Revisiting Cardiac Cellular Composition


Rationale: Accurate knowledge of the cellular composition of the heart is essential to fully understand the changes that occur during pathogenesis and to devise strategies for tissue engineering and regeneration.

Objective: To examine the relative frequency of cardiac endothelial cells, hematopoietic-derived cells, and fibroblasts in the mouse and human heart.

Methods and Results: Using a combination of genetic tools and cellular markers, we examined the occurrence of the most prominent cell types in the adult mouse heart. Immunohistochemistry revealed that endothelial cells constitute >60%, hematopoietic-derived cells 5% to 10%, and fibroblasts <20% of the nonmyocytes in the heart. A refined cell isolation protocol and an improved flow cytometry approach provided an independent means of determining the relative abundance of nonmyocytes. High-dimensional analysis and unsupervised clustering of cell populations confirmed that endothelial cells are the most abundant cell population. Interestingly, fibroblast numbers are smaller than previously estimated, and 2 commonly assigned fibroblast markers, Sca-1 and CD90, under-represent fibroblast numbers. We also describe an alternative fibroblast surface marker that more accurately identifies the resident cardiac fibroblast population.

Conclusions: This new perspective on the abundance of different cell types in the heart demonstrates that fibroblasts comprise a relatively minor population. By contrast, endothelial cells constitute the majority of noncardiomyocytes and are likely to play a greater role in physiological function and response to injury than previously appreciated. (Circ Res. 2016;118:400-409. DOI: 10.1161/CIRCRESAHA.115.307778.)

Key Words: endothelial cells ■ fibroblasts ■ flow cytometry ■ heart ■ leukocytes

Although cardiomyocytes account for ≈25% to 35% of all cells in the heart, there is a lack of consensus on the composition of the cardiac nonmyocyte cell population. In mammals, cardiomyocytes reside in close proximity to capillaries, and the calculated ratio of endothelial cells (ECs) to cardiomyocytes is 3:1.3,4 This estimated number of ECs contradicts studies that have previously characterized heart cellular composition, where findings suggested that fibroblasts constitute the principal nonmyocyte cell type.1,2,5,6 Often these analyses relied on the expression of singular proteins for identifying specific cell types; this approach may have resulted in exclusion of or loss of distinct cell populations. Because of the incongruence between these data sets and recent advances in our knowledge of heart resident immune cells, we revisited the issue of cardiac cellular composition. Using newly available genetic tracers and enhanced flow cytometry techniques to analyze the relative abundance of different cell types in the human and mouse heart, we document that fibroblasts comprise a relatively minor cell population and that ECs are the most abundant cell type.

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Methods

Cardiac Single-Cell Preparation

Mouse hearts were isolated for single-cell preparation as previously described with atria and valves removed. Isolated mouse hearts were digested using 1 of 3 protocols (designated protocol 1, 2, and 3). For protocol 1, each mouse heart was divided into ≈20 pieces, placed in 10 mL of protocol 1 digestion buffer (2-mg/mL collagenase type II in 1× HBSS [Worthington Biochemical Corporation]) in gentleMacs C-tubes (Miltenyi Biotec), dissociated using Heart 1 program of a gentleMacs Dissociator, and incubated at 37°C for 40 minutes with gentle agitation. After incubation, tissue was further dissociated using Heart 2 program before placing on ice. For protocol 2, isolated...
hearts were finely minced using forceps to ≈2-mm pieces and placed in 3 mL of protocol 2 digestion buffer (2-mg/mL collagenase type IV [Worthington Biochemical Corporation] and 1.2 U/mL dispase II [Sigma-Aldrich or Thermofisher Scientific] in Dulbecco’s phosphate-buffered saline supplemented with 0.9 mmol/L CaCl₂). Tissue was incubated at 37°C for 15 minutes with gentle rocking. After incubation, tissue digestion buffer with tissue clusters was triturated by pipetting 12 times using a 10-mL serological pipette. Dishes were again incubated at 37°C and triturated twice more (45 minutes of total digestion time). The final trituration was conducted by pipetting 30X with a p1000 pipette. For protocol 3, isolated mouse hearts were finely minced using forceps, placed in 10-mL protocol 3 digestion buffer (1.5 U/mL collagenase type X1 [Sigma-Aldrich], 60-U/mL hyaluronidase type I-S [Sigma-Aldrich], and 60-U/mL DNase 1 [Sigma-Aldrich] in Dulbecco’s phosphate-buffered saline supplemented with 0.9-mmol/mL CaCl₂ and 20-mmol/L HEPES) incubated at 37°C for 1 hour with gentle agitation, triturated 20X using a 10-mL serological pipette, and placed on ice. All cell suspensions were filtered using a 40-μm cell strainer. Filtered suspensions were placed into 50-mL tubes with 40 mL of Dulbecco’s phosphate-buffered saline and centrifuged at 200g for 20 minutes with centrifuge brakes deactivated to remove small tissue debris. Cell pellets were resuspended in 250-μL 2% FBS/HBSS solution before staining with various antibodies and reagents for flow cytometry or fluorescence activated cell sorting.

Antibody staining for specific antigens (Online Table I) was conducted in 100 μL of single-cell suspension (in 2% FBS/HBSS) using protocols 1, 2, or 3 after FC receptor blocking with CD16/CD32 antibody. After 1-hour antibody incubation at 4°C, calcein (calcein-AM or calcein-violet; Life Technologies) and Vybrant® DyeCycle™ Orange (VDO; Life Technologies) were added to antibody/cell suspensions at final concentrations of 5 and 2.5 μmol/L, respectively. Samples with dyes added were incubated in a 37°C water bath for 10 minutes, before placing samples on ice. Samples were washed with 2% FBS/HBSS and resuspended in 2% FBS/HBSS with or without the viability dyes 4,6-diamidino-2-phenylindole (DAPI) or 7-aminomycin D (7-AAD). All flow cytometry was conducted on LSR II Fortessa Flow Cytometers (BD Biosciences) or LSR II Flow Cytometers (BD Biosciences). For compensation of fluorescence spectral overlap, UltraComp eBeads (eBioscience, Inc.) were used following the manufacturer’s protocols. FACS 3.0 files generated by flow cytometry were initially processed using FlowJo Software (Tree Star, Ashland) for automated compensation. Dye-positive or dye-negative cell populations were gated and exported as new FCS 3.0 files and uploaded to the Online Data Supplement.

**Antibody, Nuclear, and Metabolically Active Cell Staining for Flow Cytometry**

Antibody staining for specific antigens (Online Table I) was conducted in 100 μL of single-cell suspension (in 2% FBS/HBSS) using protocols 1, 2, or 3 after FC receptor blocking with CD16/CD32 antibody. After 1-hour antibody incubation at 4°C, calcein (calcein-AM or calcein-violet; Life Technologies) and Vybrant® DyeCycle™ Orange (VDO; Life Technologies) were added to antibody/cell suspensions at final concentrations of 5 and 2.5 μmol/L, respectively. Samples with dyes added were incubated in a 37°C water bath for 10 minutes, before placing samples on ice. Samples were washed with 2% FBS/HBSS and resuspended in 2% FBS/HBSS with or without the viability dyes 4,6-diamidino-2-phenylindole (DAPI) or 7-aminomycin D (7-AAD). All flow cytometry was conducted on LSR II Fortessa Flow Cytometers (BD Biosciences) or LSR II Flow Cytometers (BD Biosciences). For compensation of fluorescence spectral overlap, UltraComp eBeads (eBioscience, Inc.) were used following the manufacturer’s protocols. FACS 3.0 files generated by flow cytometry were initially processed using FlowJo Software (Tree Star, Ashland) for automated compensation. Dye-positive or dye-negative cell populations were gated and exported as new FCS 3.0 files and uploaded to the Online Data Supplement.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
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<tr>
<td>RMC</td>
<td>resident mesenchymal cells</td>
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<tr>
<td>SPADE</td>
<td>spanning-tree progression analysis of density-normalized events</td>
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<tr>
<td>VDO</td>
<td>Vybrant® DyeCycle™ Orange</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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**Statistical Analyses**

All statistical analyses were conducted using Prism 6 for Windows software (GraphPad Software, Inc., La Jolla, CA). Statistical variability expressed as means±SD. Additional information is available in the Online Data Supplement.

**Results**

**Immunohistochemical Analysis of Cardiac Cellular Composition**

To determine the proportions of the major types of nonmyocyte cells in the adult murine heart, we conducted histological analysis of cardiac tissue from adult Col1a1-GFP/+ mice, which express green fluorescent protein (GFP) in monocytes, macrophages, and dendritic cells including subsets of cardiac tissue macrophages. In addition to GFP, we stained tissue sections with a hematopoietic lineage antibody cocktail (Lin1) targeting leukocyte antigens: CD45, Mrc1, B220, and major histocompatibility complex class II, to distinguish a broad cross section of cardiac leukocytes (Online Figure IA). Cardiomyocytes and ECs were identified by wheat germ agglutinin (WGA) and isolectin B4 (IB4), respectively. Using this combination of reagents, we were able to examine regional differences in cardiac cellular composition (cardiomyocytes, ECs, and leukocytes) in both ventricles and the interventricular septum by immunohistochemistry (Figure 1A and IB; Online Figures IA and IIA–IIC). We found that 31.0±4.2% of nuclei were cardiomyocytes, 43.6±4.1% ECs, and 4.7±1.5% leukocytes. These proportions remained similar in all regions of the heart (Online Figure IIB). Unmarked cells accounted for 20.7±4.5% of nuclei. When considering only nonmyocytes (Online Figure IIC), our analysis indicates that 63.3±5.4% are ECs, 6.8±2.1% are leukocytes, and 29.9±5.9% were unmarked.

As many studies have suggested that fibroblasts constitute the majority of nonmyocytes in the heart, similar analyses were performed in cardiac tissues from mice containing either a nuclear localized H2B-eGFP expressed from the platelet-derived growth factor receptor alpha (PDGFRα) locus, PDGFRαGFP, (Figure 1C; Online Figure IB) or GFP driven by a Collα1 promotor (3.2 kb) and HS 4,5 enhancer (1.7 kb), Collα1-GFP. These 2 mouse lines drive expression of GFP in both epicardial and endocardial lineages of fibroblasts. Using these 2 strains and focusing on the left ventricle, we independently stained for cardiomyocytes (ACTN2), ECs (isolectin B4 and dachshund homolog 1 [DACH1]), hematopoietic lineage cocktail (Lin2), which contains anti-CD5, anti-CD11b, anti-B220, anti-7-4, anti-Gr-1 (Ly6G/C), and anti–Ter-119 antibodies, and vascular smooth muscle cells (VSMCs/pericytes, (anti–platelet-derived growth factor receptor beta [PDGFRβ]), (Figure 1C; Online Figure IB) and data not shown). 32.6±4.8% of nuclei corresponded to cardiomyocytes, 55.0±6.0% to ECs, 5.7±1.1% to VSMCs/pericytes, 8.5±1.5% to leukocytes, and 12.6±2.5%, and 12.7±3.6% to fibroblasts. 63.3±5.4% are ECs, 6.8±2.1% are leukocytes, and 29.9±5.9% were unmarked. Using these 2 strains and focusing on the left ventricle, we independently stained for cardiomyocytes (ACTN2), ECs (isolectin B4 and dachshund homolog 1 [DACH1]), hematopoietic lineage cocktail (Lin2), which contains anti-CD5, anti-CD11b, anti-B220, anti-7-4, anti-Gr-1 (Ly6G/C), and anti–Ter-119 antibodies, and vascular smooth muscle cells (VSMCs/pericytes, (anti–platelet-derived growth factor receptor beta [PDGFRβ]), (Figure 1C; Online Figure IB) and data not shown). 32.6±4.8% of nuclei corresponded to cardiomyocytes, 55.0±6.0% to ECs, 5.7±1.1% to VSMCs/pericytes, 8.5±1.5% to leukocytes, and 12.6±2.5%, and 12.7±3.6% to fibroblasts. 63.3±5.4% are ECs, 6.8±2.1% are leukocytes, and 29.9±5.9% were unmarked.
illustrates the frequency of ECs and fibroblasts relative to other nuclei. DACH1+ ECs constituted 51.8±3.9% of the nuclei. The sum of these data may be >100% because of inaccuracies in attributing nuclei to cells stained for plasma membrane components. Nonetheless, these independent data sets demonstrate that ECs are the major cell type in the adult murine ventricle. Surprisingly, these results also show that fibroblasts, marked by 2 unrelated mouse GFP reporter lines, contributed to <20% of total nonmyocyte nuclei.

To determine whether human tissue mirrors these findings, we analyzed healthy, adult human heart samples, using antibodies that recognize cardiomyocytes, ECs, and leukocytes (Figure 1E; Online Figures IC and IID). Unfortunately, none of the antibodies surveyed consistently delineated human RMC. Examination of human tissues demonstrated that 31.2±5.6% of nuclei correspond to cardiomyocytes (ACTN2), 53.8±6.4% to ECs (CD31), and 2.8±1.2% to leukocytes (CD45). DACH1 also identified human ECs, and 51.1±2.9% of the nuclei in the human heart were DACH1+ (Online Figure IID). Thus, estimations of endothelial abundance in the human heart reflect those observed in the murine heart.

Flow Cytometry Analysis of Nucleated Metabolically Active Cells

We next performed flow cytometry analysis of single-cell preparations as an alternative approach for evaluating cardiac cellular composition. When using conventional methods, many technical issues limit the accurate assessment of relative cell proportions by flow cytometry. These issues include the ability to clearly discriminate viable, nucleated cells from tissue debris and cell clusters. Normally, this issue is mitigated by using light-scattering properties of cells such as forward- and side-scatter. However, such approaches can skew data on the relative proportions of cell types. Therefore, for analysis of relative proportions of broad cardiac nonmyocyte cell types (ECs, leukocytes, and RMC), we developed a reliable approach for resolving nucleated cells from tissue debris using Vybrant DyeCycle Orange (VDO), calcein-AM (metabolically active cells) and DAPI (cells with compromised membranes; Online Figure IIIA). To determine the correct gating position for nucleated VDO+ cells within the DAPI+, calcein+ gated population (Online Figure IIIIB), we isolated and analyzed cells from 4 regions (R1–4) with differential VDO labeling (Online Figure IIIIB–IIIID) by fluorescence activated cell sorting. Microscopy showed that R1 and R4 contained predominantly small cell fragments or clusters, whereas R2 and R3 contained mainly nucleated single cells. Analysis of forward scatter-height and forward scatter-area parameters confirmed that R1 and R4 comprise small and large elements, respectively; R2 and R3 were cells with a narrower size distribution (Online Figure IIIIC). Further analysis of R1 events showed that almost all of these entities are CD31+ and CD105+ (data not shown), indicating that a potentially significant proportion of these entities may be non-nucleated endothelial microparticles or apoptotic bodies.21 These results point to...
reliable criteria for distinguishing dead cells and debris from viable, single cells. However, it should be noted that the VDO component could be replaced by conventional singlet gating with some contamination of R1 and R4 elements. In consideration of the enhanced accuracy conferred by the incorporation of the nuclear-staining dye, we have used it for flow cytometric survey of all nucleated nonmyocytes in the myocardium.

We used this approach to identify a tissue dissociation protocol that yields the greatest number of viable, nucleated cells. Three cardiac single-cell suspension protocols with distinct dissociation enzyme cocktails were compared: protocol 1, protocol 2, and protocol 3. The total number of nucleated cells isolated per milligram of tissue was 4241.8±517.4, 11223.3±1194.3, and 6728.9±491.7 for protocols 1, 2, and 3, respectively (Online Figure IVA). All 3 protocols demonstrated >95% calcein labeling in nucleated/viable cells (Online Figure IVB). Given the high yield of nucleated/viable cells, we used protocol 2 for subsequent analyses. It should be noted that during the process of protocol screening, we assessed Langendorff perfusion for cell isolation using several commonly referenced digestion cocktails. We found that these methods yielded fewer viable noncardiomycocytes and contained contamination by valvular interstitial cells (data not shown).

Two identification methods were used to determine whether mural cells (such as VSMCs and pericytes) could be recovered from protocol 2 isolates. To mark VSMCs, we used a lineage tagging system in a transgenic mouse that expresses Cre controlled by an α SMA promoter (SMACreERT2) and a Cre reporter (ROSA26RcreERT2). An anti-NG2 antibody was used to identify pericytes. We found that VSMCs and pericytes could be observed after isolation with protocol 2 (Online Figure V), suggesting that this population of cells will be included when determining relative cardiac cell proportions.

**Cardiac Nonmyocyte Cellular Composition**

To determine cardiac nonmyocyte cell composition, we conducted flow cytometry with antibodies for ECs (CD31 and CD102), leukocytes (CD45 and CD11b), and RMC (CD90 and Sca-1) in addition to nuclear and metabolic activity stains described above. Flow cytometric analyses with manual gating of nucleated, calcein+ cells indicated that CD31+CD45+ cells (ECs) constituted 62.1±3.9%, CD45+ cells (leukocytes) 9.6±1.3%, and CD31−CD45− cells (RMC) 27.3±5.3% of all nonmyocytes (Figure 2A).

To better define cell populations represented by this antibody panel (Figure 2B), we conducted high-dimensional analysis of our flow cytometry data using the SPADE algorithm. SPADE uses agglomerative clustering to identify groups of cells (referred to as nodes) that are phenotypically similar based on multiple surface or genetic markers (described in detail previously). Schematically, nodes are represented as colored circles that are interconnected within a dendrogram. Direct connection of 1 node to another signifies phenotypic similarity between the 2 nodes, and their size represents the number of cells within the node. Node color may indicate a range of statistical parameters. For example, all nodes within the dendrogram may be colored relative to the expression level of specific markers that define cell populations. This enables systematic annotation and categorization of individual nodes or groups of nodes as specific cell types. After annotation, nodes may be selected to derive statistical data, such as relative cell proportion.

After generation of SPADE dendrograms for cardiac cells using the aforementioned markers (Figure 2B), dendrogram branches corresponding to leukocytes, ECs, and RMC were manually annotated based on marker expression levels (Figure 2C and 2D). Branches with high CD45 expression, encompassing nodes and branches with high CD11b (myeloid cells) and CD90 (T cells), were classified as leukocytes (Figure 2D). Similarly, branches expressing CD31 and CD102 but not CD45 were identified as ECs. Remaining nodes, including nodes with high expression of commonly used fibroblast/mesenchymal markers, Sca-1 and CD90, were classified as RMC. Statistical analysis of SPADE branches identified 63.9±3.4% of cardiac cells as ECs, 9.4±1.6% as leukocytes, and 26.7±4.0% as RMC (Figure 2E). These values were consistent with histological analyses described above. Moreover, the SPADE data are consistent with the flow cytometry analyses conducted on nucleated cardiac cells manually gated for ECs (CD31+CD45− cells), leukocytes (CD45+), and RMC (CD31−CD45− Figure 2A). Together, these findings confirm that ECs are the most prevalent cell type in the adult murine heart.

**Endothelial and Leukocyte Diversity**

To determine the diversity of cardiac ECs and leukocytes, we also conducted SPADE analysis after gating on viable, nucleated CD31+CD45− ECs or CD45+ leukocytes. ECs were clustered based on surface expression of CD102, CD105, podoplanin, and CD90. We found that 94.3±0.7% corresponded to vascular ECs (CD102+CD105+ nodes) and 5.2±0.7% corresponded to lymphatic ECs (podoplanin+ nodes; Figure 3A–3C). To characterize leukocyte subsets, we clustered CD45+ cells based on surface expression of CD11b, CD64, B220, IgM, CD3ε, and CD90 (Figure 3D and 3E). Myeloid cells (CD11b+ nodes), B cells (CD11b B220− nodes), T cells (CD11b CD3ε− nodes), and nonmyeloid/lymphoid (CD11b+CD20+CD3ε− nodes) cells were clearly identified within dendrograms. Consistent with cell identity, CD64, IgM, and CD90 were highly expressed in nodes corresponding to myeloid cell, B-cell, and T-cell clusters, respectively. Statistical analysis showed that 81.4±1.4%, 8.9±0.6%, 3.1±0.4%, and 6.6±0.6% of leukocytes corresponded to myeloid cells (CD11b+), B cells (B220), T cells (CD3ε−), and nonmyeloid/lymphoid cells (CD11b+CD20+CD3ε−), respectively (Figure 3F).

**RMC Composition**

Although ECs and leukocytes can be identified using a variety of antibodies that recognize surface proteins, reagents for classifying fibroblasts and mural cells (VSMC and pericytes) are limited. Consequently, we attempted to identify uniform and specific antibodies suitable for flow cytometric identification of these 2 populations. In the murine context, an anti-PDGFRβ antibody would be a candidate for mural cells. Flow cytometry demonstrated that the PDGFRβ monoclonal antibody, APB5, did not label primary cells to a satisfactory level (data not shown for 6 independent sources) although the same monoclonal antibody can be used for immunohistochemistry.

Unlike the mural cell population, we were able to clearly identify the proportion of RMC that were cardiac fibroblasts.
by using genetically tagged $Col1a1\text{-GFP}$, $PDGFR\alpha^{\text{GFP/+}}$, and $Col1a1\text{-GFP};PDGFR\alpha^{\text{GFP/+}}$ transgenic mice that express GFP in resident fibroblasts as described above. By gating on the CD45$^+$CD31$^-$ population, we observed that more than half of the RMC were GFP$^+$ (Figure 3G and 3H). To determine whether GFP cells from $Col1a1\text{-GFP}$ and $PDGFR\alpha^{\text{GFP/+}}$
mice represent similar cardiac cell populations, we conducted SPADE analysis by clustering on CD31, CD102, CD45, CD11b, CD90, and Sca-1. A high degree of overlap occurred when comparing dendrograms for Col1a1-GFP and PDGFRα<sup>GFP</sup> labeled cells (Online Figure VI). Although cells from the 2 GFP fibroblast mouse lines were convenient for categorizing and surveying the fibroblast population, the use of these transgenic mice as a universal tool to analyze fibroblasts is not always feasible. Because antibodies validated for flow cytometry were readily available for 3...
commonly used fibroblast surface antigens, Sca-1, CD90, and PDGFRα, we surveyed these by flow cytometry. PDGFRα antibodies were assessed on NIH3T3 fibroblasts, mouse embryonic fibroblasts (MEFs), including PDGFRα null MEFs, and primary cardiac single-cell isolates from wild-type and PDGFRαGFP/+ mice. None of the PDGFRα antibodies examined clearly and uniquely distinguished a positive cell population (Online Table I and data not shown) although PDGFRα expression was observed in cardiac fibroblasts by immunohistochemistry (Online Figure VII). Interestingly, after unsupervised SPADE clustering of RMC from Col1a1-GFP mice, we found that most GFP+ nodes were also MEFSK4+ (Figure 3I). Notably, similar correlation of gene expression within these nodes was not observed for CD90 or Sca-1. The MEFSK4 antibody has been used to identify and remove MEFs during culture of induced pluripotent stem cells and embryonic stem cells. This antibody identifies MEFs from C57BL/6, CF1, CF6, and DR4 mouse strains and an epitope on NIH3T3 cells.30 The close correlation of GFP expression and MEFSK4 detection was further underscored when comparing GFP expression with MEFSK4+ RMC from Col1a1-GFP, PDGFRαGFP/+ mice, or Col1a1-GFP;PDGFRαGFP/+ double transgenic mice were MEFSK4+, and most MEFSK4+ RMC were GFP+ (Online Figure VIII). MEFSK4 antigen expression was independent of activation status, as surface staining was not decreased after transforming growth factor-β1 stimulation of MEFs or after myocardial infarction (data not shown). It should be noted, however, that the antibody also stains a sub-population of CD11b+ leukocytes (data not shown). Therefore, this population must be excluded from analysis before considering the fibroblast lineage by flow cytometry. We have not been successful in tissue staining with the MEFSK4 antibody. To further investigate the MEFSK4+ RMC population by flow cytometry, we costained for the pericyte marker, NG2. NG2+ cells express the MEFSK4 epitope at lower levels than GFP+ cells, and NG2+ cells account for a majority of the MEFSK4+ GFP+ RMC population (Online Figure IX). To further define MEFSK4+ cells, we isolated CD31+CD45−, and MEFSK4+ cells by fluorescence activated cell sorting and found that fibroblast gene expression is enriched in this cell population when compared to whole ventricle (Online Figure X). These findings indicate that Sca-1 and CD90 are suboptimal markers of resident cardiac fibroblasts and that MEFSK4 may be a suitable surrogate marker for cardiac fibroblasts.

Cardiac Fibroblast Diversity

To further examine the spectrum of fibroblasts labeled by PDGFRαGFP, Col1a1-GFP, and MEFSK4, we performed lineage tracing by intercrossing Tcf21iCre/+; ROSA26RtdTomato mice with the fibroblast GFP-expressing mouse lines. In the Tcf21iCre mouse model, resident fibroblasts and their descendants are indelibly tagged with tdTomato fluorescence after Cre induction.15,31 We induced Cre-mediated recombination at either embryonic day 16.5 (Tcf21iCre/+; ROSA26RtdTomato;Col1a1-GFP) or in the adult (Tcf21iCre/+; ROSA26RtdTomato;Col1a1-GFP) mice with the fibroblast GFP-expressing mouse lines.
and conducted flow cytometry 2 to 6 months after induction to determine the proportion of tdTomato+ cells (cardiac fibroblasts) that were GFP+. Gating on the tdTomato+ cells revealed that >95% were positive for GFP and MEFSK4 (Figure 4, data not shown), demonstrating that cardiac GFP+ cells from PDGFRαGFP+ and Col1a1-GFP mice comprise Tcf21 lineage fibroblasts. Taken together, these data suggest that the population of cells identified as fibroblasts is relatively uniform as determined by 4 independent markers (PDGFRαGFP, Col1a1-GFP, MEFSK4, and Tcf21 lineage). Furthermore, our analysis indicates that this cell population constitutes 15% of the nonmyocyte cell pool, equating to ≈11% of the total cells of the heart when assuming ≈30% of the cells are cardiomyocytes.

Discussion

A comprehensive understanding of cardiac cellular composition will guide development of therapeutics that promote heart repair and regeneration. To date, attempts to survey the cellular composition of the heart, even in model organisms such as the mouse and rat, have been hindered by difficulties in cell identification and isolation. Here, using recently developed techniques and genetic tools, we demonstrate that ECs outnumber other cell types in the adult mouse heart ventricles (Figure 5) and that the cardiac fibroblast population is much smaller than previously reported. Nonetheless, classic fibroblast markers such as CD90 and Sca-1 under-represent the resident fibroblast population.

Although the accuracy and reliability of immunohistochemical data depend on the specificity and sensitivity of the antibodies used, it can be a powerful approach for evaluating cell populations within a tissue. Here, 2 histological approaches in separate colonies of mice generated highly analogous results that were further corroborated by the analysis of human tissue sections. Using histological analyses, however, we were unable to evaluate the proportion of myocytes relative to nonmyocytes with a high level of precision. This is because of the variability and dynamism of the number of nuclei per cardiomyocyte in addition to the differences between rodents and humans—most murine cardiomyocytes are binucleated, whereas most human cardiomyocytes are mononucleated. Therefore, estimations of myocyte to nonmyocyte ratios will differ depending on the species and age.

There are many explanations as to why our findings on the number and proportion of ECs in the heart differ from those of previous studies. First, access to new and enhanced reagents enabled more accurate identification of cell populations than was previously possible. Second, the use of an isolation protocol that yielded the greatest number of viable cells at the expense of cardiomyocytes may explain why flow cytometry results strongly correlated with results from immunohistochemical analyses but produced different outcomes when compared with previous studies. Many published protocols use digestion conditions that favor the isolation of cardiomyocytes with noncardiomyocytes being byproducts of the isolation. Third, establishment of the criteria of cell nucleation and vitality to determine cell frequency enabled us to perform a more accurate analysis. This approach eliminates the dependency on using light-scattering (forward- and side-scatter) properties of cells for identifying viable cells, a practice that could lead to over- or under-representation of cell types and inclusion of tissue debris as cellular events. Fourth, in classifying cell populations, we used an unsupervised clustering algorithm (SPADE) after objective cell gating. SPADE and similar clustering algorithms enable superimposition of expression data for multiple cellular markers to visualize and quantify nonmyocyte cardiac cell populations. The identity of ECs was confirmed using multiple endothelial markers including CD31, CD102, and CD105, in addition to exclusion of leukocyte markers, CD45 and CD11b.

Although the recovery of cell populations and flow cytometry data presented here correlates with the cellular distribution documented by immunohistochemistry, the possibility remains that not all cells have been included in the current assessment. These may include cells that are more sensitive to the digestion protocol or are not recovered because of insufficient digestion. Notably, we have not tested the efficacy of the nuclear and metabolic activity (VDO and calcein) staining approach to distinguish between activated, proliferating, and quiescent cell populations.

The discrepancy in leukocyte proportions determined by flow cytometry and immunohistochemistry may be attributed to inefficiencies in antigen detection in fixed tissues. Indeed, this is likely for leukocyte staining using anti-CD45 antibodies, which do not reveal stellate-shaped myeloid cells in fixed human or mouse tissue. The use of antibody cocktails for staining mouse cardiac tissue may overcome this issue; nevertheless, immunohistochemical analyses of leukocytes may still fail to detect all cells. By flow cytometry analysis—focusing on nucleated metabolically active cells and SPADE—we have estimated that leukocytes comprise 7% to 10% of all nonmyocytes. The vast majority of these cells were identified as myeloid cells and, in particular, macrophages. Several

Figure 5. Distribution of major nonmyocyte cardiac cell types. Combined data demonstrating relative cell numbers determined by flow cytometry with exclusion of cardiomyocytes.
lymphocytes were also detected although at a much lower prevalence. Histological and flow cytometric analysis also suggests that the relative number of fibroblasts in the heart may have been overestimated in the past. The lack of a clear definition for fibroblasts has been at the root of many difficulties in quantifying and tracking these cells in vivo. In some cases, cardiac fibroblasts have been quantified by excluding cells that lack structural elements corresponding to ECs, VSMCs, or cardiomyocytes. According to this definition, pericytes and leukocytes would also be classified as fibroblasts. Another confounding factor is that markers such as DDR2, CD90, Sca-1, and vimentin are not unique to fibroblasts, and not all fibroblasts express these proteins. Although resident cardiac fibroblasts derive from 2 embryonic sources, the consensus is that PDGFRα is expressed in both populations. The use of CD90 for identification of fibroblasts, on the contrary, remains a point of debate. Previously, we have shown that cardiac Tcf21 lineage cells, and GFP+ cells from PDGFRαGFP and Col1a1-GFP mouse hearts have similar levels of fibroblast gene expression including Col3a1, Col6a1, Dcn, and MMP2 with lack of expression of smooth muscle, endothelial, and cardiomyocyte genes. As flow cytometric results demonstrate that the MEFSK4 antibody detects Tcf21 lineage cells and GFP+ fibroblasts in mouse hearts, when excluding leukocytes, this antibody can be used to identify cardiac fibroblasts by flow cytometry when use of the genetic systems is not feasible. Our results also suggest that CD90 and Sca-1 only capture a fraction of the resident fibroblasts. This analysis represents the most comprehensive estimation of cardiac fibroblasts to date. Recent reports have proposed that pericytes contribute significantly to the cardiovascular remodeling process. In the uninjured heart, we show that these cells account for 5% of noncardiomyocytes and that they also express MEFSK4, albeit at lower levels than fibroblasts. Further investigation will be required to determine MEFSK4 expression in the activated pericyte population. The establishment of these cellular proportions at baseline will pave the way for future work examining how these populations vary in the postnatal, aged, and injured heart. Although the role of the EC as a dynamic regulator of tissue responses is increasingly recognized, their abundance and roles in the heart are commonly underappreciated. The proximity of ECs to the coronary circulation and cardiomyocytes provides an access point for therapeutic manipulation. Therefore, a more comprehensive understanding of these potential cellular interactions will be required. Taken together, our findings redefine the cellular composition of the adult murine and human heart and point to the cardiac EC as a potentially important protagonist in cardiac homeostasis, disease, and aging.

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Disclosures

None.

References

Accurate characterization of the cell types found in the heart is essential for understanding cardiac development, homeostasis, aging, and injury responses. This study provides the first comprehensive survey of major cell types found in the human and mouse heart and their relative abundance. We found that endothelial cells are the most abundant cell type in both the mouse and human heart, and that fibroblasts comprise a much smaller proportion of noncardiomyocyte cells than previously thought. In addition, we demonstrate a standardized approach for identifying cell types in the heart that permits simultaneous surveying of multiple noncardiomyocyte cell populations. The findings of this study fundamentally redefine our understanding of the cellular composition of the heart and may have implications for studies concerned with cardiac cellular biology in a range of developmental and disease contexts.
Revisiting Cardiac Cellular Composition


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Supplemental Material

Online Materials and Methods

Mice
Monash University (Melbourne, Australia)- All mice were maintained on the C57BL/6J background in a specific pathogen-free (SPF) facility and fed standard mouse diet ad libitum. All procedures conducted were approved by Monash University Animal Research Platform 2 (MARP2) ethics committee. Cx3Cr1GFP/+ mice1 were backcrossed over 10 generations to C57BL/6J and analyses were performed using 10 week old males. University of Hawaii (Honolulu, HI, USA)- Mice were maintained in a specific pathogen-free (SPF) facility and fed standard mouse diet ad libitum unless tamoxifen chow was used as noted. All procedures conducted were approved by University of Hawaii IACUC. All experiments using PDGFRαGFP and/or Colla1-GFP mice2,3 were performed using animals 2-7 months of age and of mixed genders. Postnatal day 4 SMACreERT2Tg;ROSA26RtdTomato animals4,5 were induced by gastric injection with 300 μg of tamoxifen (5 mg/mL) (MP Biomedicals, 156738) in sunflower seed oil (Sigma, S5007). Tcf21Cre/+;ROSA26RtdTomato mice were induced with tamoxifen either by oral gavage of a timed mating female at embryonic day 16.5 (100 μg/g body weight (20 mg/mL tamoxifen)) or feeding tamoxifen chow for two weeks (Harlan Laboratories, TD.130856) All genetically engineered animals with the exception of Colla1-GFP mice have been backcrossed for a minimum of 7 generations to B6.Cg-Gt(ROSA)26Sor tm14(CAG-tdTomato)Hze/J (ROSA26RtdTomato), Jax [007914], and contain the Nnt deletion.

Human material
Human cardiac tissue was obtained after informed consent and used per Institutional Review Board approval of the University of Hawaii at Manoa (CHS #23245). Human tissues were from mixed gender, healthy individuals age 35-55.

Antibodies
See Online Table I for antibody information.

Immunostaining and microscopy
The two sets of mouse histological data (those from Cx3Cr1GFP/+ and the fibroblast GFP lines) were gathered independently of each other.

Staining and analysis of formaldehyde-fixed 150 μm cardiac sections for confocal microscopy. Isolation of cardiac tissue, staining, and microscopy were previously described6. Briefly, mice were euthanized by CO2 asphyxiation and perfused with PBS and freshly prepared 4% formaldehyde (PFA). After overnight fixation of isolated hearts, 150 μm heart sections were prepared using a vibrating blade microtome (Leica Microsystems). Sections were permeabilized in 0.2% Triton-X-100 (Sigma-Aldrich)/PBS solution, prior to blocking (2% goat serum, 1% BSA, 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% Tween-20, 0.05% sodium azide, 0.01M PBS, pH 7.2). Tissue staining was performed in blocking solution using a range of antibodies and reagents summarized in Online Table 1. Fluorescence microscopy images were obtained using a Leica SP5 confocal laser scanning microscope. For quantification of nuclei corresponding to cardiomyocytes, endothelial cells, leukocytes or non-endothelial cell/leukocytes (unstained) we obtained 0.969 μm optical sections using 40X objective lens. Nuclei were manually counted using Imaris software (Bitplane). The minimum number of nuclei counted per image was 125, with a mean of 269.8 nuclei per field of view. For Figure 1A/B and Online Figure IIB/C, nuclei corresponding to cardiomyocytes, Lin1+ cells, endothelial cells and unmarked cells were classified according to the following criteria: cardiomyocyte nuclei, nuclei associated with large cell bodies (indicated by large cytoplasmic area outlined by WGA staining); Lin1+ cell nuclei, nuclei with Lin1 antibody cocktail staining within
nuclei or on surrounding plasma membrane; endothelial cell nuclei, nuclei surrounded by IB4 stained plasma membrane; unmarked nuclei, nuclei that do not conform to any of the criteria above.

**Formaldehyde-fixed 10 µm cryosections – Mouse.** 5-12 week old Coll1-GFP or PDGFRαGFP/+ mice were sacrificed by CO₂ asphyxiation, perfused with PBS and fixed using freshly prepared 4% formaldehyde for 1 hour at room temperature. The fixed tissues were submerged in 30% sucrose/PBS for 2 hours followed by embedding with Optimal Cutting Temperature (OCT). 10 µm thick sections were prepared before permeabilization with 0.1% Triton-X-100/PBS for 30 min. Sections were blocked with blocking solution (3% BSA, 1.5% normal donkey serum and 0.1% Triton-X-100 in PBS) and incubated overnight at 4°C with primary antibodies; PDGFRβ (CD140b), isolectin B4-Biotin, sarcomeric-α-actinin (ACTN2), and cell lineage-biotin (Lin2). The sections were then washed and incubated with relevant conjugated secondary antibodies for 1 hour before mounting and imaging with a Zeiss Axiosvert 200M microscope. As ACTN2 positive nuclei were considered independent events, cardiomyocyte estimations may be overestimated due to multinucleated cells. When choosing images for quantification those containing large coronary arteries were excluded to avoid over representation of any single cell type. In most sections, a maximum of 2-4 cells were observed that exhibited overlapping expression of GFP and the cell type specific antigen being analyzed. The minimum number of nuclei counted per image was 250 with a mean of 314 nuclei per field of view. n=3 sections from 4-5 hearts.

**Formaldehyde-fixed 10 µm cryosections – Human.** Cardiac tissue was flash frozen and stored at -80°C. Samples were thawed and fixed in 4% paraformaldehyde (PFA) at 4°C for 1 hour, cryoprotected in 30% sucrose/PBS at 4°C for 2 hours, embedded in OCT, and sectioned. Immunostaining was performed on 10 µm sections of human cardiac tissue. Sections were permeabilized in 0.1% Triton X-100 for 15 min, blocked in 5% normal donkey serum for 1 hour and incubated in primary antibody overnight at 4°C. Nuclei were stained by DAPI. Corresponding cardiomyocytes, endothelial cells, and leukocytes were quantified within 10 µm-thick sections imaged with a Zeiss Axiosvert 200M microscope. As ACTN2 positive nuclei were considered independent events, cardiomyocyte estimations may be overestimated due to multinucleated cells. The minimum number of nuclei counted per image was 86, with a mean of 149 per field of view. n=4-6 sections from 3 hearts.

**Microscopy of isolated cell populations**

After FACS, cells were plated directly in wells of 96-well tissue culture dishes and imaged 30-60 minutes after plating. Images were acquired using a Nikon eclipse Ti-S widefield fluorescence microscope using a 20X objective lens with appropriate filters. For immunocytochemistry cells were isolated according to protocol 2. Cells from a SMACreERT2;ROSA26RtdTomato heart were fixed in 4% PFA for 10 minutes and imaged immediately after DAPI staining and plating. Cells were plated in a 35 mm dish or on a chamber slide coated with attachment factor (Life Technologies, S-006-100) for 30 minutes at 37°C and washed with PBS. 2 hours after plating, cells in the chamber slide were fixed in 4% PFA for 10 minutes. The remaining cells were passaged and fixed at 48 hours. For immunocytochemistry, fixed cells were blocked with 1% BSA in PBS and incubated for one hour at room temperature with primary antibody NG2, kindly provided by Dr. William Stallcup. Cells were washed, stained with secondary antibodies for 30 minutes and imaged with a Zeiss Axiosvert 200M microscope.

**Quantitative Real Time PCR (qRT-PCR)**

RNA was isolated from cardiac ventricles of 10-15 week old C57BL6/J mice using TRIzol® LS Reagent (Ambion). Briefly, hearts were perfused with ice-cold 1×HBSS (Gibco) for ~2 min before homogenization of tissue in 1 ml of TRIzol® LS Reagent using a Polytron™ tissue homogenizer (Kinematica). Following
homogenization, RNA was extracted according to manufacturer’s instructions. MEFSK4+ RMC were sorted using a BD Influx Sorter (BD Biosciences) by gating on DAPI-CD45-CD31-MEFSK4+ single cells prepared using Protocol 2 (described above). RNA from MEFSK4+ RMC was isolated using RNAqueous®-4PCR Total RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer’s instructions, with the addition that MEFSK4+ RMC were directly sorted into lysis buffer. RNA quality and concentration was determined by spectrophotometry using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific). SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) was used to perform reverse transcription as per manufacturer’s instructions. Quantitative PCR analysis was performed on RNA from cardiac ventricle (n=4) or MEFSK4+ RMC (n=3, where each biological replicate refers to a pool of 3 heart ventricles) using LightCycler® 480 SYBR Green I Master Mix (Roche) and a LightCycler® 480 instrument (Roche). The $2^{-\Delta\Delta C_t}$ method was employed for determining relative gene expression levels, using the geometric mean of 3 housekeeping genes for normalization of input RNA: TATA-binding protein (TBP), hypoxantine-guanyl ribosyltransferase (HPRT) and actin-β. Primer sequences used in qRT-PCR gene expression analysis are summarised in Online Table II.

Online References
Online Table I. Cell-specific reagents for tissue/cell staining.

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FC: flow cytometry; TS: tissue staining; ICC: immunocytochemistry

Note that multiple clones of CD90 antibodies were tested with similar results for all clones and company sources.
# Online Table II. Primers used for qRT-PCR.

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<th>Gene</th>
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| Platelet derived growth factor receptor alpha (PDGFRα) | Forward: GGAAGGACTGGAAGCTTGGGGC  
Reverse: AGATGAGGCCCGGGCCCTGTGAGG |
| Collagen 1 alpha1 (Col1α1)                | Forward: AATGGCAGGCTGTGTGCGA   
Reverse: AACGGGTCCCCCTTGGGCCTT |
| Collagen 1 alpha1 (Col1α2)                | Forward: GGCCCCCTGGTATGACTGGCT  
Reverse: CGCACCAGGGGACCACAATC |
| Cluster of differentiation 90 (CD90)      | Forward: TGGGTGAGCAACTGGAGGC   
Reverse: CTCGGGACACACTGCAAGACTGA |
| Discoidin domain receptor 2 (DDR2)        | Forward: TTCCCTGCCAGCGAGTCAGCA  
Reverse: ACCACTGCACCTGACTCCTCC |
| Tenascin C (Ten C)                        | Forward: TCCCCGGGACTGTAGCCAGC   
Reverse: CGTGGCAGTCAGCTGGGCAG |
| Myosin heavy chain 6 (Myh6)               | Forward: ACGCTAGAGGCCAGCCAGCC   
Reverse: CCGCTCGTGCAGAAGGCT |
| TATA-binding protein (TBP)                | Forward: AGATGTGCTGAGGCCTGCGG   
Reverse: AAATAGTGATGCTGGGCACCTGCG |
| Hypoxantine-guanyl ribosyltransferase (HPRT) | Forward: GCGAGGAGACGTGGGCTGG   
Reverse: CATCATGCTAAATCAGCACCTGCG |
| Actin-beta (actin-β)                      | Forward: ACAGAGCCCTGCTGGTGGCC   
Reverse: ACCCATGCCACCACATCACGC |
Online Figure I. Representative micrographs of cardiac tissue stained for indicated markers. (A) Representative confocal microscopy optical section (0.969 µm) of cardiac tissue stained with individual leukocyte antibodies used for quantification of leukocytes in Fig 1A-B. (B) Left panel; representative image from 10 µm sections of PDGFRα<sup>GFP</sup> heart stained with IB4 and DAPI. Right panel, image demonstrating DACH1 nuclear co-staining of IB4<sup>+</sup> endothelial vessels in mouse heart. (C) Representative fluorescence micrographs of 10 µm human cardiac left ventricle stained for ACTN2, CD31, and CD45.
Online Figure II. Distribution of major cardiac cell types. (A) Schematic of cardiac sections and fields of view analyzed for enumerating cardiac cell types in Figure 1A-B. Sections isolated from mid point (mid) or apex were imaged at the left ventricle (LV), interventricular septum (IS) or right ventricle (RV) by confocal microscopy before analysis. (B) Bar graphs display proportion of cardiomyocytes (CM), endothelial cells (ECs), leukocytes (Lin1) and unstained nuclei for each locus, or (C) means of total non-myocyte populations derived from images from all loci. n=8 per loci derived from four mouse hearts (2 images/loci/heart). (D) Quantification of cardiomyocytes (ACTN2), endothelial cells (CD31), endothelial nuclei (DACH1) and hematopoietic cells (CD45) in designated heart regions in human samples. n=11-18 images per cell type from 3 human hearts.
Online Figure III. Identification of viable, nucleated and metabolically active cells by flow cytometry. (A) Gating strategy for identification of calcein\(^+\) DAPI\(^-\) cells and analysis of VDO\(^+\) or VDO\(^-\) cell populations, and (B) analysis regions 1-4 (R1-4) of VDO-stained elements. (C) Microscopy images displaying sorted cells or clusters from regions 1-4 (R1-4) indicated in B, imaged after plating for 30 min. Data representative of at least two similar experiments. All images captured using 20X objective. (D) Flow cytometry scatter plots of FSC-H and FSC-A of cells with regions 1-4 indicated in B.
Online Figure IV. Determination of optimal tissue dissociation protocol for isolation of viable nucleated cells. (A) Number of viable (DAPI\textsuperscript{lo/-}) nucleated cells (VDO\textsuperscript{+}) isolated using Protocols 1-3. (B) Percent of metabolically active (calcein\textsuperscript{+}) nucleated cells. Data is representative of multiple experiments. n=4 per protocol.
Online Figure V. Recovery of VSMCs and pericytes. (A-C) Microscopy and immunohistochemistry of cells using dissociation protocol 2. (A) Fluorescence of VSMCs (red) isolated from a tamoxifen-induced SMACreERT2;ROSA26R<sup>tdTomato</sup> heart (aSMα<sup>Cri</sup>). (B) Isolated adherent cells were stained for pericyte marker (NG2) 2 hours after culture. Fibroblasts (GFP). (C) Pericytes (NG2) after passaging cells obtained from a wild type heart.
Online Figure VI. Clustering of GFP nodes in cardiac cells from *Col1a1-GFP* and *PDGFRα*GFP/+ mice. Cardiac cells were isolated from *Col1a1-GFP* and *PDGFRα*GFP/+ mice and were clustered using SPADE based on expression of CD31, CD45, CD11b, Sca-1, CD90 and CD102. Cells were pre-gated on calcein+7-AAD- single cells.
Online Figure VII. PDGFRα expression in cardiac fibroblasts. Immunohistochemistry for PDGFRα in heart sections from (A) PDGFRαGFP/+ and (B) Col1a1-GFP mice.
Online Figure VIII. Correlation of MEFSK4 and GFP expression in cardiac cells of Col1a1-GFP, PDGFRαGFP/+ and Col1a1-GFP;PDGFRαGFP/+ transgenic mouse RMC.
Data derived from flow cytometry data where cell populations were gated on calcein⁺7-AAD⁻ singlets, which were also CD31⁻CD45⁻ (n=4).
Online Figure IX. NG2^+ population in RMC. Flow cytometry contour plots displaying NG2 expression in RMC relative to MEFSK4 staining and GFP expression. Representative of two replicates. Cardiac cells isolated from PDGFRα^{GFP/+} mice.
Online Figure X. Fibroblast gene signature of MEFSK4⁺ RMC. qRT-PCR gene expression analysis of MEFSK4⁺ RMC for fibroblast (PDGFRα, Col1a1, Col1a2, CD90, DDR2, TEN C) and cardiomyocyte (MYH6) genes. Expression levels shown relative to cardiac ventricle (n=4 and 3 biologic replicates for cardiac ventricle and MEFSK4⁺ RMC, respectively). PDGFRα: platelet derived growth factor receptor alpha; Coll1a1: collagen 1 alpha 1; Coll1a2: collagen 1 alpha 2; CD90: cluster of differentiation 90; DDR2: discoidin domain receptor 2, Ten C: tenascin C; MYH6: myosin heavy chain 6.