. All experiments were open-label. All measurements were performed in a non-blinded fashion. The number of replicates used in each experiment is indicated in the figure legends.

Supplementary information for this article is available online:
http://embomolmed.embopress.org

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Author Contributions
KM established the hypotheses, designed the study, performed experiments, analyzed data, and wrote the paper. AI assisted with immunocytochemistry, and histology. ET assisted with 4OH-tamoxifen pulsing of mice. WL performed immunocytochemistry, immunohistochemistry, and animal genotyping. BS performed mouse surgeries. RCM performed RT-qPCR, immunocytochemistry, immunohistochemistry, histology, ELISA and analyzed data. JS performed flow cytometry, epifluorescence microscopy, immunocytochemistry, immunohistochemistry, histology, animal genotyping, RNA isolation and analyzed data. LW designed the bone marrow transplantation experiments and performed irradiation of mice and bone marrow transplantation. BGS designed the bone marrow transplantation experiments. EM established the hypotheses, designed the study, co-analyzed data, and wrote the paper.

Conflict of interest
EM is a founder and equity holder in Capricor, Inc. KM has received consulting fees from Capricor, Inc. Capricor, Inc., provided no funding for this study. The remaining authors declare that they have no conflict of interest.

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Heart regeneration by endogenous cardioblasts

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