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Genetic Lineage Tracing of Sca-1+ Cells Reveals Endothelial but Not Myogenic Contribution to the Murine Heart

Ronald J. Vagnozzi, PhD[1], **Michelle A. Sargent, BS**[1], **Suh-Chin J. Lin, PhD**[1], **Nathan J. Palpant, PhD**[2], **Charles E. Murry, MD, PhD**[3], **Jeffery D. Molkentin, PhD**[1,4]

[1]Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA;

[2]Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia;

[3]Institute for Stem Cell and Regenerative Medicine, University of Washington School of Medicine, Seattle, WA, USA;

[4]Howard Hughes Medical Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

Abstract

Background -—The adult mammalian heart displays a cardiomyocyte turnover rate of ~1% per year throughout postnatal life and after injuries such as myocardial infarction (MI), but the question of which cell types drive this low level of new cardiomyocyte formation remains contentious. Cardiac-resident stem cells marked by stem cell antigen-1 (Sca-1, gene name *Ly6a*) have been proposed as an important source of cardiomyocyte renewal. However, the *in vivo* contribution of endogenous Sca-1$^+$ cells to the heart at baseline or after MI has not been investigated.

Methods -—Here we generated *Ly6a* gene-targeted mice containing either a constitutive or inducible Cre recombinase to perform genetic lineage tracing of Sca-1$^+$ cells *in vivo*.

Results -—We observed that the contribution of endogenous Sca-1$^+$ cells to the cardiomyocyte population in the heart was <0.005% throughout all of cardiac development, aging or after MI. In contrast, Sca-1$^+$ cells abundantly contributed to the cardiac vasculature in mice during physiological growth and in the post-MI heart during cardiac remodeling. Specifically, Sca-1 lineage-traced endothelial cells expanded postnatally in the mouse heart after birth and into adulthood. Moreover, pulse-labeling of Sca-1$^+$ cells using an inducible *Ly6a*-MerCreMer allele also revealed a preferential expansion of Sca-1 lineage-traced endothelial cells following MI injury in the mouse.

Address for Correspondence: Jeffery D. Molkentin, Howard Hughes Medical Institute, Cincinnati Children's Hospital Medical Center, 240 Albert Sabin Way, Cincinnati, OH 45229. jeff.molkentin@cchmc.org.

Author contributions

R.J.V. conducted experiments and acquired the data. M.A.S. performed MI surgeries and provided technical assistance. S-C. J. L. designed the *Ly6a*$^{+/Cre}$ targeting construct and generated the *Ly6a*$^{+/Cre}$ and *Ly6a*$^{+/MerCreMer}$ gene targeted mice. N.J.P. and C.E.M. designed and provided the *Ly6a*$^{+/MerCreMer}$ targeting construct. J.D.M. and R.J.V. designed the experiments, analyzed the data, and wrote the manuscript. J.D.M. directed and supervised the study.

Competing financial interests

The authors declare no competing financial interests.

Conclusions -—Cardiac-resident Sca-1$^+$ cells are not significant contributors to cardiomyocyte renewal *in vivo*. However, cardiac Sca-1$^+$ cells represent a subset of vascular endothelial cells that expand postnatally with enhanced responsiveness to pathological stress *in vivo*.

Keywords

endothelial cell; lineage tracing; myocardial infarction; myocardial regeneration; stem cell

Introduction

Studies of cardiac regeneration over the past 2 decades have offered hope in potentially restoring new cardiomyocytes to the heart after acute myocardial infarction (MI) injury or during progressive heart failure that is characterized by continuous cellular attrition[1, 2]. However, the intrinsic regenerative potential of the adult mammalian heart is limited, with multiple genetic, biochemical and radioisotope decay studies[3–5] arriving at a consensus rate of approximately 1% new cardiomyocytes per year in rodents and humans[6]. Given this low rate of inherent cardiomyocyte renewal it remains unlikely that the adult heart contains a bona fide stem cell destined to generate new cardiomyocytes[7]. Moreover, the most recent studies have suggested that this low rate of endogenous cardiomyocyte renewal is primarily due to cell cycle activity of existing cardiomyocytes[6].

Despite this, the concept that endogenous progenitor-like cells exist within the adult heart as a source for new cardiomyocyte formation remains firmly rooted in the literature[8]. Progenitor cells expressing the c-Kit stem cell factor surface receptor or the stem cell antigen-1 (Sca-1) marker define cells with purportedly profound cardiomyogenic potential in culture, or when injected into the injured mouse or rat heart[9–12]. However, lineage tracing approaches for the c-Kit marker using *Kit* allele Cre recombinase gene-targeted mice showed that these cells do not contribute significantly to new cardiomyocyte formation during development, with aging or after acute injury[13–16].

Sca-1$^+$ cells were originally reported to possess myogenic differentiation capacity upon isolation and reinjection into murine blastocysts or ischemic adult mouse hearts[12, 17, 18]. Subsequent studies have dissected the molecular signature of Sca-1$^+$ cells, which showed more cardiogenic progenitor-like features *in vitro*[19]. One previous study even suggested that several percent of new cardiomyocytes in the heart after injury are derived from endogenous Sca-1$^+$ cells[20]. Thus, there remains a pressing need to better understand the intrinsic biological function of Sca-1$^+$ cells in the heart to address whether these cells truly have myogenic capacity *in vivo*.

Methods

An expanded methods section is available in the online-only Data Supplement.

All materials, datasets, and protocols used in this study will be made available to investigators upon request. *Ly6a*$^{+/Cre}$ and *Ly6a*$^{+/MerCreMer}$ knock-in mice will be made available under a material transfer agreement (MTA) between Cincinnati Children’s Hospital

(CCHMC) and the recipient institution. Requests for resources and reagents should be directed to and will be fulfilled by the corresponding author.

Experimental Animals (Mice) and Animal Procedures

All procedures were performed according to institutional guidelines and governmental regulations (PHS Animal Welfare Assurance number D16–00068 [A3108–01]). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children's Hospital under protocol number IACUC2015–0047.

MI was induced in mice via permanent ligation of the left coronary artery as previously described[21]. Blinding was not performed because only one genotype was used in each experiment (either *Ly6a*$^{+/Cre}$ × *R26-TdT* or *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP*).

Immunohistochemistry and confocal microscopy

Tissues from *Ly6a*$^{+/Cre}$ × *R26-TdT* or *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice were isolated and processed for immunohistochemistry as described in Supplemental Methods.

Flow Cytometry

For flow cytometry analysis of dissociated cardiac non-myocytes from *Ly6a*$^{+/+}$ × *R26-TdT*, *Ly6a*$^{+/Cre}$ × *R26-TdT*, or *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice, single-cell suspensions were prepared via repeated rounds of enzymatic digestion and trituration using collagenase type IV (Worthington #LS004188) and dispase II (Roche #10165859001) according to "Protocol #2" from Pinto et. al.[22]. For flow cytometry analysis of whole bone marrow from *Ly6a*$^{+/+}$ × *R26-TdT*, *Ly6a*$^{+/Cre}$ × *R26-TdT*, Ly*6a*$^{+/+}$, or *Ly6a*$^{+/MerCreMer}$ mice, cells were isolated by cannulating dissected tibias and femurs with a 25-gauge needle and flushing with 5–10 mL of flow cytometry staining buffer as previously described[23].

Quantification and Statistical Analysis

For all experiments, the exact number (*n*) of mice used in each experiment is reported in the figure legends for each data panel. For experiments involving MI surgery, the number of animals that received surgery was determined based on prior experimentation in the lab, which demonstrated a peri-operative surgical mortality rate of 20%. Only animals that did not survive the surgical procedure were excluded from analysis. Randomization was not necessary because the mice were genetically identical within a given group. All data subjected to statistical analysis were determined to follow a Gaussian distribution via the Shapiro-Wilk normality test with α=0.05. Comparisons between exactly two groups were made using an unpaired two-tailed t-test. Comparisons between more than two groups were made using a one-way ANOVA with Tukey's multiple comparisons test. p-values for each comparison are shown in each respective figure and $p<0.05$ was considered statistically significant. All statistical tests were performed using GraphPad Prism 7.0d.

Results

Cardiac Sca-1$^+$ Cells are Predominantly Endothelial Cells in the Postnatal Heart

To assess the role that endogenous Sca-1$^+$ progenitor cells might play in the heart, here we generated gene-targeted mice with Cre recombinase inserted into the *Ly6a* gene locus. These mice were crossed with Cre-responsive *Rosa26-TdTomato* (*R26-TdT*) reporter mice to permanently label Sca-1$^+$ cells and their progeny throughout development and postnatal growth (Figure 1A). No TdTomato$^+$ cells were observed in the hearts or bone marrow of control *R26-TdT* mice lacking *Ly6a*$^{+/Cre}$, indicating the absence of reporter leak (Supplemental Figure 1). We first performed histological assessment of the hearts from *Ly6a*$^{+/Cre}$ × *R26-TdT* mice over a time course of postnatal growth. TdTomato$^+$ cells were observed as early as postnatal day 1 (p1) in the hearts of these mice, primarily located in distinct clusters scattered throughout. By 1.5 months of age, these TdTomato$^+$ cells expanded evenly throughout the heart in *Ly6a*$^{+/Cre}$ × *R26-TdT* mice, which was more notable by 3 months of age (Figure 1B). Further analysis of these hearts by immunohistochemistry revealed that the vast majority of TdTomato+ cells were positive for the vascular endothelial cell marker CD31, both at p1 (Figure 1C) and at 1.5 months (Figure 1D). To more quantitatively assess the identity of Sca-1 lineage-traced cells we also performed flow cytometry on dissociated heart preparations from *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at 3 months of age. We again observed robust labeling of the endothelium, as ~70% of Sca-1 antibody-positive cells that were CD31$^+$ also were TdTomato$^+$. In contrast, significantly fewer Sca-1$^+$ cells that were CD31$^-$ expressed TdTomato (~8–9%; Figure 1E–F). Additional flow cytometry assessment demonstrated that the majority of these Sca-1$^+$ CD31$^-$ cells (>90%) were resident PDGFRα$^+$ cardiac fibroblasts (Supplemental Figure 2A–B). Taken together these data suggest a cumulative contribution of Sca-1$^+$ cells to the cardiac vasculature during physiological growth in the mouse.

Sca-1$^+$ Cells Contribute Few Cardiomyocytes to the Heart Over Postnatal Growth

To assess the cardiomyogenic contribution of Sca-1$^+$ cells *in vivo* throughout development and postnatal growth, we performed immunohistochemistry studies in *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at 3 months of age using antibodies against sarcomeric α-actinin and PCM1 to label the cytoplasm and nuclei[24] of cardiomyocytes, respectively (Figure 2A–B). We observed rare single TdTomato$^+$ cardiomyocytes in the hearts of *Ly6a*$^{+/Cre}$ × *R26-TdT* mice (Figure 2C), which when quantified accounted for ~0.0035% of cardiomyocytes in these hearts (Figure 2D). Given previous observations from our lab and others demonstrating that leukocytes are a known source of false labeling in lineage tracing studies due to fusion with endogenous cardiomyocytes[13, 25], we also examined the bone marrow of *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at 3 months of age by flow cytometry. We observed that ~30% of total bone marrow cells were TdTomato$^+$ (Figure 2E), and these were mostly mature hematopoietic cells as indicated by surface staining for the pan-leukocyte marker CD45 (Figure 2F). This suggests an even further reduction in the potential for Sca-1$^+$ cells to form *de novo* cardiomyocytes as some of the TdTomato$^+$ cardiomyocytes we observed may be the result of fusion events from lineage-traced leukocytes. Taken together, our results indicate that the cardiomyocyte contribution from endogenous Sca-1$^+$ cells during development and physiological growth in mice is extremely rare and likely of no physiologic significance.

Generation of an Inducible Lineage Tracing Model for Adult Sca-1$^+$ Cells

To specifically examine the contribution of endogenous Sca-1$^+$ cells to the adult heart at baseline or after injury, we generated a second mouse line in which a cDNA encoding the tamoxifen-regulated MerCreMer (modified Cre-recombinase) fusion protein was targeted to the endogenous *Ly6a* locus. These mice were crossed with Cre-responsive *Rosa26-eGFP* (*R26-eGFP*) reporter mice to permanently label Sca-1$^+$ cells and their progeny coincident with tamoxifen administration (Figure 3A). Without tamoxifen, no eGFP$^+$ cells were observed in these mice, again confirming the absence of reporter leak (Figure 3B). *Ly6a* $^{+/MerCreMer}$ × *R26-eGFP* mice treated with tamoxifen throughout adulthood starting at 3 months of age showed endothelial cell labeling across multiple tissues examined (Figure 3C), although other cell types with known Sca-1 expression were also labeled, including liver hepatic cells[26], parenchymal cells of the kidney[27, 28] and splenocytes[27, 29, 30].

Ly6a Heterozygosity due to Targeted MerCreMer Insertion Does Not Impact Endogenous Sca-1 Function

To determine whether introduction of the MerCreMer cDNA at the endogenous *Ly6a* locus resulted in heterozygosity which could impact normal Sca-1 function, we performed additional analysis of the bone marrow compartment of *Ly6a*$^{+/MerCreMer}$ or *Ly6a*$^{+/+}$ mice at 9-months of age. Although the biological roles of Sca-1 remain poorly defined, the hematopoietic system is the best-characterized tissue in terms of Sca-1 function in mice, as animals lacking all Sca-1 protein show a pronounced reduction in basal production of megakaryocytes *in vivo* as well as deficiencies in hematopoietic stem cell and T-cell responsiveness *ex vivo*[31, 32]. However, we observed that bone marrow isolated from *Ly6a* $^{+/MerCreMer}$ mice had comparable amounts of megakaryocytes and monocytes versus *Ly6a*$^{+/+}$ controls, and only a modest decrease in granulocytes (Supplemental Figure 3A–B). More importantly, *Ly6a*$^{+/MerCreMer}$ mice showed no differences in total bone marrow Sca-1$^+$ cell content, or Sca-1 surface staining, compared to *Ly6a*$^{+/+}$ controls. Thus, the genetic systems used to lineage trace Sca-1$^+$ cells by introduction of Cre recombinase driven from the endogenous *Ly6a* locus do not alter Sca-1 surface expression or cell composition in the bone marrow, which is the major site of Sca-1 functionality in vivo.

Adult Sca-1$^+$ Endothelial Cells Expand During Aging and Post-MI Remodeling

In the heart, *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice again showed predominantly endothelial cell labeling by immunohistochemistry, thus we went on to further characterize the endothelial contribution of Sca-1$^+$ cells to the heart specifically after the completion of physiological growth or following myocardial infarction (MI) (Figure 4A). *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice treated with tamoxifen starting at 3 months of age showed predominant labeling of the cardiac endothelium as indicated by CD31 immunohistochemistry (Figure 4B), although examples of larger vessels lacking eGFP positivity were observed (Figure 4C), suggesting that Sca-1$^+$ cells mostly contribute to the microvascular capillary network of the heart. By flow cytometry, >80% of Sca-1 antibody-positive CD31$^+$ cells but significantly fewer (~10%) Sca-1 antibody-positive CD31$^-$ cells were labeled with eGFP (Figure 4D). We again observed very rare eGFP$^+$ cardiomyocytes in these mice, which by histological quantitation accounted for less than 0.002% of all myocytes analyzed (Figure 4E).

Finally, to examine the role of Sca-1$^+$ endothelial cells in the cardiac injury response, we subjected *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice to permanent occlusion MI injury following a 4-wk regimen of tamoxifen to pulse-label a subset of Sca-1$^+$ cells. Prior to MI, isolated eGFP$^+$ CD31$^+$ endothelial cells were observed throughout the heart with tamoxifen administration (Figure 4F). Post-MI, we observed a gradual increase in the proportion of CD31$^+$ endothelial cells labeled with eGFP (Figure 4G). By 8-wks post-MI, branching eGFP$^+$ vascular clusters were also observed (Figure 4H). Quantitation of total endothelial cell content revealed an increase in cardiac capillary density post-MI, consistent with compensatory vascular remodeling[33, 34] (Figure 4I). Moreover, the percentage of CD31$^+$ endothelial cells that were eGFP$^+$ significantly increased, roughly doubling during this time despite the prior withdrawal of tamoxifen. These results suggest an angiogenic expansion of previously pulse-labeled Sca-1$^+$ vessels during vascular remodeling of the post-MI heart. Of note, we were unable to detect any eGFP$^+$ cardiomyocytes using this tamoxifen pulse labeling protocol at any of the time points examined post-MI, indicating that MI did not increase the cardiomyogenic potential of Sca-1+ cells *in vivo*.

Discussion

In this study we generated 2 genetic lineage tracing mouse models to examine the contribution of endogenous Sca-1$^+$ cells to the heart under physiological and pathological conditions. We observed that cardiac Sca-1$^+$ cells contribute robustly to the vasculature but to less than 0.005% of the total cardiomyocyte pool in mice throughout development and aging. This percentage is likely even much lower given 80% fusion rates in the heart from labeled leukocytes using a similar lineage tracing approach[13]. After MI injury, this apparent rate of new cardiomyocyte generation from Sca-1$^+$ cells did not change and remained exceedingly low, however expansion and vascular branching of Sca-1$^+$ endothelial cells were observed, collectively indicating that the Sca-1$^+$ marker reflects endothelial cell identity in the murine heart *in vivo* and not a subset of endogenous stem cells.

One caveat of our current study is that only 8–10% of Sca-1$^+$ CD31$^-$ cells were recombined with either Cre line. Thus, our observed values of Sca-1$^+$ cell-derived myocytes may be underestimated as not all presumed “progenitor-like” Sca-1$^+$ cells would be labeled. However, not all non-myocytes and non-CD31 cells in the heart that express Sca-1 are progenitor in nature, such as the known expression of this marker on a select pool of fibroblasts (Supplemental Figure 2 and[22]). Moreover, there is a large family of closely related *Ly6* gene members in the mouse[35, 36] that might be obscuring the difference between lineage tracing from the *Ly6a* locus and antibody-based detection of cells by flow cytometry if the antibodies employed are not exquisitely specific for just Sca-1 (*Ly6a* gene) protein. Another consideration is that the apparent cardiomyogenic frequency of Sca-1$^+$ lineage-traced cells was 10-times lower than what we previously observed with c-Kit$^+$ cell-dependent lineage tracing[13]. Notwithstanding, the rates of Sca-1$^+$ or c-Kit$^+$ cells contributing to apparent new cardiomyocyte formation remain several orders of magnitude below accepted rates of new cardiomyocyte formation during aging or after injury, rates which are thought to arise primarily through cardiomyocyte cell cycle activity[6]. Finally, a very recent study that employed a dual recombination genetic labeling system with Cre and Dre recombinases in the mouse, which labels all non-myocytes independent of the *Kit* or

Ly6a allele, showed no contribution of nonmyocytes, progenitor cell or otherwise, in generating myocytes within the adult heart, further proving that the adult heart lacks a myocyte-producing stem cell of any sort[37]. However, we showed that Sca-1 cells in the heart do have the capacity to produce additional endothelial cells, suggesting a potential application for therapeutic revascularization strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Becher UM, Tiyerili V, Skowasch D, Nickenig G and Werner N. Personalized cardiac regeneration by stem cells-Hype or hope? EPMA J. 2011;2:119–30. [PubMed: 23199133]
2. Nguyen PK, Rhee JW and Wu JC. Adult Stem Cell Therapy and Heart Failure, 2000 to 2016: A Systematic Review. JAMA Cardiol. 2016;1:831–841. [PubMed: 27557438]
3. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP and Lee RT. Mammalian heart renewal by pre-existing cardiomyocytes. Nature. 2013;493:433–6. [PubMed: 23222518]
4. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S and Frisen J. Evidence for cardiomyocyte renewal in humans. Science. 2009;324:98–102. [PubMed: 19342590]
5. Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C, Malm T, Andra M, Jashari R, Nyengaard JR, Possnert G, Jovinge S, Druid H and Frisen J. Dynamics of Cell Generation and Turnover in the Human Heart. Cell. 2015;161:1566–75. [PubMed: 26073943]
6. Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, Frisen J, Giacca M, Hare JM, Houser S, Lee RT, Marban E, Martin JF, Molkentin JD, Murry CE, Riley PR, Ruiz-Lozano P, Sadek HA, Sussman MA and Hill JA. Cardiomyocyte Regeneration: A Consensus Statement. Circulation. 2017;136:680–686. [PubMed: 28684531]
7. Cai CL and Molkentin JD. The Elusive Progenitor Cell in Cardiac Regeneration: Slip Slidin' Away. Circ Res. 2017;120:400–406. [PubMed: 28104772]
8. Leri A, Kajstura J and Anversa P. Role of cardiac stem cells in cardiac pathophysiology: a paradigm shift in human myocardial biology. Circ Res. 2011;109:941–61. [PubMed: 21960726]
9. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B and Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003;114:763–76. [PubMed: 14505575]
10. Ellison GM, Vicinanza C, Smith AJ, Aquila I, Leone A, Waring CD, Henning BJ, Stirparo GG, Papait R, Scarfo M, Agosti V, Viglietto G, Condorelli G, Indolfi C, Ottolenghi S, Torella D and Nadal-Ginard B. Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. Cell. 2013;154:827–42. [PubMed: 23953114]
11. Liang SX, Tan TY, Gaudry L and Chong B. Differentiation and migration of Sca1+/CD31- cardiac side population cells in a murine myocardial ischemic model. Int J Cardiol. 2010;138:40–9. [PubMed: 19254813]

12. Oh H, Chi X, Bradfute SB, Mishina Y, Pocius J, Michael LH, Behringer RR, Schwartz RJ, Entman ML and Schneider MD. Cardiac muscle plasticity in adult and embryo by heart-derived progenitor cells. Ann N Y Acad Sci. 2004;1015:182–9. [PubMed: 15201159]

13. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marban E and Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. Nature. 2014;509:337–41. [PubMed: 24805242]

14. Hatzistergos KE, Takeuchi LM, Saur D, Seidler B, Dymecki SM, Mai JJ, White IA, Balkan W, Kanashiro-Takeuchi RM, Schally AV and Hare JM. cKit+ cardiac progenitors of neural crest origin. Proc Natl Acad Sci U S A. 2015;112:13051–6. [PubMed: 26438843]

15. Sultana N, Zhang L, Yan J, Chen J, Cai W, Razzaque S, Jeong D, Sheng W, Bu L, Xu M, Huang GY, Hajjar RJ, Zhou B, Moon A and Cai CL. Resident c-kit(+) cells in the heart are not cardiac stem cells. Nat Commun. 2015;6:8701. [PubMed: 26515110]

16. Liu Q, Yang R, Huang X, Zhang H, He L, Zhang L, Tian X, Nie Y, Hu S, Yan Y, Zhang L, Qiao Z, Wang QD, Lui KO and Zhou B. Genetic lineage tracing identifies in situ Kit-expressing cardiomyocytes. Cell Res. 2016;26:119–30. [PubMed: 26634606]

17. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML and Schneider MD. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A. 2003;100:12313–8. [PubMed: 14530411]

18. Wang X, Hu Q, Nakamura Y, Lee J, Zhang G, From AH and Zhang J. The role of the sca-1+/CD31- cardiac progenitor cell population in postinfarction left ventricular remodeling. Stem cells. 2006;24:1779–88. [PubMed: 16614004]

19. Noseda M, Harada M, McSweeney S, Leja T, Belian E, Stuckey DJ, Abreu Paiva MS, Habib J, Macaulay I, de Smith AJ, al-Beidh F, Sampson R, Lumbers RT, Rao P, Harding SE, Blakemore AI, Jacobsen SE, Barahona M and Schneider MD. PDGFRalpha demarcates the cardiogenic clonogenic Sca1+ stem/progenitor cell in adult murine myocardium. Nat Commun. 2015;6:6930. [PubMed: 25980517]

20. Uchida S, De Gaspari P, Kostin S, Jenniches K, Kilic A, Izumiya Y, Shiojima I, Grosse Kreymborg K, Renz H, Walsh K and Braun T. Sca1-derived cells are a source of myocardial renewal in the murine adult heart. Stem Cell Reports. 2013;1:397–410. [PubMed: 24286028]

21. Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF and Molkentin JD. Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. J Biol Chem. 2004;279:15524–30. [PubMed: 14749328]

22. Pinto AR, Ilinykh A, Ivey MJ, Kuwabara JT, D'Antoni ML, Debuque R, Chandran A, Wang L, Arora K, Rosenthal NA and Tallquist MD. Revisiting Cardiac Cellular Composition. Circ Res. 2016;118:400–9. [PubMed: 26635390]

23. Schwanekamp JA, Lorts A, Vagnozzi RJ, Vanhoutte D and Molkentin JD. Deletion of Periostin Protects Against Atherosclerosis in Mice by Altering Inflammation and Extracellular Matrix Remodeling. Arterioscler Thromb Vasc Biol. 2016;36:60–8. [PubMed: 26564821]

24. Bergmann O, Zdunek S, Alkass K, Druid H, Bernard S and Frisen J. Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. Exp Cell Res. 2011;317:188–94. [PubMed: 20828558]

25. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ and Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature. 2003;425:968–73. [PubMed: 14555960]

26. Petersen BE, Grossbard B, Hatch H, Pi L, Deng J and Scott EW. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology. 2003;37:632–40. [PubMed: 12601361]

27. van de Rijn M, Heimfeld S, Spangrude GJ and Weissman IL. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc Natl Acad Sci U S A. 1989;86:4634–8. [PubMed: 2660142]

28. Dekel B, Zangi L, Shezen E, Reich-Zeliger S, Eventov-Friedman S, Katchman H, Jacob-Hirsch J, Amariglio N, Rechavi G, Margalit R and Reisner Y. Isolation and characterization of nontubular

sca-1+lin- multipotent stem/progenitor cells from adult mouse kidney. J Am Soc Nephrol. 2006;17:3300–14. [PubMed: 17093069]

29. Trevisan M and Iscove NN. Phenotypic analysis of murine long-term hemopoietic reconstituting cells quantitated competitively in vivo and comparison with more advanced colony-forming progeny. J Exp Med. 1995;181:93–103. [PubMed: 7807027]
30. Holmes C and Stanford WL. Concise review: stem cell antigen-1: expression, function, and enigma. Stem cells. 2007;25:1339–47. [PubMed: 17379763]
31. Stanford WL, Haque S, Alexander R, Liu X, Latour AM, Snodgrass HR, Koller BH and Flood PM. Altered proliferative response by T lymphocytes of Ly-6A (Sca-1) null mice. J Exp Med. 1997;186:705–17. [PubMed: 9271586]
32. Ito CY, Li CY, Bernstein A, Dick JE and Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood. 2003;101:517–23. [PubMed: 12393491]
33. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW and Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med. 2000;342:626–33. [PubMed: 10699162]
34. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS and Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. J Clin Invest. 2005;115:2108–18. [PubMed: 16075055]
35. Lee PY, Wang JX, Parisini E, Dascher CC and Nigrovic PA. Ly6 family proteins in neutrophil biology. J Leukoc Biol. 2013;94:585–94. [PubMed: 23543767]
36. Loughner CL, Bruford EA, McAndrews MS, Delp EE, Swamynathan S and Swamynathan SK. Organization, evolution and functions of the human and mouse Ly6/uPAR family genes. Hum Genomics. 2016;10:10. [PubMed: 27098205]
37. Li Y, He L, Huang X, Bhaloo SI, Zhao H, Zhang S, Pu W, Tian X, Li Y, Liu Q, Yu W, Zhang L, Liu X, Liu K, Tang J, Zhang H, Cai D, Ralf AH, Xu Q, Lui KO, Zhou B. Genetic Lineage Tracing of Non-myocyte Population by Dual Recombinases. Circulation. 2018; Apr 26. pii: CIRCULATIONAHA.118.034250. doi: 10.1161/CIRCULATIONAHA.118.034250. [Epub ahead of print

Clinical Perspective

What is new?

- This is the first study to perform genetic lineage tracing of endogenous Sca-1$^+$ (gene name *Ly6a*) cells throughout postnatal cardiac growth or after cardiac injury in mice.
- Cardiac Sca-1$^+$ cells predominantly contribute to the cardiac vasculature, particularly in the endothelial cell expansion and remodeling that occurs early after birth or following myocardial infarction (MI).
- Cardiac Sca-1$^+$ lineage traced cells can contribute to the cardiomyocyte pool of the adult mouse heart but at a level that is extremely low and physiologically insignificant (1 in 20,000), and likely due to fusion of cardiomyocytes with Sca-1-lineage traced leukocytes.

What are the clinical implications?

- Although there is no known human homologue of Sca-1 (*Ly6a*), clinical application of patient-derived cells for heart regeneration has been proposed based partly on characterization of comparable populations of Sca-1$^+$ cells in mouse models.
- Our data suggest that Sca-1 is not a marker of cells with enhanced stem or progenitor-like activity in the heart.
- While not cardiomyogenic, Sca-1 cells in the heart appear to represent a subset of cardiac endothelial cells that are more responsive to injury, which may impact human treatment if comparable cells can be isolated.

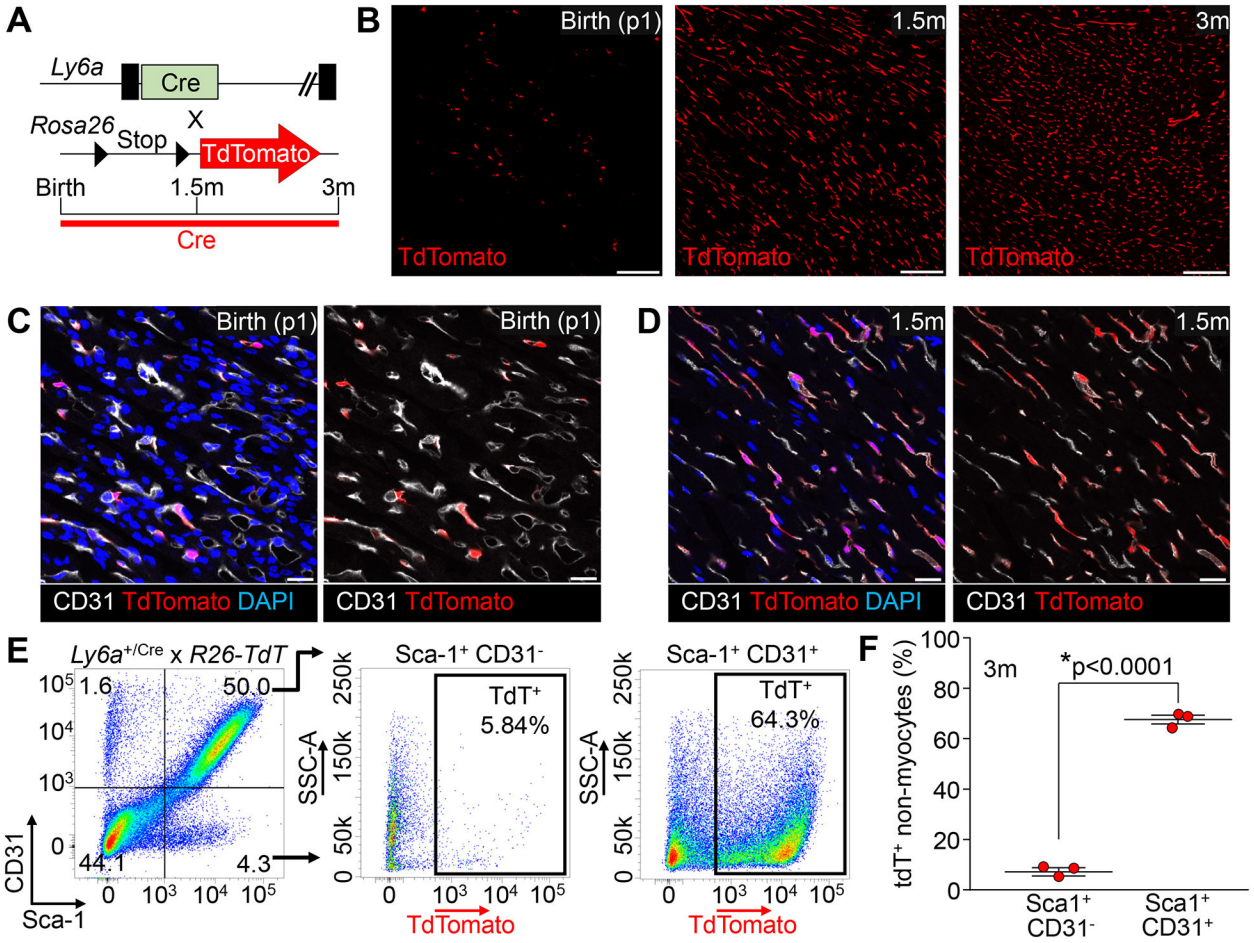

Figure 1: Cardiac Sca-1$^+$ Cells Contribute to the Vasculature Throughout Postnatal Growth.

A. Experimental scheme and timeline for genetic lineage tracing studies in this figure using constitutive Sca-1 (gene name *Ly6a)* Cre gene-targeted mice. **B.** Representative confocal micrographs of histological sections from hearts of *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at either postnatal day 1, 1.5 months of age, or 3 months of age, showing continual expansion of TdTomato$^+$ cells (red) in the heart. Scale bars = 100 μm. **C, D.** Immunohistochemistry on cardiac histological sections was performed to detect CD31 (white) along with endogenous TdTomato fluorescence (red). DAPI (blue) was used to visualize nuclei. TdTomato$^+$ endothelial cells were seen in small clusters at postnatal day 1 (**C**) and throughout the heart at 1.5 months of age (**D**). Scale bars = 10 μm. **E, F.** Representative flow cytometry plots (**E**) and quantitation (**F**) from dissociated *Ly6a*$^{+/Cre}$ × *R26-TdT* mouse hearts at 3 months of age (n=3) analyzed using antibodies against Sca-1 and CD31. The first plot shows Sca-1 positivity by fluorochrome-conjugated antibody staining (Sca-1), versus CD31 positivity also by antibody (CD31). The second plot shows TdTomato$^+$ cells (endogenous TdTomato fluorescence) versus side scatter within the Sca-1$^+$ CD31$^-$ gate (lower right quadrant) shown in the first plot as indicated. The final plot shows TdTomato$^+$ cells (endogenous TdTomato fluorescence) versus side scatter within the Sca-1$^+$ CD31$^+$ gate (upper right quadrant) shown in the first plot as indicated.

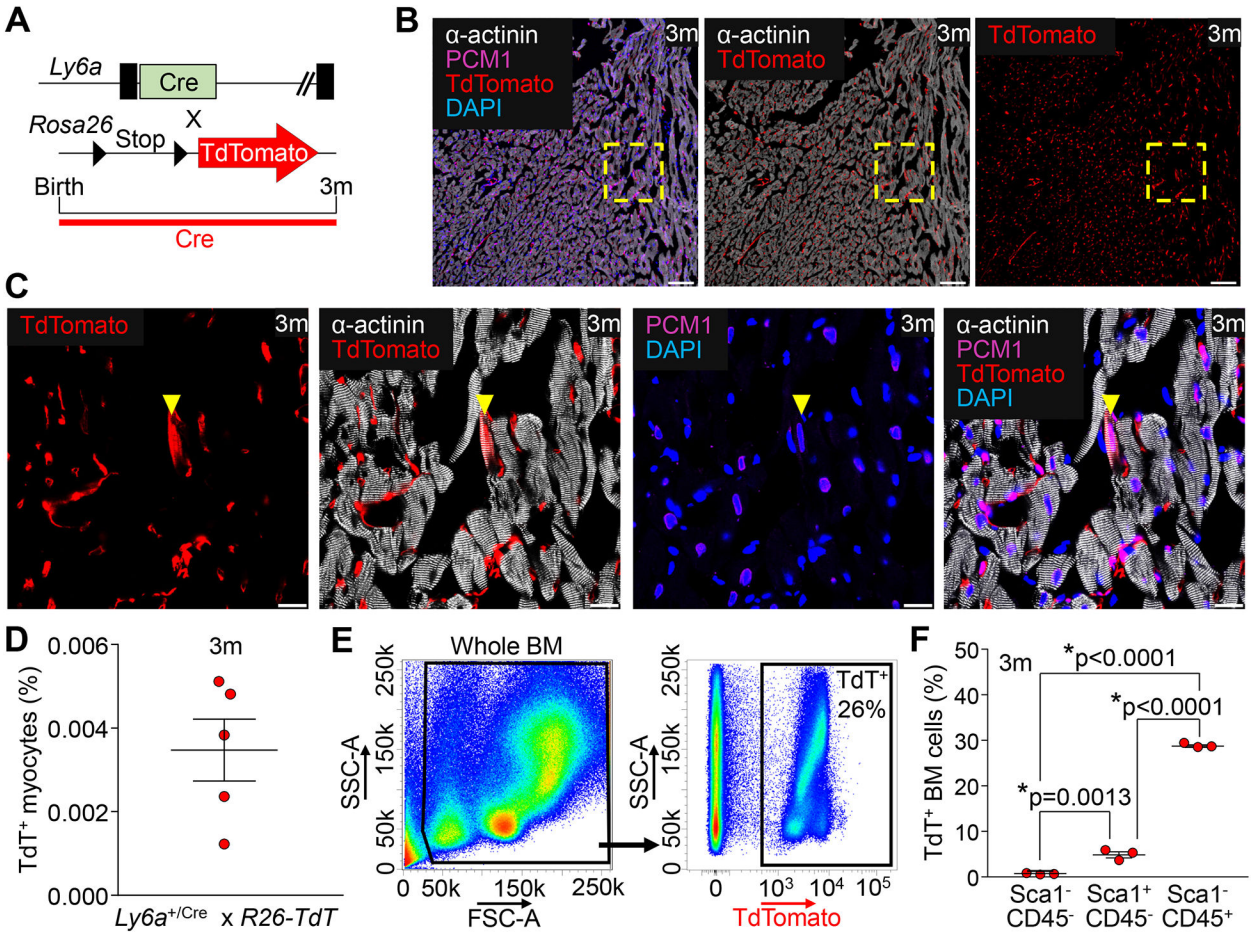

Figure 2: Cardiac Sca-1^{+} Cells Contribute Few Cardiomyocytes Throughout Postnatal Growth.

A. Experimental scheme and timeline for genetic lineage tracing studies in this figure using *Ly6a*-Cre gene-targeted mice. **B.** Immunohistochemistry was performed on cardiac histological sections from *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at 3 months of age using antibodies against sarcomeric α-actinin (white) and PCM1 (purple). DAPI (blue) was used to visualize nuclei. Representative confocal micrograph shows a rare TdTomato^{+} cardiomyocyte (yellow box). Scale bar: 100 μm. **C.** High-magnification confocal micrographs of the yellow boxed area denoted in **B**. Yellow arrowhead indicates a TdTomato^{+} cardiomyocyte (α-actinin^{+} PCM1^{+}). Scale bars = 10 μm. **D.** Quantitation of TdTomato^{+} cardiomyocytes from histological sections of hearts from *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at 3 months of age. Quantitation is shown from hearts of n=5 mice over 144 histological sections and 303260 total cardiomyocytes counted. **E, F.** Representative flow cytometry plots (**E**) and quantitation (**F**) from the bone marrow of *Ly6a*$^{+/Cre}$ × *R26-TdT* mice at 3 months of age (n=3). The first plot shows forward (FSC-A) versus side (SSC-A) scatter to determine size distribution of the bone marrow. The second plot shows TdTomato^{+} cells (endogenous TdTomato fluorescence) versus side scatter from the gate shown in the first plot as indicated (**E**). Cells within this second gate were stained with antibodies against Sca-1 and CD45, which showed primarily mature CD45^{+} leukocytes that were TdTomato^{+} (**F**).

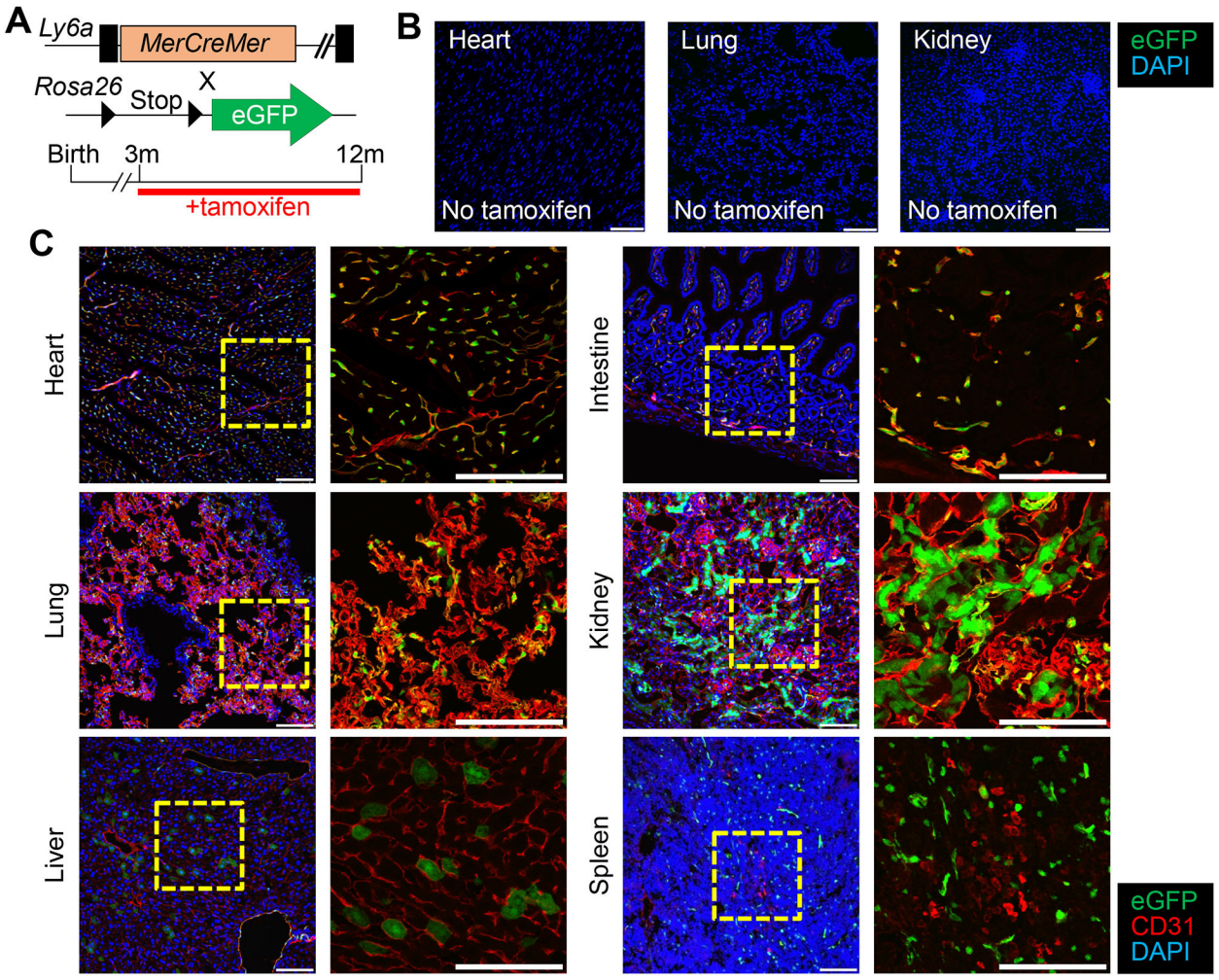

Figure 3: Inducible Genetic Lineage Tracing in the Adult Mouse Tracks Sca-1$^+$ Cells During Aging.

A. Experimental scheme for genetic lineage tracing studies in this figure using a tamoxifen-inducible *Ly6a*-MerCreMer gene-targeted mouse line and the R26-eGFP reporter line. **B.** eGFP reporter expression is dependent on tamoxifen induction, as untreated *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice aged for one year do not show eGFP$^+$ cells across multiple tissues surveyed. **C.** *Ly6a*$^{+/MerCreMer}$ × *R26-eGFP* mice treated with tamoxifen starting at adulthood (3 mos) out to 1 yr of age showed endothelial cell labeling (CD31, red) of eGFP$^+$ cells (green) throughout the heart, lung, liver, intestine, and kidney. Yellow boxes indicate areas shown to the right of each image in an enlarged view. Other cell types previously reported to express Sca-1 are also labeled with eGFP. DAPI (blue) was used to visualize nuclei. Scale bars = 100 μm.

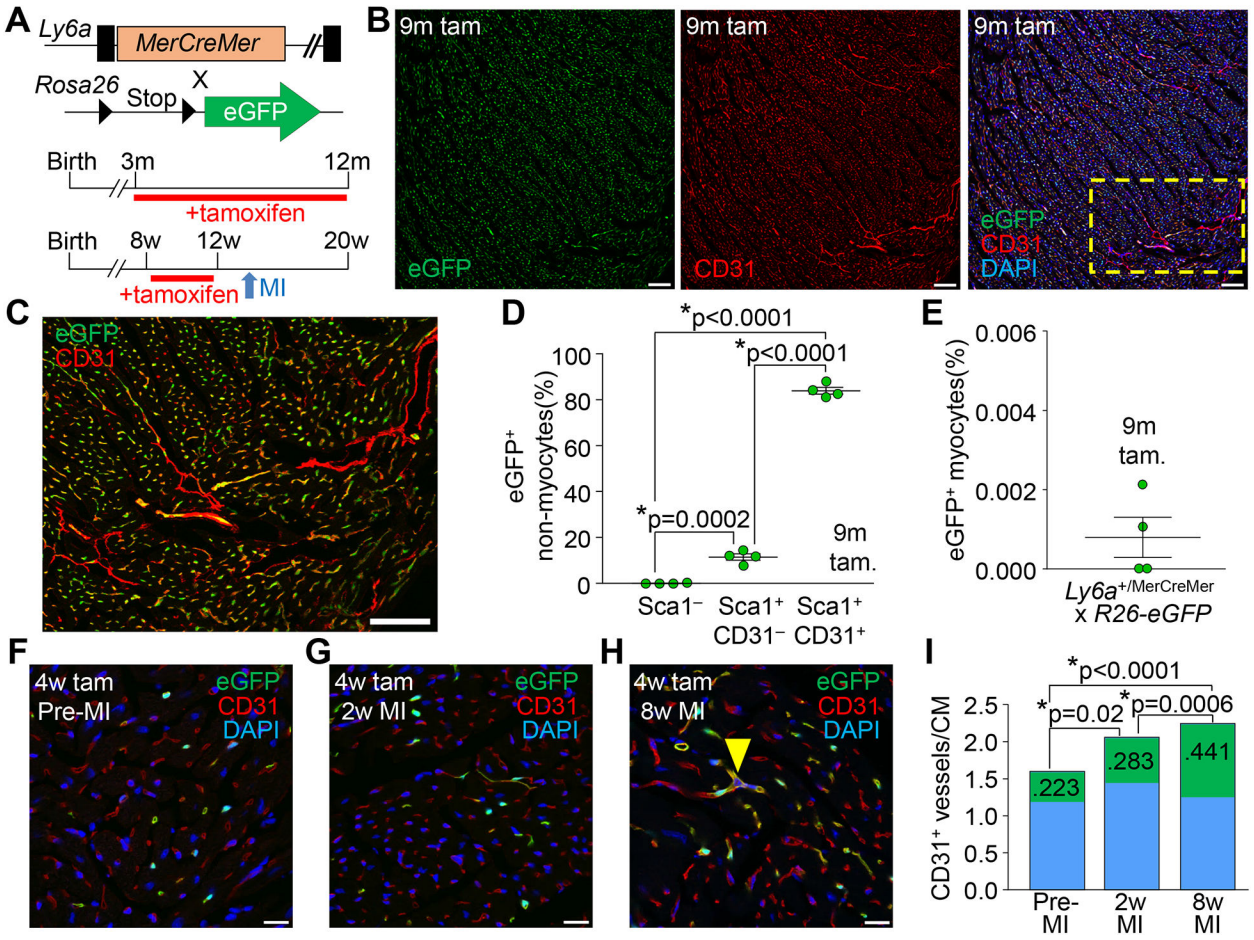

Figure 4: Sca-1^{+} Cells Contribute to the Vasculature in the Murine Heart During Adulthood and After Injury.

A. Experimental scheme for genetic lineage tracing studies in this figure using *Ly6a*-MerCreMer mice along with the R26-eGFP reporter line during aging (Timeline #1) or post-MI (Timeline #2). **B, C.** Representative confocal micrographs (**B**) showing co-localization of CD31 (red) and eGFP (green) in cardiac histological sections in *Ly6a*$^{+/\text{MerCreMer}}$ × *R26-eGFP* mice continually treated with tamoxifen from 3 months to 1 year of age (9 m of labeling). Scale bars = 100 μm. Yellow box denotes area shown in enlarged view in (**C**), demonstrating eGFP^{+} capillaries and small vessels adjacent to eGFP^{-} large vessels. Scale bar = 100 μm. **D.** Quantitation of eGFP^{+} cells from dissociated *Ly6a*$^{+/\text{MerCreMer}}$ × *R26-eGFP* hearts (n=4) by flow cytometry using antibodies against Sca-1 and CD31. **E.** Quantitation of eGFP^{+} cardiomyocytes from hearts of *Ly6a*$^{+/\text{MerCreMer}}$ *x R26-eGFP* mice over 9 m of tamoxifen labeling in adulthood. Quantitation was from n=4 mice and 374832 total cardiomyocytes counted from histological sections. **F-I.** *Ly6a*$^{+/\text{MerCreMer}}$ × *R26-eGFP* mice were given tamoxifen for 4 weeks followed by MI injury. Representative high-magnification confocal micrographs of heart histological sections labeled with CD31 (red) and DAPI (blue) are shown prior to MI (**F**) or at 2 wks post-MI (**G**) and at 8 wks post-MI (**H**). Yellow arrowhead in **H** indicates a vascular branching event by previously pulse-labeled eGFP^{+} endothelial cells. Scale bars = 10 μm. **I.** Quantitation of overall cardiac capillary density and

the percentage of $CD31^+$ endothelial cells labeled with eGFP in *Ly6a*$^{+/MerCreMer}$ *x R26-eGFP* mice post-MI. Green bars represent the fraction of $CD31^+$ endothelial cells that were $eGFP^+$, relative to total endothelial cells (green plus blue bars). n=4–5 mice per time point.