Cardiac progenitor cells are important for maintenance of myocardial structure and function, but genetic deletion of Sca-1 causes primary cardiac defects in myocardial contractility and repair. To understand consequences of stem cell antigen-1 (Sca-1) deletion on functional properties of c-kit+ cardiac progenitor cells and myocardial performance using a Sca-1 knock-out/green fluorescent protein knock-in reporter mouse (ScaKI).

Methods and Results: Genetic deletion of Sca-1 results in early-onset cardiac contractile deficiency as determined by echocardiography and hemodynamics as well as age-associated hypertrophy. Resident cardiac progenitor cells in ScaKI mice do not respond to pathological damage in vivo, consistent with observations of impaired growth and survival of ScaKI cardiac progenitor cells in vitro. The molecular basis of the defect in ScaKI cardiac progenitor cells is associated with increased canonical Wnt signaling pathway activation consistent with molecular characteristics of lineage commitment.

Conclusions: Genetic deletion of Sca-1 causes primary cardiac defects in myocardial contractility and repair consistent with impairment of resident cardiac progenitor cell proliferative capacity associated with altered canonical Wnt signaling. (Circ Res. 2012;111:750-760.)

Key Words: Sca-1  c-kit  heart  cardiac progenitor cell  infarction  myocardium  Sca-1 knock-out  β-catenin  cardiac development

Identification of multipotent stem cells in adult tissues such as the myocardium rests with stem cell markers originally developed to define cells of hematopoietic lineage. The c-kit cell surface receptor is commonly used to mark stem/progenitor cells. c-kit has been identified on small nonmyocytes within the myocardium referred to as cardiac progenitor cells (CPCs) that mediate myocardial repair after ischemic injury.1-3 CPCs also express the cell surface marker stem cell antigen-1 (Sca-1) both in vitro1 and in vivo.4 Sca-1 is an 18-kDa glycosyl phosphatidylinositol–anchored cell surface protein member of the Ly-6 antigen family with cell signaling and adhesion properties5 present on the surface of tissue-specific stem cells in the hematopoietic,6 mammary gland,7 and vascular niche8 in the mouse. Although typically associated with tissue stem or progenitor cells, Sca-1 is also expressed on a variety of fully differentiated cell types.9

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Roles of Sca-1 protein in stem cell development and function primarily defined in the hematopoietic lineage initially described over 15 years ago in the mouse10 led to use for marking and enrichment of stem cells. Functions of Sca-1 include promotion of adhesion and proliferation that are critical for optimal hematopoietic activity.6 Furthermore, Sca-1 is a part of a family of proteins that contain a urokinase plasminogen activator reporter (UPAR) domain involved in cellular adhesion, migration by modulating integrin function, and degradation of the extracellular matrix.6 In addition, Sca-1 has been implicated in signaling for cellular differentiation.11 Isolated mesenchymal stem cells positive for both Sca-1 and c-kit differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells on injection into the heart.12 The number of Sca-1-positive cells increases in the left ventricle of the heart after myocardial infarction (MI)13 and progenitor cells from bone marrow also migrate to injured myocardium after infarction and contribute to repair14 suggesting that Sca-1+ cells contribute to regeneration and repair after an MI. Collectively, these characteristics make Sca-1 a critical protein for evaluating stem cells in the heart.
Consequences of Sca-1 deletion on the functional properties of myocardial c-kit+ CPCs and myocardial performance were assessed in the present study using Sca-1 reporter mice with a single allele GFP knock-in, Sca-1<sup>GFP/+</sup>, which labels Sca-1+ cells in peripheral blood with GFP. Findings were generated using this Sca-1 knock-in (ScaKI) mouse model with both alleles of the Sca-1 protein coding sequence replaced with GFP downstream of the endogenous Sca-1 promoter, generating a mouse with a genetic deletion of ScaKI possessing the genotype of Sca-1<sup>GFP/GFP</sup>. Prior studies reveal the ScaKI mouse exhibits its serial bone marrow stem cell repopulating defects but has not been previously studied for a cardiac phenotype, although an unrelated murine model of Sca-1 deletion has been postulated to possess an impaired response to pathological challenge due to defective Nkx2.5-positive non-myocytes. In comparison, our findings using the ScaKI line reveal deleterious effects of Sca-1 deletion on c-kit+ CPCs and associated underlying signaling mechanisms.

**Methods**

**Transgenic Animal Models**

Sca-1 knock-in transgenic mice were a generous gift from Dr Timothy Graubert. A construct encoding GFP was inserted into the second exon of the Sca-1 gene at the start codon, functionally eliminating Sca-1 protein expression and creating a mouse in which GFP was expressed under the control of the endogenous Sca-1 promoter. These animals were then back-crossed into a C57/Bl6 background to create the ScaKI mice used in these experiments, which are homologous for the recombined genes resulting in a transgenic line which is negative for Sca-1 but positive for GFP. Prior studies reveal the ScaKI mouse exhibits its serial bone marrow stem cell repopulating defects but has not been previously studied for a cardiac phenotype, although an unrelated murine model of Sca-1 deletion has been postulated to possess an impaired response to pathological challenge due to defective Nkx2.5-positive non-myocytes. In comparison, our findings using the ScaKI line reveal deleterious effects of Sca-1 deletion on c-kit+ CPCs and associated underlying signaling mechanisms.

**Surgical Methods**

Echocardiography was performed under minimal (1.25%) isoflurane anesthesia with 2.5% supplemental oxygen using an Acuson Sequoia C256 instrument. Myocardial infarctions were carried out under 1.5% isoflurane anesthesia by ligating the left descending coronary artery with 8-0 suture. To confirm adequate ligation, cyanosis and akinesia of the affected left ventricle were observed. Sham operations were performed by opening and closing the chest. Postoperative palliative care was administered immediately and at 12 hours. Hemodynamic measurements were accomplished with a closed chest by inserting a pressure/volume catheter through the left ventricle under chloral hydrate sedation. Data were collected and analyzed using a Scisense microtip pressure processor. To generate paraffin-embedded cardiac samples, mice were deeply sedated with chloral hydrate, and the hearts were arrested in diastole by catheterizing the abdominal aorta and flushing the heart with a high potassium chloride/cadmium chloride solution. Ten percent neutral buffered formalin (NBF) fixative was perfused into the coronary arteries at systolic pressure while the left ventricle was filled with NBF at diastolic pressure. Retroperfused hearts were then removed from the chest cavity and placed in NBF overnight, followed by processing for paraffin embedding using a Leica TP1050 automated tissue processor.

**Immunofluorescence Microscopy**

Paraffin-embedded tissue sections, cut at 5 μm, were prepared from mouse hearts that had been processed as described above. Immunohistochemistry protocol is as previously described and is detailed in the online-only Data Supplement. Antibody information is detailed in Online Table I. Confocal images and counts were acquired using a Leica TCS SP2 confocal microscope.

**Paraffin-Embedded Cell Counts**

Hearts were harvested at the specific developmental time points described in the text and paraffin embedded. At least 3 nonsequential sections were cut and stained per heart. The area included within the count was measured using the Leica LCS Lite (Leica AG) software.

**Cardiac Stem Cell Isolation and Culture**

CPCs were isolated and cultured as previously described. Briefly, 2 mice per preparation were anesthetized using ketamine-xylazine solution, and the heart was cannulated through the aortic arch and perfused at 37°C in oxygenated basic buffer (J-MEM, 0.7 g/L HEPES, 1.25 g/L taurine, 20 U/L insulin, Pen/Strep/Glutamine, Amphotericin, Gentamicin, pH 7.3) on a Radnotti apparatus. The heart was then digested for 12 minutes at 37°C in 320 units/Ml collagenase II in oxygenated basic buffer. Afterward, the heart was minced in basic buffer containing 0.5% bovine serum albumin and the cardiomyocytes were pelleted for 1 minute at 100g and discarded. Remaining cells in the supernatant were passed through a 25-μm filter and pelleted. The cell pellet was resuspended and incubated with anti–c-kit (CD117) Miltenyi beads in PBS+0.5% bovine serum albumin, washed, and isolated on a Miltenyi magnetic column to extract c-kit+ CPCs according to manufacturer’s instructions. CPCs were cultured according to standard tissue culture protocols in CPC media (DMEM/F12, 10% embryonic stem cell–grade fetal bovine serum (FBS), PSG, insulin-transferrin-selenium, 1000 U/mL LIF, 40 ng/mL EGF, 20 ng/mL bFGF).

**Flow Cytometry and Apoptosis Analysis**

Cells were grown in complete media and analyzed by flow cytometry after nonenzymatic removal from the culture plates. The cells were resuspended at 100 000 cells in 100 μL PBS+2% FBS, stained with directly conjugated antibodies, and analyzed after 2 wash steps. For apoptosis treatment and analysis, CPCs were seeded into 6-well plates in CPC media for at least 12 hours before treatment. Treated cells received the indicated amount of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for

### Non-standard Abbreviations and Acronyms

- αSMA: α-smooth muscle actin
- AWD: anterior wall dimension
- βcat-I: β-catenin inhibitor
- Ca-Actin: cardiac α-actinin
- CPC: cardiac progenitor cell
- FBS: fetal bovine serum
- GFP/EGFP: green fluorescent protein/enhanced green fluorescent protein
- GSK-I: GSK3β inhibitor
- MI: myocardial infarction
- NBF: neutral buffered formalin
- NTG: nontransgenic control mouse line, refers to C57/BL6 strain from which the Sca-1 knock-out/GFP knock in mouse was derived
- PWD: posterior wall dimension
- RLU: relative luminescence units
- Sca-1: stem cell antigen-1
- ScaKI: Sca-1 knock-out/GFP knock-in mouse
- qPCR: quantitative PCR
5 hours. After peroxide incubation, treated cells and untreated controls were labeled with Annexin V-APC Apoptosis Detection Kit (BD Pharmingen) according to manufacturer’s instructions. Briefly, cells were suspended at 100,000 cells per 100 μL in Annexin V binding buffer. Each set of treated and untreated cells was incubated for 20 minutes at room temperature in the dark with 0.75 μL Annexin V and 2 μL 7-AAD per 100,000 cells; 200 μL of binding buffer was added post incubation and the cells were analyzed by flow cytometry with a BD FACSAria instrument. Unstained and single stain controls were used to establish baseline fluorescence levels for the samples.

Trypan Blue and Cyquant Assay
Cell cultures were counted after passage using 2% trypan blue solution by hemocytometer determination. For trypan blue assay, 15,000 cells per well were seeded at day 0 in a 6-well plate and counted on a hemocytometer at the indicated days post plating. For Cyquant assay, 1000 cells per well were seeded in triplicate day 0 early in the morning in 100 μL of full-growth media. In the evening of day 0 through day 5, the growth media was gently removed from the appropriate wells and replaced with 1× HBSS containing Cyquant NF reagent diluted 1:500. Fluorescence was measured 30 minutes later on a Tecan Spectraflour Plus plate reader at an excitation/emission of 485/535 with a standard gain of 70.

Quantitative PCR
mRNA was isolated from cells using Zymo Research quick RNA miniprep according to manufacturer’s instructions. Briefly, at least 100,000 cells per sample were collected in ZR RNA buffer, applied to a binding column, and centrifuged at 12,000 rpm for 1 minute. Flow-through was discarded and washed once with RNA wash buffer. The RNA was eluted in DNase/RNase-free water and quantified by spectrophotometer. RT-PCR was accomplished using the Bio-Rad iScript cDNA synthesis kit according to manufacturer’s instructions. Briefly, 1 μg of RNA per sample was added to 4 μL of 5X Master Mix, 1 μL of 20X reverse transcriptase, and RNase-free water up to 20 μL. The RT-PCR reaction conditions were 5 minutes at 25°C, 30 minutes at 42°C, followed by 5 minutes at 85°C. cDNA was diluted at least 1:60 in RNase/DNase-free water. Quantitative (qPCR) was accomplished using 20 to 30 μg of cDNA, 3 μmol/L forward and reverse primers, and 2X Bio-Rad Sybr Green master mix at a final volume of 15 μL. qPCR was performed on a Bio-Rad MJ research Opticon instrument with amplification protocol: 10 minutes at 95°C; then 15 seconds at 95°C, 60 seconds at 60°C, then plate-read for 50 cycles followed by a melt curve analysis for product specificity. qPCR primer sequences are detailed in Online Table II. Fold difference analysis was calculated using the 2–ΔΔCt method, using GAPDH as housekeeping gene control. qPCR arrays were accomplished according to manufacturer instructions by adding 25 μL per well of 2X Bio-Rad Sybr Green master mix and 25 μL per well of cDNA. SA Biosciences qPCR array primer sequences (RT2 Profiler PCR Array Mouse Signal Transduction PathwayFinder, PAMM-014A) are proprietary information.

siRNA
CPCs derived from FVB/N mice were plated in 10 mL full-growth media for 12 hours at 500,000 cells per 10 cm plate. The media was replaced with transfection media consisting of CPC growth media containing 0% FBS. siRNA for mouse Sca-1 (Ly6A) was obtained from Bioneer (110454, proprietary sequence) and resuspended at 100 μmol/L in DNase/RNase-free water. Transfection mix containing 425 μL of transfection media, 50 μL of HiPerfect (Qiagen) transfection reagent, and 15 μL of 20 μmol/L siRNA was added to the 10-cm plate and incubated for 24 hours. The media was then changed to DMEM/F12 with 2.5% FBS and incubated for an additional 24 hours. Cells were then passaged and plated in full CPC media for the indicated assays.

SuperTopFlash Luciferase Assays
The SuperTopFlash 7X TCF/Lef-luciferase reporter in a lentiviral expression plasmid was a kind gift of Dr Karl Willert at the University of California, San Diego. CPC lines were infected with an equal amount of SuperTopFlash lentivirus and passaged several times to insure stable expression. Cells were plated overnight and then treated for 24 hours with GSK3β inhibitor (Santa Cruz 202636) at 1 μmol/L or β-catenin inhibitor (EMD 219330) at 7.5 μmol/L. Luciferase expression level was assessed using the Promega Bright-Glo luciferase assay according to manufacturer’s instructions. Luminescence was quantified on a Tecan Spectroflour Plus plate reader.

Statistical Analysis
All probability values were determined using a paired, 2-tailed Student t test with at least 3 samples per group. Significance for qPCR analysis was determined by comparing ΔCt values of experimental versus control samples using a paired, 2-tailed Student t test with at least 3 samples per group.

Results
Depressed Cardiac Function in ScaKI Mice and Hypertrophy in Older Animals
Phenotypic characterization was performed to assess defects in myocardial structure and function by echocardiography, using sex-matched ScaKI and nontransgenic control mice at 12 to 18 weeks (males) as well as 12 to 14 months (male and female groups). Both young and old ScaKI mice exhibit significantly larger end-systolic diameter measurements relative to nontransgenic controls (Figure 1A and 1B). In vivo hemodynamic measurements confirm poor cardiac function in ScaKI hearts as measured by change in pressure over change in time (dP/dT), where maximum pressure was significantly lower and minimum pressure was significantly higher in ScaKI hearts (Figure 1C and 1D). Fractional shortening (FS) and ejection fraction (EF) are both significantly lower in 1-year-old ScaKI mice, which also exhibited significantly larger hearts as measured by echocardiography of anterior wall dimension (AWD) and posterior wall dimension (PWD) (Figure 1B) and heart weight/body weight ratio (Figure 1F). Increased weight of old ScaKI hearts and size can be attributed to cardiomyocyte hypertrophy due to an increase in average cardiomyocyte cross-sectional area as well as significantly increased AWD and PWD (Figure 1B and 1G).

ScaKI mice also exhibit increased numbers of myocytes expressing atrial natriuretic peptide, a maker of hypertrophy, in both younger older animals (Online Figure 1). Significantly reduced survival is observed in ScaKI mice relative to normal control subjects when challenged with a very large infarction (Online Figure II). Significantly fewer myocytes are labeled for BrdU in 28- to 30-week-old ScaKI mice relative to normal controls (Online Figure III), consistent with loss of proliferative responsiveness in aged ScaKI hearts. Thus, hypertrophic remodeling in older animals may be accentuated in response to stress due in part to impaired cellular replacement.

Diminished Number of CPCs in ScaKI Mice
c-kit-positive CPCs are present in normal or infarcted myocardium from both nontransgenic controls as well as ScaKI
Figure 1. ScaKI mice have depressed cardiac function in younger and older animals and hypertrophy in older animals. Echocardiographic measurements of NTG and ScaKI mice at 12 to 16 weeks old (n=6) (A) and 12 to 14 months old (n=6) (B) are shown. End-systolic diameter (ESD), end-diastolic diameter (EDD), anterior wall dimension (AWD), and posterior wall dimension (PWD) are shown. Hemodynamic measurements of change in pressure over change in time (dP/dT) in NTG and ScaKI mice at 12 to 16 weeks old (n=3) (C) and 12 to 14 months old (n=3) (D) are shown. E, Calculated measurements of cardiac function, fractional shortening (FS), and ejection fraction (EF) in younger and older NTG and ScaKI mice. F, Heart weight-to-body weight ratios of 12- to 16-week old (n=5) and 12- to 14-month-old NTG and ScaKI mice (n=3). G, Cardiomyocyte cross-sectional area in the left ventricle of 12- to 14-month-old C57 and ScaKI animals. Error bars represent SEM. *P<0.05, #P<0.02; NS, not significant compared with age-matched NTG controls.
tissue samples (Figure 2A through 2C). However, significantly fewer total c-kit+ CPCs are present in ScaKI myocardial sections from normal tissue (1.5±0.6/mm²) compared with nontransgenic controls (2.6±0.8/mm²; Figure 2B). CPC migration to the zone of pathological damage increases in response to MI, peaking between 4 to 10 days after MI. As true for normal myocardium, significantly fewer CPCs are present within the infarct region of ScaKI hearts (2.4±1.0/mm²) relative to nontransgenic controls (8.7±1.2/mm²; Figure 2C), although infarct size is not significantly different between the groups as measured by percent of scarred left ventricular free wall (LVFW) at 7 days. Confocal images of proliferating c-kit+ cells at 7 days after MI in NTG (left) and ScaKI mice (right); c-kit (green), desmin (red), and proliferating cell nuclear antigen (PCNA, white). Scale bar is 40 μm. 

**Figure 2.** ScaKI mice have fewer c-kit+ cells in normal myocardium and in the infarct zone (IZ) and border zone (BZ) of a 7-day MI. A, Confocal images of c-kit+ cells in the normal myocardium of NTG (left) and ScaKI (right) mice. B, Scans in the IZ of a 7-day MI in an NTG (left) heart and an ScaKI heart (right); c-kit (green), tropomyosin (“Tmyo,” red), and topro (blue). C, Quantification of c-kit+ cells/mm² in normal and infarcted myocardium of NTG and ScaKI mice. D, Infarct area as measured by percentage of scarred left ventricular free wall (LVFW) at 7 days. E, Confocal images of proliferating c-kit+ cells at 7 days after MI in NTG (left) and ScaKI mice (right); c-kit (green), desmin (red), and proliferating cell nuclear antigen (PCNA, white). Scale bar is 40 μm. F, Quantification of proliferating c-kit+ cells as percentage of PCNA+/c-kit+ cells over total c-kit+ cells. Arrowheads indicate c-kit+ cells. Error bars represent SEM. *P<0.05, #P<0.02, NS indicates not significant.

Consequences of Sca-1 Knockout on c-kit+ Cells During Postnatal Development

The relationship between cardiac development, CPCs, and Sca-1–driven or Sca-1–driven GFP expression in the postnatal heart was determined in a developmental time course encompassing 2 days, 1 week, 2 weeks, and 12 weeks of age.
Figure 3. Sca-1 knockout consequences for c-kit+ cells during development. Confocal images of adult heart sections of a NTG animal depicting a c-kit+/Sca-1− cell (A) and c-kit+/Sca-1+ cell (B); c-kit (white), Sca-1 (green), desmin (red), and topro (blue). Confocal images of adult heart sections are shown of a ScaKI animal depicting a c-kit+/GFP− cell (C) and a c-kit+/GFP+ cell (D); c-kit (white), GFP (green), tropomyosin (“Tmyo,” red), and topro (blue). Arrowheads indicate c-kit+ cells. E, Quantification of total cells expressing c-kit at the indicated ages. F, Quantification of c-kit+ cells in the myocardium with respect to Sca-1 or GFP expression. G, Quantification of either Sca-1+ only or GFP+ only cells in the myocardium at indicated ages. Error bars represent SEM. *P<0.05, #P<0.02; NS indicates not significant versus NTG animals at the comparable age.
Presence of CPCs phenotyped as c-kit+ only as well as c-kit+/Sca-1+ were observed in normal nontransgenic control myocardial sections from 2 days up to adulthood (Figure 3A and 3B). Similarly, c-kit+ only and c-kit+/GFP+ CPCs were also observed in the ScaKI myocardium throughout all assessed time points (Figure 3C and 3D). Significantly fewer total c-kit+ CPCs were present in ScaKI hearts at 2 weeks and 12 weeks of age relative to nontransgenic controls (Figure 3E), but this difference was not present at 2 days and 1 week of age. Further characterization of the collective c-kit+ CPC population was performed by subdivision to determine the percentage of dual positive c-kit+/Sca-1+ CPCs in normal nontransgenic samples versus c-kit+/GFP+ CPCs in ScaKI sections (necessitated as GFP+ due to homozygous deletion of Sca-1 antigen in the ScaKI line). Significantly fewer c-kit+/GFP+ cells were present at postnatal day 2 in the ScaKI hearts relative to c-kit+/Sca-1+ cells in the normal control, with a possibly compensatory concurrent increase of c-kit+ only cells in ScaKI myocardium. Shortly thereafter at postnatal day 7, differences vanished between the controls and ScaKI for both c-kit+ alone and dual positive c-kit+/Sca-1+ cell categories. However by 2 weeks of age onward, ScaKI hearts had significantly fewer dual positive CPCs compared with nontransgenic controls. Since significant differences were not evident for the number c-kit+ only CPCs, decreases in the total c-kit+ CPC population can be attributed to fewer dual positive c-kit+/GFP+ CPCs in the ScaKI samples (Figure 3F) that are the counterparts to the dual positive c-kit+/Sca-1+ population in normal tissue. The number of Sca-1+ only or GFP+ only cells in the heart was comparable between the groups at all time points examined (Figure 3G). Vascular endothelium in the heart also expresses Sca-1, but knock-in of GFP in the ScaKI line does not overtly affect the number or gross morphology of vessels and capillaries observed in histological sections (Online Figure VIII).

**Stem Cell Marker Expression, Decreased Proliferation, and Survival in Adult ScaKI CPCs**

CPCs were isolated using the c-kit marker from both ScaKI and nontransgenic control mice to determine phenotypic properties in vitro. Resulting cultures exhibit activation of the Sca1 promoter as evidenced by GFP expression in ScaKI (37.2%, background corrected) or Sca-1 protein in nontransgenic controls (51.1%, background corrected) by flow cytometry (Figure 4A). Proliferation of ScaKI CPCs is depressed relative to normal CPCs (Figure 4B). ScaKI CPCs also show increased sensitivity to oxidative stress induced by H2O2 exposure leading to apoptosis (Figure 4C).

Impaired proliferative phenotypes generated by genetic deletion of Sca-1 were confirmed using siRNA to Sca-1 to knockdown Sca-1 in normal CPCs generated from wild-type FVB/N mice. The number of Sca-1+ CPCs in the culture was reduced from an average of approximately 55% in siScramble transfected cells to 10% in siSca-1 transfected cells from days 1 to 3 after plating (Figure 4D). Proliferative responses of siSca-1 cells are significantly reduced compared with siScramble cells by either trypan blue or Cyquant analyses (Figure 4E and 4F).

**Altered β-Catenin Signaling in ScaKI CPCs**

The mechanisms of Sca-1 signaling and specific genes affected by Sca-1 expression have yet to be described. However, phenotypic deficiencies in proliferation and survival in ScaKI CPCs (Figures 2–4) suggest an underlying molecular basis. Screening assays by qPCR analysis using cDNA derived from each cell line under optimal growth conditions assessed activation of multiple signaling cascades (Figure 5A). The canonical Wnt pathway exhibited the greatest number genes significantly changed between ScaKI CPCs versus nontransgenic controls. Several genes in the Wnt pathway showed significant changes: WNT1-inducible signaling pathway protein 1 (Wisp1), cadherin 1 (Cdhl1), and lymphoid enhancer binding factor 1 (Lef1) were decreased, whereas Axin2 expression was significantly increased. Decreased expression of additional non–Wnt-related genes for vascular cell adhesion molecule 1 (VCAM1) and NLR family, apoptosis inhibitory protein 1 (Naip1/Birc1a), was also statistically significant. Quantitative changes in gene expression were confirmed by qPCR of 3 additional cDNA samples from nontransgenic control and ScaKI CPCs performed in duplicate (Figure 5B).

Wnt/β-catenin gene expression alterations prompted further examination of pathway activation in ScaKI CPCs, revealing significantly elevated β-catenin–mediated transcriptional activity increased by 2-fold in ScaKI CPCs compared with NTG control CPCs (Figure 5C). Inhibition of β-catenin phosphorylation and ubiquitination by the pharmaceutical inhibitor GSK3β increased β-catenin/TCF/Lef transcriptional activity in NTG control CPCs (1.5-fold) and ScaKI CPCs (2-fold), confirming functional activity of this pathway in the cultured cells (Figure 5C). β-Catenin/TCF/Lef signaling is involved in switching from self-renewal to differentiated phenotypes in embryonic stem cells and, similarly, increased β-catenin signaling in ScaKI CPCs is associated with significant elevation of MEF2c, cardiac α-Actinin (Co-Actinin), α-smooth muscle actin (αSMA), and SM22 relative to NTG control CPCs by qPCR analysis (Figure 5D). Participation of β-catenin in promoting commitment correlates with significant decreases in MEF2c and Co-actinin were in ScaKI CPCs after 3 days of β-catenin inhibitor treatment (Figure 5E; 7.5 µmol/L), calibrated to reduce SuperTopFlash luciferase activity but not impair proliferation (Online Figure IX). Similar to induction of cardiac-specific transcripts in CPCs on lineage commitment, the impaired proliferation and increased cardiogenic commitment of ScaKI CPCs are consistent with enhancement of a lineage-committed phenotype that appears linked to aberrant canonical Wnt signaling involving β-catenin.

**Discussion**

Maintenance and repair of myocardial structure and function depends on CPCs; therefore understanding the molecular biology of CPCs will be essential for expanding our repertoire of methodological approaches for enhancing cellular therapeutic approaches. Although Sca-1 is a widely accepted marker of stem cells in the hematopoietic system, downstream molecular signaling and consequences of Sca-1 dysregulation are poorly understood in
Figure 4. Adult ScaKI CPCs express stem cell markers and demonstrate decreased proliferation and survival in vitro. 

**A**, Flow cytometric analysis of CPCs derived from NTG (left) and ScaKI (right) hearts and analyzed for GFP expression (top) or stained for Sca-1 expression (bottom). 

**B**, Growth curve of n=3 NTG and ScaKI CPCs using trypan blue exclusion assay. 

**C**, NTG and ScaKI CPCs treated with H$_2$O$_2$, stained with AnnexinV/7-AAD, and analyzed with flow cytometry, n=4. 

**D**, Flow cytometric analysis of Sca-1+ CPCs after siRNA of Sca-1 or scrambled RNA at the indicated time points. 

**E**, Cyquant proliferation assay of siSca-1 versus siScramble CPCs at the indicated time points. 

**F**, Growth curve of siSca-1 and siScramble CPCs using trypan blue exclusion assay. Error bars represent SEM, *P<0.05.
the hematopoietic context and essentially unknown in the myocardial environment. Therefore, this study was undertaken to characterize Sca-1/CPCs in particular with respect to development, function, maintenance and repair of the heart. Sca-1 has been previously used by other groups to mark mouse and human CPCs. Lack of Sca-1 in the adult mouse heart results in minor developmental contractile defects in agreement with observations from prior studies as well as age-associated hypertrophy that has not been previously described (Figure 1).

A critical role for Sca-1 expression for optimal myocardial and CPC function is now clear from findings in this study, although prior publications examining the hematologic system of the ScaKI mice failed to reveal an overt phenotype. Since bone marrow of older (13 months old) ScaKI mice possess fewer c-kit+CPCs (Online Figure X), we hypothesized that Sca-1 deletion would be consequential for the CPC population but only manifest in adult animals with exacerbation by aging or pathological challenge.

Myocardial function in ScaKI mice is characterized by reduced function in early adulthood and hypertrophy evolving with age. Enlarged myocytes are present in young adult ScaKI animals without a concomitant increase in heart weight/body weight ratio (Figure 1). This age-associated increase in average myocyte size within the ScaKI heart is presumably due to decreased new myocyte formation by CPCs (Online Figure III). Fewer c-kit+CPCs are present in ScaKI mice in normal or infarcted myocardium (Figure 2), consistent with the premise of a functionally compromised stem cell population in the ScaKI heart.

Cardiac impairment in young adult mice prompted a developmental time course analysis of CPCs spanning postnatal life (2 days) until adulthood (12 weeks). Deficiencies in c-kit+CPCs of ScaKI hearts were significant at 2 weeks of age with lower c-kit+CPC number, due primarily to fewer c-kit+/GFP+ in ScaKI hearts compared with c-kit+/Sca-1+CPCs in NTG controls (Figure 3). Based on these observations, it is tempting to speculate that c-kit+/Sca-1+CPCs and c-kit+/Sca-1- CPCs may act as distinct cell types, since if c-kit+CPCs in the heart were derived from c-kit+/Sca-1+CPCs, then both groups of Sca-1 and Sca-1- CPCs should be concomitantly reduced.

Cultured CPCs were successfully established from ScaKI myocardium despite lower numbers of CPCs exhibiting deficiencies in growth and survival (Figure 4) similar to prior reported findings in the noncardiac context. Poor growth potential of ScaKI CPCs observed in vitro (Figure 4) parallels the in vivo observation of impaired CPC response to infarction (Figure 2). Taken together, these results indicate that phenotypic characteristics of the ScaKI heart probably involve functional impairment of CPCs lacking Sca-1.

It is important to note that 2 other types of cells that normally express Sca-1 may affect the overall cardiac phenotype of the ScaKI mice: the vasculature and a subpopulation of circulating hematopoietic cells. A structural quantification of the baseline and post-MI vasculature did not reveal any overt differences in the ScaKI heart in either baseline
The molecular defect underpinning defective performance of Sca-1– CPCs appears to involve canonical Wnt signaling where β-catenin/TCF/Lef is the primary transcriptional effectors for growth and survival (Figure 5). TCF/Lef also has non–Wnt-associated functions25 and, in the absence of activated β-catenin, functions as a transcriptional repressor.26 Wnt signaling is associated with adult stem/progenitor cell proliferation, survival, and differentiation,27 as well as cardiac development in embryogenesis.28,29 ScaKI CPCs show higher β-catenin signaling associated with phenotypic characteristics of lineage commitment at the mRNA level (Figure 5). Indeed, increased β-catenin signaling promotes differentiation in several other types of progenitor cells, such as when epithelial progenitor cells exit cell cycle and initially differentiate into mature hair follicles.30 Strength of β-catenin signaling differentially determines differentiation of mesenchymal progenitor cells to either a chondrocyte or osteoblast fate,31 and β-catenin signaling inhibits proliferation together with promotion of neuronal differentiation.32 A crucial role for Sca-1 in lineage commitment and c-kit expression has also been reported for hematopoietic stem cells.6 A recent publication on cardiac side population cells, which are Sca-1+ and are a putative CPC, has revealed that an excess of β-catenin signaling results in fewer cardiac side population cells after infarction, which is consistent with our findings.33 Thus, overactive β-catenin signaling probably contributes, at least in part, to impairment of proliferation and up-regulation of cardiovascular lineage markers. Future studies will need to elaborate on the role of Wnt pathway signaling downstream of Sca-1 in CPCs.

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Disclosures

None.

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Novelty and Significance

**What Is Known?**

- Cardiac progenitor cells (CPCs) exist within the myocardium of multiple species, including humans, and contribute to repair and regeneration of the heart after injury.
- CPCs are small cycling cells in the myocardium, which may express the cell surface markers c-kit and Sca-1.
- CPCs can be isolated from cardiac tissue, expanded and manipulated in culture, then reintroduced to provide therapeutic benefits after a heart attack.

**What New Information Does This Article Contribute?**

- Sca-1 in CPCs increases canonical Wnt/β-catenin signaling, resulting in reduced cell cycling and premature differentiation of CPCs.

The rationale of this study is to describe the function of Sca-1 in the heart, using the phenotypic consequences of Sca-1 deletion on cardiac structure and function. Sca-1 is a marker of stem cells and the vasculature in many tissues and its functions outside of the hematopoietic system have yet to be thoroughly investigated. Likewise, the intracellular signaling pathway for Sca-1 has yet to be elucidated. Our novel findings include that Sca-1 is important for the optimal cardiac function as well as the resident CPCs. The findings are noteworthy because CPCs are an emerging clinical therapy for heart attack and cardiomyopathy. This study is the first to indicate that canonical Wnt/β-catenin signaling is affected by deletion of Sca-1 in CPCs. Sca-1 is a surface protein and therefore a potential target for intervention to affect CPC function. Overall, our data confirm the importance of Sca-1 to maximal CPC function and the importance of CPC activity to optimal heart function.
Sca-1 Knockout Impairs Myocardial and Cardiac Progenitor Cell Function
Brandi Bailey, Jenna Fransioli, Natalie A. Gude, Roberto Alvarez, Jr, Xiaoxue Zhan, Åsa B. Gustafsson and Mark A. Sussman

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Supplemental Material

Supplemental Methods

Immunofluorescence Microscopy

Paraffin embedded tissue sections, cut at 5μm, were prepared from mouse hearts that had been processed as described in the Methods section. Sections of were deparaffinized in xylene and rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was then performed in 10mmol/L citrate, pH 6.0, using 1100-W microwave oven for 3 minutes at high power and 12 minutes at 50% power. The slides were allowed to cool for 15 minutes at 4°C, then washed in 3 times in TN buffer (NaCl 150 mmol/L, Tris 100mmol/L, pH 7.5) and quenched with 3% hydrogen peroxide in TN buffer for 20 minutes to remove endogenous peroxidase activity. Slides were then washed 3 times in TN buffer and blocked for 1 hour in TNB (TN buffer containing 0.5% Blocking Buffer, proprietary formula from TSA™ kit, Perkin Elmer/NEN). Slides were incubated at 4°C overnight with primary antibodies diluted in TNB. Slides were then washed 3 times for 5 minutes per wash in 1X TN and incubated for 2 hours at room temperature in the dark with species-specific secondary antibodies conjugated to fluorophores in TNB. After secondary labeling, slides were washed 3 times in TN. Nuclei were stained for 20 minutes with either To-pro-3-Iodide (Topro) at 1/10,000 in TN or Sytox Blue at 1/200 in TN. Slides were mounted for viewing in Vectashield medium. Detection of some antigens required signal amplification using biotin-streptavidin and/or horseradish peroxidase (HRP) conjugated antibodies followed by reaction with a fluorescent tyramide substrate according to manufacturer’s instructions. Antibody information is detailed in Online Table I. Confocal images and counts were acquired using a Leica TCS SP2 confocal microscope.

Bone Marrow Isolation

Bone marrow cells (BMC) were flushed from both tibias and femurs of each mouse using PBS + 1%FBS buffer. BMCs were separated from red blood cells by density centrifugation using Histopaque reagent according to manufacturer’s instructions. The BMCs were assayed by FACS analysis fresh from isolation.

Vascular Casting

Microfil injection was performed according to previously described protocol¹. Briefly, mice were sedated with ketamine/xylazine solution and the right common carotid artery was cannulated and injected slowly with microfil solution. The hearts were placed into PBS overnight and then dehydrated in increasing ethanol solutions and cleared in methyl salicylate according to manufacturer’s instructions.
BrdU Injections

Normal nontransgenic controls and ScaKI males at 28-30 weeks of age were injected IP once daily with sterile 20mg/kg BrdU in PBS for two weeks.

Supplemental Results

Decreased MI survival in ScaKI mice

Despite modest remodeling in the young ScaKI hearts, significantly fewer ScaKI mice survive a major myocardial infarction (MI) for 14 days relative to nontransgenic controls as shown by Kaplan-Meyer analysis (Supplemental Figure II.II). Postmortem investigation indicates cardiac rupture as the primary cause of death (4 cardiac rupture, 2 undetermined) in ScaKI mice after infarction challenge.

Vascular endothelial layers in the myocardium express Sca-1 antigen or Sca-1 driven GFP

In addition to serving as a hematopoietic stem cell marker, Sca-1 also labels vasculature in several organ systems\(^2,3\). Due to possible presence of Sca-1 on vascular structures in the heart, the genetic deletion of Sca-1 in ScaKI mice may manifest an altered vasculature. The gross vascular pattern, as assessed by latex perfusion of the heart, appears similar in ScaKI and NTG hearts (Supplemental Figure III.IA). Sca-1 is indeed expressed on the vascular endothelial surface as evidenced by colocalization adjacent to the vascular marker \(\alpha\)-Smooth Muscle Actin (\(\alpha\)SMA) (Supplemental Figure IIB, left) and colocalization with von Willebrand’s Factor (vWF) (Figure 3.1, left) in myocardial sections of nontransgenic mice. In comparison, GFP expression occurs throughout the vascular cell and colocalizes adjacent to \(\alpha\)SMA in ScaKI myocardial sections (Supplemental Figure III.I, right). Comparable to Sca-1+/vWF+ cells in nontransgenic controls, GFP+/vWF+ cells are observed in ScaKI mice (Supplemental Figure III.IC, right). Thus, Sca-1 expression is associated with vasculature of the myocardium.

To examine whether absence of Sca-1 affected vascular density, CD31+ vessels in both nontransgenic controls and ScaKI myocardial sections were assessed and quantitated by immunohistochemical staining. Overt appearance of vasculature marked by CD31 immunoreactivity is comparable between nontransgenic controls and ScaKI (Supplemental Figure III.ID). Assessment of capillary density revealed no significant difference in the myocardium between the control and the ScaKI groups (Supplemental Figure III.IE).
12-14 month old ScaKI mice have fewer stem cells as labeled by c-kit expression in the bone marrow

Levels of stem cell marker c-kit+ were analyzed from either normal nontransgenic controls or ScaKI bone marrow at the ages of 16 weeks and 12 months of age. Whole bone marrow was isolated and red blood cells were removed. Staining and FACS analysis (Online Figure XA) of Sca-1 and c-kit markers in normal nontransgenic controls mice and GFP fluorescence and c-kit in ScaKI mice revealed that although the levels are c-kit+ cells are equal in younger mice, older ScaKI mice have fewer c-kit+ cells in the bone marrow (Supplemental Figure V.IIB). Thus, ScaKI mice show increases in GFP+ cells in the bone marrow relative to normal nontransgenic controls likely due to increased half life turnover rate for GFP protein relative to Sca-1 (Online Figure X-A).
Online Tables

**Online Table I.** Primary antibody listing

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Amplification (paraffin only)</th>
<th>Marker</th>
<th>Manufacturer</th>
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<td>c-kit</td>
<td>Goat</td>
<td>1:50</td>
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<td>Stem cells</td>
<td>R&amp;D Systems</td>
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<td>GFP</td>
<td>Rabbit</td>
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<td>Molecular Probes</td>
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<td>Ki67</td>
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<tr>
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<td>Desmin</td>
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<td>Vessel</td>
<td>Invitrogen</td>
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**Online Table II.** qPCR primer sequences

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<th>Reverse Primer 5’-3’</th>
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<td>Birc1a</td>
<td>AAACCGGATGTGCTCTCAGTCTTT</td>
<td>AGCACAGCCATGAGAGAGTGACAA</td>
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<tr>
<td>GAPDH</td>
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<td>CATACAGGAAATCAGCTTACAA</td>
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<tr>
<td>Lef1</td>
<td>AGTGACTCTAGTACGCTGACTTT</td>
<td>TAGCGTGACAGCTAGCTCGACAT</td>
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<tr>
<td>Sca-1</td>
<td>TCAGGAGGCAGCAGTTATATTGGA</td>
<td>TACATCGACAGCTTCACCTC</td>
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<tr>
<td>Vcam1</td>
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<td>Wisp1</td>
<td>GCGCTTTACTGACATGGCTTGCCTT</td>
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<tr>
<td>Gata4</td>
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<td>AGCTGCTTTCCCCTC</td>
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<td>α-SMA</td>
<td>GTTCAGTGGCTCCTCTGTA</td>
<td>ACTGGGACAGCATGGAAAAG</td>
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<td>SM22</td>
<td>GACTGACAATTCTCCTGGCTCAT</td>
<td>CCGAAGCTACTTCTCCTC</td>
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4
Online Figure I. Confocal images of sections immunolabeled with Atrial Natriuritic Peptide (ANP) from a (A) 13 week old NTG mouse (B) 13 week old ScaKI mouse (C) 13 month old NTG mouse (D) 13 month ScaKI mouse and (E) 3 week post Trans-Aortic Constriction (TAC) NTG mouse. ANP (green), Desmin (red) and Topro (blue). Arrows indicate ANP+ myocytes.
**Online Figure II.** Kaplan-Meyer MI survival analysis of n=6 normal nontransgenic control or ScaKI mice 20 to 26 weeks of age after a large myocardial infarction. # p<0.02.

**Online Figure III.** Confocal images of sections immunolabeled for BrdU from normal nontransgenic control (A) or (B) ScaKI mice at 28-30 weeks of age. BrdU (green), Desmin (red) and Topro (blue). (C) Quantitation of BrdU+ myocytes from both NTG and ScaKI sections. Error bars represent SEM. # p<0.02.
Online Figure IV. ScaKI mice possess fewer Ki67+/c-kit+ positive cells in unchallenged myocardium as well as infarct and border zone at 7 days following myocardial infarction. Confocal images of c-kit+ cells in the 7 day infarct region of normal nontransgenic control (A, Ki67+/c-kit+) or ScaKI (B, Ki67-/c-kit+) mice. Ki-67 (white), c-kit (green), tropomyosin (“Tmyo”, red) and Topro (blue). (C) Quantitation of Ki67+/c-kit+ cells/mm² in the MI and border zone of the infarcted myocardium of normal nontransgenic control or ScaKI mice.

Online Figure V. Fewer c-kit positive cells in ScaKI mice in the infarct and border zone at 7 days post-myocardial infarction. Confocal images of PCNA+/c-kit+ cells in the normal myocardium of normal nontransgenic control (A) and ScaKI (B) mice. PCNA (white), c-kit (green), desmin (red) and Topro (blue). (C) Quantitation of PCNA+/c-kit+ positive cells/mm² in myocardium of normal nontransgenic control and ScaKI mice.
Online Figure VI. Confocal images of sections immunolabeled with antibodies for c-kit and the mast cell marker, tryptase. Typtase (green), c-kit (red), desmin (blue) and Topro (white).

Online Figure VII. Confocal images of sections stained for Isolectin B4 positive vessels 7 days post MI from normal nontransgenic control (A) or ScaKI mice (B). (C) Quantitation of Isolectin B4+ vessels from both NTG and ScaKI sections. IsolectinB4 (green), tropomyosin (red), and topro (blue). Error bars represent SEM. NS – not significant.
Online Figure VIII. Sca-1, or Sca-1 driven GFP, is expressed on the vascular endothelial surface and lack of Sca-1 does not alter vessel density in the heart. (A) Latex vascular castings of normal nontransgenic control and ScaKI adult hearts. “Left” indicates left ventricle with the LAD featured prominently. “Right” indicates right ventricle. (B) Sca-1 in normal nontransgenic control (left) and GFP in ScaKI (right) animals co-localize with α-SMA (white), GFP or Sca-1 (green), Tropomyosin (“Tmyo”, red), and Sytox Blue (“Sytox”, blue). (C) Sca-1 in normal nontransgenic control (left) and GFP in ScaKI (right) animals co-localize with vonWillebrand’s Factor in the adult myocardium; vonWillebrand’s Factor (white), GFP or Sca-1 (green), Tropomyosin (red), and Sytox Blue (blue). (D) Normal nontransgenic control (left) and ScaKI (center) heart sections stained with CD31 (green), Desmin (red) and Topro (blue). Quantitation (right) of CD31 vessels per mm² in both normal nontransgenic control and ScaKI hearts. (E) NTG (left) and ScaKI (center) heart sections stained with IsolectinB4 (green), Desmin (red) and Topro (blue). Quantitation (right) of isolectinB4+ vessels per mm² in both NTG and ScaKI hearts. All animals are 12-16 weeks of age. Scale bar is 40μm. Error bars represent standard error of the mean (SEM).
Online Figure IX. β-catenin inhibitor decreases luciferase activity and at a higher dose decreases cell proliferation. (A) Luciferase SuperTopFlash assay of untreated, 7.5µmol/L and 15µmol/L β-catenin inhibitor (Bcat-I) treated SuperTopFlash transduced normal nontransgenic control (NTG STF) and ScaKI (ScaKI STF) CPCs. (B) Cyquant assay of untreated, 7.5µmol/L and 15 µmol/L β-catenin inhibitor (Bcat-I) treated SuperTopFlash transduced normal nontransgenic control (C57 STF) and ScaKI (ScaKI STF) CPCs.

Online Figure X. 12-14 month old ScaKI mice have fewer stem cells as labeled by c-kit expression in the bone marrow. (A) Scatter plots of bone marrow isolated and immunolabeled for either c-kit and Sca-1 in C57 animals or c-kit alone combined with Sca-1 driven GFP fluorescence in ScaKI animals. (B) Quantitation of the percent c-kit+ cells in the bone marrow of n=3 animals at indicated ages. Error bars represent SEM. # p<0.02.
Supplemental References


