Expertise of cardiac progenitor cells may, in fact, exist.6 hearts after myocardial injury has raised the possibility that the presence of immature cycling cardiomyocytes in human differentiated organ without regenerative capacity. However, work has held the notion that the adult heart is a terminally compensate through a hypertrophic response.2–5 Previous apoptosis, and an inability of remaining cardiomyocytes to function is a decrease in the number of viable cardiomyocytes. Recent observations have suggested that the adult heart may contain a progenitor cell population. Side population (SP) cells, characterized by a distinct Hoechst dye efflux pattern, have been shown to exist in multiple tissues and are capable of tissue-specific differentiation. In this report, we confirm the existence of a cardiac SP cell population, immunophenotypically distinct from bone marrow SP cells. Moreover, we demonstrate that among cardiac SP cells, the greatest potential for cardiomyogenic differentiation is restricted to cells negative for CD31 expression and positive for stem cell antigen 1 (Sca1) expression (CD31−/Sca1+). Furthermore, we determine that CD31−/Sca1+ cardiac SP cells are capable of both biochemical and functional cardiomyogenic differentiation into mature cardiomyocytes, with expression of cardiomyocyte-specific transcription factors and contractile proteins, as well as stimulated cellular contraction and intracellular calcium transients indistinguishable from adult cardiomyocytes. We also determine the necessity of cell-extrinsic signaling through coupling, although not fusion, with adult cardiomyocytes in regulating cardiomyogenic differentiation of cardiac SP cells. We, therefore, conclude that CD31−/Sca1+ cardiac SP cells represent a distinct cardiac progenitor cell population, capable of cardiomyogenic differentiation into mature cardiomyocytes through a process mediated by cellular coupling with adult cardiomyocytes. (Circ Res. 2005;97:52-61.)

Key Words: cardiac side population cells • bone marrow side population cells • cardiomyogenic differentiation • cardiomyogenesis • CD31 • stem cell antigen 1

Heart failure remains a leading cause of morbidity and mortality in developed countries.1 The central cellular mechanism underlying the development of myocardial dysfunction is a decrease in the number of viable cardiomyocytes, secondary to either acute ischemic injury or chronic apoptosis, and an inability of remaining cardiomyocytes to compensate through a hypertrophic response.2–5 Previous work has held the notion that the adult heart is a terminally differentiated organ without regenerative capacity. However, the presence of immature cycling cardiomyocytes in human hearts after myocardial injury has raised the possibility that cardiac progenitor cells may, in fact, exist.6

More recently, cells expressing the stem cell antigen, Sca1, the receptor for stem cell factor, c-kit, the homeodomain transcription factor, islet-1, or undifferentiated cells that grow as self-adherent clusters (cardiospheres), have been identified in the adult heart and have been suggested to be capable of differentiation into cardiomyocytes.7–11 In addition to these cell types, an alternative and distinct tissue-specific progenitor cell population has also been identified.12 These so-called side population (SP) cells are characterized by their intrinsic capacity to efflux Hoechst dye through an ATP-binding cassette transporter.13 First isolated from bone marrow,14 SP cells proved to be enriched for long-term repopulating hematopoietic stem cells.15 SP cells have also been isolated from extrahematopoietic tissues including lung, mammary gland, testes, kidney, skin, and skeletal muscle.12 Whereas the function and differentiation capacity of SP cells isolated from extrahematopoietic tissues remain controversial, in skeletal muscle, SP cells have been suggested to give rise to myocytes following intramuscular implantation and to contribute to muscle regeneration.16 More recently, cells with the SP phenotype have been isolated from adult cardiac tissue.17,18 The characteristics and cardiomyogenic potential of these cardiac SP (CSP) cells, however, have not yet been extensively evaluated.9 In this article, we confirm the presence of a heterogenous CSP cell population within the adult heart. Bone marrow SP (BMSP) cells typically express CD31,19 Thus, in comparing CSP cells with BMSP cells, CD31 expression was assessed. We show that CD31−/Sca1+ CSP cells represent a specific cardiac progenitor cell subpopulation, immunophenotypically distinct from BMSP cells, and capable of self-renewal and biochemical and functional cardiomyogenic differentiation into mature, contracting car-
diomyocytes. Moreover, we determine the necessity of cell-extrinsic signaling through coupling, not fusion, with adult cardiomyocytes in regulating cardiomyogenic differentiation of CSP cells.

Materials and Methods

Animals
Cardiac cell suspensions were prepared from mice (8 to 12 weeks of age) of the following background: C57BL/6J, C57BL/6-Tg (ACTB-Bgeo/GFP) 21Lbe/J (The Jackson Laboratory). Animal studies were conducted according to NIH-approved guidelines, as well as approved guidelines of the institutional animal care and use committee of Boston University School of Medicine.

Isolation and Preparation of Cardiac and Bone Marrow SP Cells
Cardiomyocyte-depleted cardiac cell suspensions were prepared as previously described with modification. Briefly, minced cardiac tissue was digested with 0.1% collagenase B (Roche Molecular Biochemicals), 2.4 U/mL dispase II (Roche Molecular Biochemicals), and 2.5 mmol/L CaCl_2 at 37°C for 30 minutes, filtered, and with washed with Hanks’ balanced salt solution (HBSS) buffer with 2% fetal calf serum and 10 mmol/L HEPES. Bone marrow cell suspensions were obtained as previously described. Cell suspensions were incubated with Hoechst 33342 (5 μg/mL) (Sigma) at 37°C for 90 minutes in DMEM (Cellgro) (2%) fetal calf serum, 10 mmol/L HEPES at a concentration of 10^6 nucleated cells/mL and washed in cold HBSS before cell surface antigen staining. Cell surface antigen staining was performed at 4°C for 30 minutes using fluorochrome-conjugated monoclonal rat anti-mouse antibodies reactive to Sca-1, CD34, CD45, and CD45 (all from Pharmingen). Respective isotype controls (Pharmingen) were used as negative controls. Propidium iodide (PI) (2 μg/mL) was added before fluorescence-activated cell sorting (FACS) to exclude dead cells.

FACS Analysis
FACS was performed using MoFlo (Cytomation, Inc) equipped with triple lasers. The Hoechst dye was excited using an argon multiline UV (333 to 363 nm) laser, fluorescence emission was collected with a 405/30 band-pass filter (Hoechst blue), and a 660 long-pass filter (Hoechst red). Phycerothrin (PE), green fluorescence protein (GFP), and PI fluorescence was detected using a 488-nm argon laser. SP cells were identified as described previously and sorted for negative control. Immunostaining was visualized using laser scanning confocal microscopy.

Reverse Transcription–Polymerase Chain Reaction
RNA was extracted from purified CSP cells using a commercially available RNasey Mini Kit (Qiagen), and cDNA was generated using a reverse transcription kit (Promega). Polymerase chain reaction (PCR) was performed on cDNA using the following primers: Nkx2.5 (5’-CCGAGAAATTTGCAATGCTCCATTGTGGTCTGTCAGGAGATGCGGGTACATAGGAGG-3’ and 5’-ATGAGCCAGGCCTACTCGGTA-3’); α-actinin (5’-TGGCTAGCTATGCTTCAGTCA-3’ and 5’-CGAATTTT-3’); α-actin (5’-TGGCTAGCTATGCTTCAGTCA-3’ and 5’-CGAATTTT-3’); α-myosin heavy chain (α-MHC) (5’-GGAGAGTTGACGCGCTTCAAGG-3’ and 5’-CTGCGTGAGGTATTCTCTCCTTG-3’); α-smooth muscle actin (α-SMA) (5’-AGCTTTTGGCAGGAAATGATTGGG-3’ and 5’-AAGATCATCTTGCCCTCAGAAGG-3’); Tie2 (5’-GGCCTGGACACGAGG-3’ and 5’-CCACATTCAACCTCTTCTTATTAC-3’); and Desmin (5’-AAGAGCCAGGCGCTCTCTGGCTC-3’ and 5’-GGCTCACTTCCTCAGATG-3’).

Functional Differentiation of Cardiac SP Cells

Colony Forming Unit Assay
Colony forming unit assays were performed by plating cardiac cells at an initial density of 7000 cells/mL in Methocult GF M3534 media according to the instructions of the manufacturer (Stem Cell Technologies), similar to as previously described. Cell colonies consisting of >25 cells were scored after 10 days in culture.

Immunocytochemistry
Cultured cells were washed with PBS and fixed in 3.7% formaldehyde for 20 minutes at room temperature. Cells were then permeabilized with methanol at −20°C for 20 minutes, washed with PBS, and blocked in 1% BSA for 1 hour. Immunostaining was performed using the following antibodies: connexin43 and α-actinin (Sigma); GATA4, MEF2C, troponin I, and MyoD (all from Santa Cruz Biotechnology, Inc); and anti-Cre-recombinase (Novagen). Alexa Fluor 555 (Molecular Probes) was used for secondary detection, with the exception of α-actinin, which was directly labeled with TRITC according to the instructions of the manufacturer (Pierce). Cells were all counterstained with nuclear 4’,6-diamidino-2-phenylindole (DAPI) stain. Secondary antibody alone was used as a negative control. Immunostaining was visualized using laser scanning confocal microscopy.

Cardiac SP Cell Coculture and Cellular Fusion
For coculture experiments, adult rat ventricular cardiomyocytes were isolated and cultured from male Wister rats, as described. CSP cells were cultured alone or in coculture with adult cardiomyocytes at a ratio of 1:10 for 14 to 21 days, as described in individual experiments. To assess for cellular fusion between cardiac SP cells and cocultured cardiomyocytes, cardiomyocytes were infected with adenovirus encoding Cre-recombinase (Ad5-CMV-Cre) (Gene Transfer Vector Core, University of Iowa) at 50 to 100 viral particles/cell. After washing with media, CSP cells isolated from STOCK Tg(ACTA-geo/GFP)21Lbe/J mice were cocultured with Cre-recombinase transfected cardiomyocytes. Cultures were screened for GFP-expressing cardiomyocytes after 14 days. In additional experiments, Ad5-CMV-RFP (Gene Transfer Vector Core, University of Iowa) infected cardiomyocytes expressing red fluorescence protein (RFP) were cocultured with GFP-expressing CSP cells. Double-positive fused cells were evaluated by two independent investigators after 14 days.

Cell Contractility and Intracellular Calcium Transients
Cell contractility and intracellular calcium transients were determined in CSP and adult cardiomyocytes cocultured on laminin-coated cell chambers (Cell Microcontrols). Cells were perfused with 1.2 mmol/L Ca^{2+} Tyrode solution and electrically paced at 1 Hz using field stimulation. GFP fluorescence was recorded by an intensified charge-coupled device camera. Cell contractility measurements were performed with a high-speed (240 frames per second) charge-coupled device camera in combination with real time edge-detection software (SoftEdge Acquisition System and IonWizard, IonOptix Corp). For calcium transient measurements, cells were incubated with membrane-permeable furap-2 (1 μmol/L) (Molecular Probes) and probenecid (500 μmol/L) (Sigma). Fura-2 was excited at 360/380 nm and GFP at 480 nm, and both excitation and emission band pass filters were used to prevent any overlapping fluorescence signals.

Statistical Analysis
Statistical differences between groups were evaluated using Student unpaired t test or analysis of variance, as appropriate. All data are presented as mean ± SEM. Probability values <0.05 were considered statistically significant.

Results

Immunophenotype, Self-Renewal, and Early Cardiac Gene Expression in Cardiac SP Cells
Adult hearts were subjected to collagenase/dispase digestion to obtain a cardiomyocyte-depleted nucleated cell suspension.
FACS analysis following Hoechst 33342 staining of cell suspensions revealed the presence of a Hoechst-low cell population with a fluorescent profile distinct from the main cell population (Figure 1A). Hoechst efflux in this side population of cells was completely inhibited by treatment with the ATP-binding cassette transporter inhibitor verapamil (Figure 1B), similar to as previously observed in BMSP cells,14 thereby confirming the presence of a CSP cell population in the adult mouse heart.

To further investigate this CSP cell population in relation to the well-characterized BMSP cell population,14,15 isolated CSP and BMSP were examined for expression of hematopoietic markers (CD45 and CD34), stem cell markers (Sca1 and c-kit), and cellular adhesion markers (CD31 [PECAM-1] and CD44). BMSP cells stained positive for the hematopoietic markers CD45 and CD34, as well as for the hematopoietic stem cell markers c-kit and Sca1 (Table). Additionally, they expressed the cell surface markers CD31 and CD44. In contrast, CSP cells were largely negative for CD45, CD34, and CD44 but exhibited expression of CD31, suggesting an immunophenotypically distinct population relative to BMSP cells. Notably, CSP widely expressed the stem cell antigen Sca1, but were negative for c-kit. The low expression of c-kit, however, was attributable to enzymatic cleavage during the digestion process, as treatment of c-kit

<table>
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<tr>
<th>Surface Marker</th>
<th>Cardiac SP (%)</th>
<th>Bone Marrow SP (%)</th>
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<tr>
<td>Sca1</td>
<td>84±2</td>
<td>63±4</td>
</tr>
<tr>
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<td>≤1</td>
<td>24±3</td>
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<tr>
<td>c-kit</td>
<td>≤1</td>
<td>84±2</td>
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Figure 1. Characterization of CSP cells. A, FACS analysis of a cardiomyocyte-depleted nucleated cell suspension following isolation from adult mouse hearts and staining with Hoechst 33342 dye, demonstrating distinct Hoechst extruding CSP and nonextruding MP cells. B, FACS analysis following treatment with the ATP-binding cassette transporter inhibitor verapamil, demonstrating inhibition of Hoechst efflux from CSP cells. C, Representative bright-field images of expanding cell colonies of Sca1/CSP cells. D, Sca1/MP cells following 10 days culture in methylcellulose media. E, Greater colony forming activity in Sca1/CSP cells relative to Sca1/MP cells (P<0.01), suggesting a higher potential for cell-renewal in CSP cells. F, Reverse transcription–PCR analysis of whole heart (heart) and CSP cells for expression of cardiac-specific transcription factors (Nkx2.5, GATA4), early myofilament proteins (smooth muscle actin [SMA], desmin), late myofilament proteins (α-actinin, α-MHC), and the endothelial cell marker Tie2.
digestion regimen resulted in a significant reduction in c-kit positive cells (data not shown).

Previous work has identified Sca1 as a putative marker of cardiac progenitor cells. Therefore, we examined the in vitro colony forming potential of Sca1+/CSP cells and Sca1+ cardiac non-SP, main population (MP) cells isolated from adult myocardium. Sca1+/CSP cells or Sca1+/MP cells were sorted and plated on methylcellulose media and analyzed for colony formation after 10 days in culture. As shown in Figure 1C through 1E, Sca1+/CSP cells demonstrated greater colony formation relative to Sca1+/MP cells, suggesting a higher potential for self-renewal.

To determine the expression of genes specific for cardiac development, purified Sca1+/CSP cells were examined using reverse transcription–PCR analysis (Figure 1F). Freshly isolated Sca1+/CSP cells or Sca1+/MP cells were sorted and plated on methylcellulose media and analyzed for colony formation after 10 days in culture. As shown in Figure 1C through 1E, Sca1+/CSP cells demonstrated greater colony formation relative to Sca1+/MP cells, suggesting a higher potential for self-renewal.

To identify specific subpopulations present within the CSP collection, Sca1+/CSP cells were separated according to expression of the endothelial surface marker CD31. Notably, CD31+/Sca1+ cells composed the majority of total CSP cells (73.8±2.4%), with CD31-/Sca1+ cells representing only 10.4±2.0% of total CSP cells (~500 to 1000 cells per adult heart, 8 to 12 weeks old) (Figure 2A). Analysis of CD31+ and CD31− cardiac cells revealed that these 2 subpopulations accounted for distinct portions of the SP band. CD31+/Sca1+ cardiac cells composed the sharp upper portion of the SP band, whereas CD31−/Sca1+ cells constituted the broad lower part of the band (Figure 2B). Additionally, although both populations demonstrated similar cell granularity (Figure 2C), CD31+/Sca1+ cells were larger relative to CD31-/Sca1+ by forward scatter analysis (Figure 2D).

CD31−/Sca1+/CSP Cells Exhibit Intrinsic Cardiomyogenic Potential

To identify specific subpopulations present within the CSP collection, Sca1+/CSP cells were separated according to expression of the endothelial surface marker CD31. Notably, CD31+/Sca1+ cells composed the majority of total CSP cells (73.8±2.4%), with CD31-/Sca1+ cells representing only 10.4±2.0% of total CSP cells (~500 to 1000 cells per adult heart, 8 to 12 weeks old) (Figure 2A). Analysis of CD31+ and CD31− cardiac cells revealed that these 2 subpopulations accounted for distinct portions of the SP band. CD31+/Sca1+ cardiac cells composed the sharp upper portion of the SP band, whereas CD31−/Sca1+ cells constituted the broad lower part of the band (Figure 2B). Additionally, although both populations demonstrated similar cell granularity (Figure 2C), CD31+/Sca1+ cells were larger relative to CD31-/Sca1+ by forward scatter analysis (Figure 2D).
To determine the cardiomyogenic potential of CSP cells, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP and CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP were cultured independently on laminin-coated culture plates. In culture, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells adhered to the culture plate surface (Figure 3A), spread out and progressively changed their morphology to a spindle shape (Figure 3B). In contrast, the majority of CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells remained nonadherent and free floating (Figure 3C through 3D). Whereas freshly isolated CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP exhibited limited GATA4 protein expression by immunohistochemistry, following monoculture, >30% of CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells expressed both GATA4 (Figure 4A) and the muscle-specific transcription factor MEF2C (Figure 4B). Consistent with transcription factor expression, cultured CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells also exhibited expression of cardiac-specific contractile proteins, including α-actinin (Figure 4C) and troponin I (Figure 4D). Importantly, however, α-actinin expression was disorganized (Figure 4C), without the characteristic sarcomeric organization seen in mature cardiomyocytes. Correspondingly, in monoculture, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells did not exhibit spontaneous contraction or evidence of functional maturity. In contrast to CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP and BMSP did not express cardiac transcription factors or contractile proteins when cultured under identical conditions (data not shown).

**Cellular Coupling With Adult Cardiomyocytes Is Required for the Maturation and Functional Differentiation of Cardiac SP Cells**

Previous work has suggested that in addition to cell-intrinsic signaling, important cell-extrinsic mechanisms may regulate stem cell activity and differentiation. In particular, local cellular niches or tissue microenvironments may serve as key control mechanisms in determining the cellular fate of a stem cell population. Therefore, to determine the role of extrinsic cellular signals in mediating CSP differentiation, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP and CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells were isolated from mice ubiquitously expressing GFP and cocultured with adult cardiomyocytes. Similar to as seen in monoculture, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells expressed the cardiac transcription factors GATA4 (64%) and MEF2C (35%) when cocultured with adult cardiomyocytes, with expression apparent as early as 3 days. Cocultured CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells also exhibited electromechanical coupling with adult cardiomyocytes, with clear expression of the gap junction protein connexin43 at the intercalated disk between adjacent cells (Figure 5A). Following coupling with adult cardiomyocytes, cocultured CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells demonstrated further maturity, as evidenced by the development of sarcomeric organization (10%), with ordered myofibrils and a clear striation pattern starting at 7 days (Figure 5B). Differentiated cells stained negative for the skeletal muscle-specific factor MyoD, excluding skeletal muscle differentiation (data not shown).

While these results suggest that in coculture with adult cardiomyocytes, CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells are capable of differentiation into cells with biochemical and immunohistochemical features of mature cardiomyocytes, the capacity of these cells for functional differentiation still remains unknown. Therefore, we determined the ability of differentiated CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells to undergo coordinated cellular contraction. In coculture with adult cardiomyocytes, consistent with the development of sarcomeric organization, 10% of CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP demonstrated spontaneous contractions at low frequency (<1 Hz). Furthermore, electric stimulation of CD31<sup>+</sup>/Sca1<sup>+</sup>/CSP cells resulted in rhythmic contractions synchronized with those in adjacent cocultured adult cardiomyocytes (Figure 6A through 6C). To further determine whether differentiated CSP cells underwent active cellular contraction, rather than passive mechanical coupling or tethering to cocultured adult cardiomyocytes, intracellular calcium transients were simultaneously determined, using the
intracellular calcium indicator fura-2, in CD31+/Sca1+/CSP cells and cocultured adult cardiomyocytes. In conjunction with synchronized contraction and relaxation, CD31+/Sca1+/CSP cells demonstrated intracellular calcium transients indistinguishable from those seen in cocultured adult cardiomyocytes (Figure 6D through 6F), thereby demonstrating active, rather than passive, contraction in differentiated CSP cells. These results suggest that CD31+/Sca1+/CSP cells are capable of functional differentiation into cardiomyocytes, with maturation of the contractile apparatus and cellular calcium handling proteins, through a process mediated by coupling with adult cardiomyocytes.

In contrast, Sca1+/CSP positive for CD31 (CD31+/Sca1+/CSP) did not express the cardiac transcription factors GATA4 or MEF2 (<1%) and failed to undergo cardiomyogenic differentiation in coculture with adult cardiomyocytes (data not shown). In addition, a similar culture system was used to assess the potential for cardiac differentiation in BMSP cells. In both monoculture and in coculture with adult cardiomyocytes, BMSP cells remained quiescent, without expression of cardiac transcription factors, contractile proteins or evidence for electromechanical coupling with cocultured cardiomyocytes (data not shown).

**Functional Cardiomyogenic Differentiation of Cardiac SP Cells Occurs in the Absence of Cellular Fusion**

To determine the role of cellular fusion in mediating differentiation of CSP cells, cocultured adult cardiomyocytes and CD31+/Sca1+/CSP were examined for nuclear morphology. Adult cardiomyocytes were predominantly binucleated (85%), whereas CD31+/Sca1+/CSP cells were largely mononucleated (85%). After cardiomyogenic differentiation in coculture, >75% of CD31+/Sca1+/CSP cells remained mononucleated, suggesting that cellular fusion with cocultured adult cardiomyocytes was not required for the differentiation process. The absence of significant cellular fusion was further confirmed through coculturing of CD31+/Sca1+/CSP expressing GFP with adult cardiomyocytes, infected with Ad5-CMV-RFP, and expressing RFP. In this coculture system no double positive cells could be found and spontaneous contracting CSP cells demonstrated only GFP expression (Figure 7A and 7B).

To further determine whether cellular fusion is required for cardiomyogenic differentiation of CSP, a Cre-lox-mediated reporter system was used. Under this system, adult cardiomyocytes expressing Cre-recombinase were cocultured with CD31+/Sca1+/CSP reporter cells isolated from STOCK Tg(ACTB-Bgeo/GFP)21Lbe/J transgenic mice.21 In the event of cellular fusion between cardiomyocytes and CSP reporter cells, cardiomyocyte Cre-recombinase would result in excision of a loxP-flanked Bgeo reporter, enabling the expression of GFP within fused cells.21 Adult cardiomyocytes were, therefore, infected with Ad5-CMV-Cre, resulting in robust Cre-recombinase expression (>90%) throughout the entire culture period (Figure 7C through 7D). To confirm the integrity of this reporter system, CD31+/Sca1+/CSP reporter cells, isolated from STOCK Tg(ACTB-Bgeo/GFP)21Lbe/J mice, were directly infected with Ad5-CMV-Cre, resulting in robust GFP expression (Figure 7E through 7F). With coculture of CD31+/Sca1+/CSP reporter cells and Cre-recombinase expressing adult cardiomyocytes, however, <2% of differentiated CSP (<0.2% of total CSP) were positive for GFP (Figure 7G and 7H), thereby suggesting that, although fusion may occur at very low levels, it was not required for cardiomyogenic differentiation of CD31+/Sca1+/CSP.

**Discussion**

In this article, we identify the existence of a SP cell population in the adult heart and define these cells as immunophenotypically distinct from BMSP cells. Moreover, we demonstrate that among Sca1+/CSP cells, the greatest potential for cardiomyogenic differentiation is restricted to cells negative for CD31 expression and, thereby, establish CD31+/Sca1+/CSP cells as a specific cardiac progenitor cell population. Most importantly, we provide the first evidence for the functional differentiation of CD31+/Sca1+/CSP cells into cardiomyocytes and determine the necessity of cell-extrinsic signaling through coupling, although not fusion,
with adult cardiomyocytes in regulating the cardiomyogenic differentiation of CSP cells.

Immunophenotype of Cardiac SP Cells Versus Bone Marrow SP Cells

The characterization of SP cells performed in this study identified immunophenotypically distinct SP cell populations between hematopoietic and cardiac tissues. In contrast to BMSP, CSP cells exhibited limited expression of the hematopoietic markers CD45 and CD34. Whereas previous observations of SP cell populations within extrahematopoietic tissues have suggested variable degrees of CD45 expression, our results suggest that CSP represent a unique population of cells relative to BMSP. The distinction between these cell populations is further suggested given the marked phenotypic differences between BMSP and CSP. In contrast to CSP, SP cells isolated from bone marrow showed no evidence for cardiac-specific gene expression or functional cardiomyogenic differentiation, either in monoculture or in coculture with adult cardiomyocytes. Importantly, however, these findings do not directly contradict previous observations of Goodell and colleagues, in which labeled BMSP cells were found to home to areas of injured myocardium in vivo and express cardiac-specific contractile proteins. Rather, these data suggest that BMSP cells exhibit limited intrinsic potential for cardiomyogenic differentiation under in vitro culture conditions. In vivo, circulating BMSP cells may exit the vasculature and undergo differentiation into CSP cells, with tissue-specific alterations in gene expression and cell surface protein expression before obtaining the capacity for cardiomyogenic differentiation. Such a process may occur in skeletal muscle, in which bone marrow derived stem cells have been suggested to undergo differentiation into skeletal muscle stem cells (satellite cells) before forming skeletal myocytes.

Cardiomyogenic Differentiation Is Limited to CD31−/Sca1+/CSP Cells

Detection of cells with the potential for stem cell activity has largely been achieved through identification of cell surface markers. Previous work has suggested that the stem cell antigen Sca1 represents a marker particular for cardiac progenitor cells. Importantly, however, Sca1 expression is nonspecific and is observed in a heterogeneous cell population that comprises ~15% of noncardiomyocyte nucleated cells within the heart. Although only a small percentage of total Sca1+ cardiac cells exhibit an SP phenotype, isolated SP cells are highly enriched for Sca1 expression. Our results suggest that among Sca1+ cardiac cells, the capacity for cellular renewal is attributable to Sca1+ cells with an SP phenotype, rather than Sca1+ cardiac cells as a whole. This finding is in accordance with data from other tissues, where SP cells have been shown to be enriched in colony-forming capacity compared with non-SP cells.

Additionally, limited previous work has examined the genotypic and phenotypic heterogeneity of specific SP cell subpopulations. As such, we further isolated distinct subpopulations within Sca1+/CSP cells based on the expression of the endothelial cell marker, CD31. Most Sca1+/CSP cells were found to express CD31, with CD31−/Sca1+/CSP cells...
Figure 6. Functional maturity of cardiomyogenic differentiated CSP cells. GFP-expressing CD31⁺/Sca1⁺/CSP cells cocultured with adult cardiomyocytes (CM), with fluorescent image (A) demonstrating GFP fluorescence (white) and corresponding bright field image (B). Cell shortening was determined in CSP and neighboring cardiomyocyte (CM) electrically stimulated at 1 Hz, with boxes denoting areas of measurement. Corresponding cell contraction curves (C) demonstrating stimulated contraction in CD31⁺/Sca1⁺/CSP cells (green), similar to cocultured adult cardiomyocytes (red). Similarly, fluorescent image (D) demonstrating GFP fluorescence (white) in a GFP-expressing CD31⁺/Sca1⁺/CSP cell (CSP) and cocultured adult CM. Cells were loaded with the calcium sensitive fluorophore fura-2 and fluorescence recorded in CSP and CM electrically stimulated at 1 Hz, with boxes denoting areas of measurement (E). Corresponding fura-2 fluorescence tracings (F) demonstrate comparable intracellular calcium transients in CD31⁺/Sca1⁺/CSP (green) and cocultured adult cardiomyocytes (red). Cell contractility and intracellular calcium transient measurements were assessed in 6 individual experiments, with 2 to 3 cells assessed per group per experiment.

representing only a minority subpopulation. Importantly, gene expression and cell size varied significantly between CD31⁺/Sca1⁺ and CD31⁺/Sca1⁻ CSP cells, suggesting that even among the relatively small percentage of cardiac cells that exhibit an SP phenotype, a significant heterogeneity exists. Notably, in our in vitro system, cardiomyogenic differentiation was exclusively observed in cardiac SP cells lacking CD31, suggesting additional functional differences among CSP subpopulations. We, therefore, established this relatively specific subpopulation of CD31⁺/Sca1⁺/CSP cells, as a cardiomyogenic progenitor cell population. Notably, Sca1⁻/CSP cells do not express the LIM-homeodomain transcription factor islet-1 and thus are distinct from the recently reported islet-1⁻ cardioblasts.

Although SP cells have previously been identified within adult myocardium by Martin et al and Hierlihy et al, this study represents the first to intensively investigate and demonstrate the capacity of CSP cells for functional differentiation. CD31⁺/Sca1⁺/CSP demonstrated not only biochemical differentiation, as evidenced by the expression of cardiac transcription factors and contractile proteins as well as sarcomeric organization, but also functional cardiomyogenic differentiation, as determined by cellular contraction and intracellular calcium transients indistinguishable from cocultured adult cardiomyocytes. Such functional differentiation is essential, as it suggests the functioning of sarcomeric and calcium handling systems and, as such, the maturity of differentiated CD31⁺/Sca1⁺/CSP cells.

Necessity of Cell-Extrinsic Signaling in Regulating Cardiomyogenic Differentiation

Although CD31⁺/Sca1⁺/CSP cells exhibited the potential for cardiomyogenic differentiation, this capacity was not independent of external regulators. Rather, complete differentiation was found to require both cell-intrinsic and cell-extrinsic factors. Freshly-isolated CD31⁺/Sca1⁺/CSP cells exhibited expression of cardiac-specific transcription factors Nkx2.5 and GATA-4 at baseline, and with long-term monoculture, these cells demonstrated further expression of early and late cardiac-specific contractile proteins. Despite the expression of necessary transcription factors and proteins, CD31⁺/Sca1⁻/CSP cells were incapable of the sarcomeric organization required for functional maturity. Overcoming this restriction required cell-extrinsic stimulation, in the form of coupling with adult cardiomyocytes. Such coupling allowed sarcomeric organization and functional maturation of CD31⁺/Sca1⁺/CSP cells into cardiomyocytes indistinguishable from adult cardiomyocytes. Notably, signaling by cocultured adult cardiomyocytes was alone not sufficient to induce differentiation, as evidenced by a lack of differentiation in CD31⁺/Sca1⁻/CSP and BMSP cells.

Although the decisive signaling mechanisms regulating cardiomyogenic differentiation of CD31⁺/Sca1⁺/CSP require further elucidation, our data emphasize the importance of milieu-dependent regulators. The finding that CSP cell differentiation is regulated by milieu-dependent exogenous signaling mechanisms is consistent with prior in vivo observations. Our data further suggest that this milieu-dependent
using a Cre-lox–mediated excision reporter system. This finding is in agreement with recent studies in islet-1 and c-kit expressing cardiac progenitor cells, which have demonstrated cardiomyogenic differentiation in the absence of significant cellular fusion.8,10

Cardiac progenitor cells hold enormous potential for the treatment of cardiovascular disease. Determination of the functional differentiation of CD31^+/Sca1^+/CSP progenitor cells into mature cardiomyocytes raises the possibility of harnessing these cells for therapeutic cardiac regeneration.

Acknowledgments

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CD31− but Not CD31+ Cardiac Side Population Cells Exhibit Functional Cardiomyogenic Differentiation
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SUPPLEMENTAL FIGURE LEGEND

Supplementary Figure 1: Absence of islet-1 gene expression in CD31+/Sca1+ cardiac SP cells. Reverse transcriptase PCR (rt-PCR) analysis of CD31+/Sca1+ cardiac SP cells (CSP) and brain for islet-1 with expected islet-1 expression in brain (positive control), but no expression in CSP cells. 18 S expression was used as a control for cDNA integrity. The following primer sequences were used: Islet-1 (5’-CAGAGTCATCGAGTGTGG-3’ and 5’-TCTACTGCTGCTGCTTCAAC-3’); 18S (5’-CGGCGACGACCCATTGAAC-3’ and 5’-GAATCGAACCCTGATTCCC-3’).
Supplemental Figure 1

Islet-1

18 S