**Proliferation, differentiation and migration of SCA1⁺/CD31⁺ cardiac side
population cells *in vitro* and *in vivo***

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Running head: Growth, differentiation and migration of CSP cells.

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Abstract

Background: Side-population (SP) cells, identified by their capacity to efflux Hoechst dye, are highly enriched for stem/progenitor cell activity. They are found in many mammalian tissues, including mouse heart. Studies suggest that cardiac SP (CSP) cells can be divided into SCA1⁺/CD31⁻, SCA1⁺/CD31⁺ and SCA1⁻/CD31⁻ CSP subpopulations. SCA1⁺/CD31⁻ were shown to be cardiac and endothelial stem/progenitors while SCA1⁺/CD31⁺ CSP cells are endothelial progenitors. SCA1⁻/CD31⁻ CSP cells remain to be fully characterized. In this study, we characterized SCA1⁻/CD31⁻ CSP cells in the adult mouse heart, and investigated their abilities to proliferate, differentiate and migrate *in vitro* and *in vivo*.

Methods and Results: Using fluorescence-activated cell sorting, reverse transcriptase/polymerase chain reaction, assays of cell proliferation, differentiation and migration, and a murine model of myocardial infarction we show that SCA1⁻/CD31⁻ CSP cells are located in the heart mesenchyme and express genes characteristic of stem cells and endothelial progenitors. These cells were capable of proliferation, differentiation, migration and vascularization *in vitro* and *in vivo*. Following experimental myocardial infarction, the SCA1⁻/CD31⁻ CSP cells migrated from non-infarcted areas to the infarcted region within the myocardium where they differentiated into endothelial cells forming vascular (tube-like) structures. We further demonstrated that the SDF-1 α /CXCR4 pathway may play an important role in migration of these cells after myocardial infarction.

Conclusions: Based on their gene expression profile, localization and ability to

proliferate, differentiate, migrate and vascularize *in vitro* and *in vivo*, we conclude that SCA1⁺/CD31⁺ CSP cells may serve as endothelial progenitor cells in the adult mouse heart.

Key words: stem cells; side population cells; myocardial ischemia; cell migration; CXCR4 receptor.

1. Introduction

The prevailing view of the heart, as a terminally differentiated organ, has been challenged by the discovery of resident cardiac stem cells (CSCs), potentially bringing the treatment of cardiac diseases into a new era. Various subsets of resident CSCs have been identified that are capable of self-renewal, proliferation, and differentiation into cardiomyocytes, endothelial and smooth muscle cells. These findings were based on: (i) expression of stem cell-associated markers, such as c-kit and SCA1 [1, 2], (ii) a specific culture system to obtain cardiospheres that expressed cardiac and smooth muscle genes [3], (iii) expression of the homeodomain transcription factor, Isl-1 [4], and (iv) discovery of cardiac side-population (SP) cells. After labeling with Hoechst 33342 dye, SP cells can be distinguished by their weak labeling compared to the main population (MP) of labeled cells [5-7]. This unique property of SP cells can be explained by their expression of the p-glycoprotein multidrug/ATP-binding cassette transporter protein ABCG2, which pumps Hoechst dye out of the cell [8, 9]. Side-population cells isolated from adult mammalian tissues and organs, including heart, are enriched for stem/progenitor cell activity [7, 9-11].

Studies from different groups, including ours suggested that cardiac SP (CSP) cells are capable of differentiation into cardiomyocytes as well as endothelial and smooth muscle cells [12-15]. Based on expression of SCA1 and CD31, murine CSP cells can be divided into SCA1⁺/CD31⁻, SCA1⁺/CD31⁺ and SCA1⁻/CD31⁻ cells [7, 14, 16]. *In vitro* and *in vivo* studies have shown that SCA1⁺/CD31⁻ CSP cells are able to differentiate into cardiomyocytes and endothelial cells, while SCA1⁺/CD31⁺ CSP

cells differentiate into endothelial cells [7, 14, 15]. However, the nature and differentiation potential of SCA1⁻/CD31⁻ CSP cells has remained unclear.

Cell migration is an important character of stem cells. We have shown that, after myocardial infarction (MI) in mice, SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells migrate from the healthy myocardium into the infarcted area. Our studies further suggested that the SDF-1 α /CXCR4 pathway plays an important role in this process [14, 15]. However, the capacity of SCA1⁻/CD31⁻ CSP cells to migrate and the role of SDF-1 α /CXCR4 pathway in their behavior remain unknown.

In this study, we set out to characterize SCA1⁻/CD31⁻ CSP cells in the adult mouse heart, and to investigate their capacity for proliferation, differentiation, vascularization and migration *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Animals

Wild-type C57BL/6J mice (female, 8–10 weeks) were purchased from Beijing HFK-Bio-Technology (Beijing, China). Mice were group-housed in individually-ventilated cages under pathogen-free conditions. The general health status of the animals was monitored daily. All animal experiments described herein were conducted strictly according to ethical standards approved by the institutional animal ethical committee of Jinzhou Medical University (approval ID: LY2014D001). We have consulted the ARRIVE guidelines in preparing this report [17].

133 2.2. CSP cell isolation, fluorescence-activated cell sorting and analysis, and magnetic
134 depletion of CD45⁺ cells

135 Isolation of CSP cells was performed as described [14, 15]. Briefly, cardiac tissue
136 was digested with 0.1% collagenase B, 2.4 U/ml dispase II and 2.5 mmol/l CaCl₂ for
137 45 min. Cell suspensions were incubated for 90 min with Hoechst 33342 (5 µg/ml;
138 Sigma-Aldrich, USA) at a density of 10⁶ cells/ml with or without verapamil (50 mM;
139 Sigma-Aldrich, USA) [9]. The suspension was subsequently incubated with one or
140 more fluorescently-conjugated monoclonal rat anti-mouse antibodies including
141 anti-SCA1-FITC, anti-CD31-PE and anti-CXCR4-APC (BD, USA). For negative
142 control, cells were incubated with isotope/control antibodies to establish gating
143 parameters for positively-stained cells. All incubations were performed for 30 min at
144 4°C in the dark.

145 To remove CD45⁺ cells from cell suspensions of 3-day post-MI hearts,
146 biotin-conjugated anti-CD45 antibody and streptavidin-conjugated microbeads
147 (Miltenyi Biotech, Germany) were used as described [14, 15]. Purification of the cells
148 was analyzed by flow cytometry using PE-conjugated anti-CD45 antibody (BD, USA)
149 staining. The cells were stained with Hoechst 33342 following by SCA1, CD31 and
150 CD184 as mentioned above.

151 Fluorescence-activated cell sorting (FACS) on a FACS Vantage-SE-Cell Sorter
152 (BD, USA) was used to isolate SCA1⁺/CD31⁻, SCA1⁺/CD31⁺, SCA1⁻/CD31⁻ CSP and
153 SCA1⁻/CD31⁻ main population (MP) cells for all experiments. The following 6
154 parameters were used to discriminate cells in the sorts: Hoechst 33342 (red), Hoechst

33342 (blue), forward scatter, side scatter, FITC-SCA1 and PE-CD31 reactivity (green) as described [14, 15]. This protocol is illustrated in Supplementary Fig S1.

The Hoechst dye was excited at 350 nm. A 488-nm argon laser was used for exciting PE and FITC, and a 633-nm HeNe laser for APC. Data were analyzed using BD FACSDivaTM software v4.12 (BD, USA).

2.3. Methylcellulose assay (colony forming unit assay)

SCA1⁺/CD31⁺ CSP and MP cells (7000 cells/ml) were plated in Methocult GF M3534 media following the manufacturer's instructions (StemCell Technologies, Canada) as described [5, 7, 15]. Cell colonies consisting of more than 30 cells were scored after 14 day in culture. In order to determine whether the cells retained their SCA1⁺/CD31⁺ CSP phenotype, Methocult media was cut into pieces and incubated with DMEM at 37°C with shaking for 35 min. The resuspended cells were collected and expression of the ABCG2, CD133 and SCA1 genes was examined by semi-quantitative real-time RT-PCR (qRT-PCR).

2.4. Primary cell culture

SCA1⁺/CD31⁺ CSP cells were plated into fibronectin-coated 8-well chamber slides (10,000 cells/well) and were cultured with a defined endothelial differentiation medium (EGM-2) from Cambrex (Baltimore, MD) and antimicrobial agents. They were maintained in humidified 5% CO₂/air at 37°C. Culture medium was changed regularly. After 14 days, cells were collected and expression of the

ABCG2, CD133, vWF and Tie2 genes was examined by qRT-PCR.

2.5. Immunofluorescence staining

The hearts were harvested 14 days after MI. Hearts embedded in OCT (Tissue-Tek, USA) were frozen and were cryosectioned at 5 μ m. Slides were stored at -20°C before staining. Immunofluorescence labeling of heart tissue was performed as described [11, 15]. Primary antibodies included polyclonal rabbit anti-mouse vWF, monoclonal rat anti-mouse ABCG2 (Chemicon, USA), polyclonal rabbit anti-mouse SCA1 (R&D System, USA), and monoclonal anti-mouse α -actinin FITC (Sigma-Aldrich, USA). Secondary antibody combinations included goat anti-rabbit IgG-FITC (Invitrogen, USA) goat anti-rat IgG biotin (Vector-Lab, USA) and streptavidin-Cy3 (BD, USA). Nuclei were counterstained with 4-6-diamidino-2-phenylindol-dihydrochloride (DAPI). Non-specific IgG-isotypes were employed as negative controls (Vector-Lab, USA). Slides were imaged using an Olympus-DP70 microscope and images were captured with a digital camera BX51.

2.6. Cellular uptake of diI-labeled acetylated low-density lipoprotein (DiI-Ac-LDL)

SCA1-/CD31- CSP cells were isolated and incubated for 8 hours at 37 °C with endothelial differentiation medium (EGM-2) containing 10 mg/ml of DiI-Ac-LDL (Molecular Probes, USA) in fibronectin-coated 8-well chamber slides (7,000 cells/well). After washing with PBS, cells were fixed and counterstained with DAPI as described [15]. Slides were imaged as described above.

2.7. *Semi-quantitative real-time RT-PCR*

Total RNA was extracted from distinct CSP cell isolates using a RNeasy-Mini Kit according to the manufacturer's instruction (Qiagen, Germany). Complementary DNA was generated using M-MLV MicroRNA Reverse Transcription Kit based on the manufacturer's instruction (Promega, USA). qRT-PCR was performed with Rotor-gene 3000 (Corbett-Research, Australia) and a SYBER Green qPCR-SuperMix-UDG kit (Invitrogen, USA) based on the manufacturers' instructions. Primers for the qRT-PCR were purchased from Qiagen, including ABCG2 (ID: QT00173138), VEGFR2 (ID: QT00097020), VEGFR1 (ID: QT00096292), Tie2 (ID: QT00114576), vWF (ID: QT00116795), CD133 (ID: QT01065162), Nkx2.5 (ID: QT00124810), SCA1 (ID: QT00293167), E-cadherin (ID: QT00121163) and β -actin (ID: QT01136772). The thermal cycler conditions were as follows: Cycle 1 (95°C for 3 min) X1, Cycle 2 (95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec) X45, Cycle 3 (72°C for 4 min). All samples were amplified in triplicate. Gene expression levels of ABCG2, VEGFR2, VEGFR1, Tie2, vWF, CD133, Nkx2.5, SCA1, and E-cadherin were normalized to a housekeeper gene, β -actin. Amplification data was analyzed with Corbett Rotor-gene 3000 software (Corbett Research, Australia) as described [15]. Gene expression data are presented in relative units.

2.8. *Mouse model of myocardial infarction and in vivo cell migration study*

Experimental MI was induced by permanent ligation of the left anterior descending coronary artery (LADCA) in C57BL/6J mice (female, 8-10 weeks) as we

previously described [14, 15]. Briefly, mice were anaesthetized with an intraperitoneal injection of 0.1 ml/10 g body weight of a cocktail consisting of 1 part Hypnorm, 1 part Midazolam and 2 parts distilled water by volume. Unconsciousness was monitored based upon complete suppression of the foot withdrawal full reflex. They were ventilated with a standard rodent ventilator (Harvard Ventilator) which was set to the rate of 110 ± 5 respirations per minute with a tidal volume of 2 ml. The thoracic cavity was opened by incision between the second and the third intercostal space, a rodent rib spreader was introduced to permit visualization of the heart and the LAD coronary artery was ligated with suture. Approximately 150,000 SCA1⁺/CD31⁺ CSP cells, labeled with the red fluorescence tracker dye, PKH26 (Sigma-Aldrich, USA), were injected into the (non-ischemic) right ventricle wall. The needle was directed towards the right auricle to ensure that the cells were delivered away from the ischemic area. Finally, surgical stitching was used to appose subcutaneous tissue and skin. After surgery, animals were placed on heating pads maintained at 37°C until they regained mobility. Ten hours post-operation, they received buprenorphine for analgesia (0.03 mg/kg, intraperitoneal injection). Nerve reflexes, basic sense of movement and muscle tension were regularly monitored during all the post-operation period, as previously described [14, 15]. Fourteen days later the mice were sacrificed by cervical dislocation and the hearts were harvested. In some experiments, hearts with LADCA ligation were harvested 3 days post-MI to study expression of CXCR4 or E-cadherin in CSP cells. Sham-operated mice underwent identical surgical procedures except without LADCA ligation.

2.9. Chemotaxis assay

The *in vitro* migration of SCA1⁺/CD31⁺ CSP cells was appraised using polycarbonate membrane Transwell® inserts (Millipore, USA) with 8 µm pores size as previously described [14, 15]. Briefly, 30,000 cells in 50 µl DMEM without serum were added to the upper surface of the membrane in each chemotaxis chamber. SDF-1α (R&D Systems, USA) at a concentration of 0, 50, 100 or 500 ng/ml was placed in the lower chamber. After 4 hours of incubation at 37°C in 5 % CO₂ and 95 % humidity, the upper membrane surface was scraped free of cells and debris, and gently washed with PBS. Membranes were then fixed and stained as described [14]. Cell migration was measured by counting the number of cells that had migrated through pores and adhered to the lower surface of the membrane in five adjacent high-power fields (40x). To block the SDF-1α signal, the cells were incubated with anti-CXCR4 antibody (10 µg/ml, eBioscience, USA) [14] for 30 min before being added to the membrane with SDF-1α (500 ng/ml) in the lower chamber. Experiments were performed in triplicate. Cell numbers for experimental groups were expressed as a fold-increase compared to control groups (no SDF-1α).

2.10. Statistics Analysis

For each experiment dissociated cells from eight mouse hearts were pooled to provide sufficient CSP cells for quantitation. Each experiment was repeated three times to obtain means used for statistical testing (n=3 experiments) as previously described [14]. GraphPad Prism (GraphPad Software, CA, USA) was used for

statistics analysis. Data was presented as mean $\pm$ SD. Data were compared using Student's *t*-test. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1 Isolation, gene expression of SCA1⁺/CD31⁺ CSP cells

Adult mouse hearts were enzymatically digested into a single cell suspension and subjected to FACS analysis. CSP cells can be identified on density dot plots as a distinct cell subpopulation of approximately 1% of total cells based on their ability to efflux Hoechst 33342 dye. The CSP disappeared when the ABCG2 transporter was inhibited with verapamil (Fig. 1A **up right box**). The majority of CSP cells expressed SCA1 and/or CD31 (Fig. 1B). CSP cells can be further divided into SCA1⁺/CD31⁺, SCA1⁺/CD31⁺ and SCA1⁺/CD31⁺ subpopulations [7, 14, 15]. Based on their immunophenotype, SCA1⁺/CD31⁺, SCA1⁺/CD31⁺, SCA1⁺/CD31⁺ CSP and SCA1⁺/CD31⁺ MP cells were isolated by FACS. The purity of SCA1⁺/CD31⁺ CSP cells isolated by FACS was approximately 98% (Fig. 1C).

We and others, previously found that SCA1⁺/CD31⁺ CSP cells expressed stem cell marker genes and early cardiac genes [7], while SCA1⁺/CD31⁺ CSP cells expressed stem cell marker genes plus endothelia-specific genes [15]. To further characterize SCA1⁺/CD31⁺ CSP cells, we compared their gene expression pattern to SCA1⁺/CD31⁺ and SCA1⁺/CD31⁺ CSP cells. The expression of genes specific for stem/progenitor, early cardiac development and endothelial cells were examined using qRT-PCR. As shown in Fig. 1D, three sub-population cells strongly expressed ABCG2 that is

responsible for efflux of Hoechst 33342 dye [8]. and CD133 a marker for endothelial progenitor cells [18]. In contrast, Nkx2.5 (a marker of early cardiac commitment), was detected only in SCA1⁺/CD31⁻ cells, while Tie2, VEGFR2, VEGFR1 (markers of endothelial cells) were expressed only in SCA1⁺/CD31⁺ CSP cells (Fig. 1 D). The gene expression pattern of three sub-population cells is summarized in Table 1. Expression of CD133, but not Nkx2.5 and endothelial genes (Tie2, VEGFR2, VEGFR1) and Uptake of DiI-Ac-LDL (Supplementary Fig S2) suggest that SCA1⁺/CD31⁻ CSP cells may be endothelial progenitor cells.

3.2 *In vitro* colony-formation and differentiation of SCA1⁺/CD31⁻ CSP cells

To extend our findings, we next examined the *in vitro* colony-forming potential of SCA1⁺/CD31⁻ CSP cells. Freshly isolated SCA1⁺/CD31⁻ CSP and MP cells were plated on methylcellulose media. Colony formation was compared after 14 d in culture. Figure 2A shows a typical colony formed by SCA1⁺/CD31⁻ CSP cells. A typical field of SCA1⁺/CD31⁻ MP cells is shown for comparison (Fig 2B). SCA1⁺/CD31⁻ CSP cells produced many more colonies, compared with SCA1⁺/CD31⁻ MP cells (Fig 2C). These cells retained identical expression of ABCG2 and CD133 mRNAs as was found in freshly isolated SCA1⁺/CD31⁻ CSP cells (Fig. 2D). They also retained their SCA1⁺ phenotype after culturing (Fig. 2E). These results, therefore, suggested a substantially greater potential for self-renewal in SCA1⁺/CD31⁻ CSP cells than for MP cells.

To examine their differentiation capacity, SCA1⁺/CD31⁻ CSP cells were cultured in conditions to promote endothelial differentiation. After 14 days, SCA1⁺/CD31⁻ CSP

cells formed vascular networks in culture (Fig. 3A). Next, we used qRT-PCR to compare expression of stem/progenitor cell marker genes (ABCG2 and CD133) and endothelial marker genes in freshly isolated and cultured SCA1⁺/CD31⁺ CSP cells. Expression of ABCG2 was detected in freshly isolated SCA1⁺/CD31⁺ CSP cells but not after they were cultured (Fig. 3B). Likewise CD133 was expressed in freshly isolated SCA1⁺/CD31⁺ CSP cells, but expression was greatly decreased after culture (Fig. 3B). Conversely, vWF and Tie2 were highly expressed in cultured SCA1⁺/CD31⁺ CSP cells, but were not expressed in isolated SCA1⁺/CD31⁺ CSP cells (Fig. 3B). These results suggest that, when exposed to the appropriate conditions, SCA1⁺/CD31⁺ CSP cells were able to differentiate into mature endothelial-like cells *in vitro*, consistent with SCA1⁺/CD31⁺ CSP cells being endothelial progenitor cells.

3.3 Localization of SCA1⁺/CD31⁺ CSP cells in the heart

To resolve the location of SCA1⁺/CD31⁺ CSP cells in the heart, we first used immunofluorescence with antibody specific to ABCG2 to identify CSP cells. In sections of adult mouse heart we found ABCG2-stained cells distributed randomly in the heart mesenchyme. Some were located in the vasculature (Fig. 4A-D). Comparison of adjacent serial sections permitted identification of ABCG2⁺/SCA1⁺ cells (representing SCA1⁺/CD31⁺ CSP cells). While ABCG2⁺/SCA1⁺ cells were located in the vasculature, ABCG2⁺/SCA1⁺ cells were randomly distributed in the heart mesenchyme (Fig. 4E-H).

3.4. Migration and differentiation of SCA1⁺/CD31⁺ CSP cells in the heart

We have found that after MI, SCA1⁺/CD31⁺ and SCA1⁺/CD31⁺ CSP cells migrated from healthy myocardium to the ischemic infarct region where SCA1⁺/CD31⁺ CSP cells differentiated into cardiomyocyte and endothelial cells, while SCA1⁺/CD31⁺ CSP cells differentiated into endothelial cells [14,15]. We tested the prediction that SCA1⁺/CD31⁺ CSP would similarly migrate into the ischemia region after MI. Based upon their *in vitro* behavior we further predicted that they would then differentiate into endothelial cells. To test these ideas, SCA1⁺/CD31⁺ CSP cells (purity of 98%, Fig 1C) were labeled with PKH26 dye and were injected into the right ventricle wall of hearts. Two weeks post-MI, transplanted cells had indeed migrated to the infarcted region in the left ventricle wall. Some of them formed tube-like microvascular structures (Figs. 5A-D, arrow in D). The injection site identified by the visible needle tract confirmed that the cells had been injected into healthy myocardium of the right ventricle wall (Figs. 5E-G). This indicates that the transplanted SCA1⁺/CD31⁺ CSP cells had migrated from the healthy right ventricle wall to the infarct region in the left ventricle wall. These cells that had migrated into the infarcted area were also labeled by antibodies against vWF (Figs. 6A-D). Our results, therefore, suggest that after MI, SCA1⁺/CD31⁺ CSP cells migrate from non-infarct area of myocardium into the ischemic region where they differentiate into endothelial cells and formed tube-like vascular structures.

3.5. Increasing expression of CXCR4 and decreasing expression of E-cadherin on

SCA1⁻/CD31⁻ CSP cells in post-ischemic hearts

We found that expression of CXCR4 was up-regulated in SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells, with evidence that the SDF-1 α /CXCR4 pathway might play an important role in migration of these types of CSP cells from healthy myocardium to the ischemic region after MI [14,15]. We therefore, hypothesized that expression of CXCR4 on SCA1⁻/CD31⁻ CSP cells might be similarly up-regulated after MI. To test this we isolated SCA1⁻/CD31⁻ CSP cells from hearts 3 days post-MI. However, bone marrow-derived CD45⁺ SP cells increased to 35% of total heart SP cells 3 days post-MI [14]. To minimize contamination by these bone-derived SP cells we first depleted CD45⁺ cells using magnetic bead separation as described [14], which yielded 95% purity of CD45⁻ CSP cells from suspensions of post-MI heart cells (Supplementary Fig S3). FACS analysis was then used to assess CXCR4 expression on the SCA1⁻/CD31⁻ CSP cells. In the day 3 post-MI heart the proportion of SCA1⁻/CD31⁻ CSP cells expressing CXCR4 was increased 6-fold compared to the same subpopulation of cells isolated from control hearts (Figs. 7A-G). The detection of CD45-depleted CSP cells was blocked by verapamil (50 mM), confirming that CD45 depletion did not alter the SP phenotype (Fig. 7D).

Since CSP cells migrate after MI we questioned whether they might alter their expression of the major cell-cell adhesion molecule, epithelial cadherin (E-cadherin). To test this idea SCA1⁻/CD31⁻, SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells from 3-day infarcted and sham-operated hearts were isolated by FACS and subjected to qRT-PCR analysis. Notably, after MI the expression of E-cadherin was significantly

decreased in SCA1⁻/CD31⁻ cells, by comparison with cells isolated from control hearts. Similar results were found for the SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP subpopulations (Fig. 7H). Our findings are consistent with previous studies that suggested that down-regulation of E-cadherin might convert immobile cells into migratory cells [19].

3.6. Migration of SCA1⁻/CD31⁻ CSP cells was induced by SDF-1 α via CXCR4 *in vitro*

SDF-1 α was shown to act via its receptor CXCR4 to induce chemotaxis in SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells [14, 15]. Since SCA1⁻/CD31⁻ CSP cells express CXCR4, we speculated that SDF-1 α might induce chemotaxis of SCA1⁻/CD31⁻ CSP cells in a similar manner. To examine this, an *in vitro* chemotaxis assay was performed, which revealed a dose-dependent migratory response of SCA1⁻/CD31⁻ CSP cells towards a SDF-1 α gradient (Fig. 8). The chemotactic response to SDF-1 α was significantly blocked by an anti-CXCR4 antibody, confirming the role of SDF-1 α /CXCR4 interaction in this migratory response (Fig. 8).

4. Discussion

This is the first study to characterize SCA1⁻/CD31⁻ CSP cells in the adult mouse heart. We and others, previously reported that SCA1⁺/CD31⁻ CSP cell were capable of differentiation into cardiomyocyte and endothelial cells *in vitro* and *in vivo* [7, 14]. Later, we reported that SCA1⁺/CD31⁺ CSP cells served as cardiac endothelia progenitor cells [15]. The role of SCA1⁻/CD31⁻ CSP cells has remained unknown.

Since SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells are enriched for stem/progenitor cell activity [7, 14, 15], we speculated that SCA1⁻/CD31⁻ CSP cells might represent another population of stem/progenitor cells in the adult mouse heart. In order to characterize these cells, we compared the gene expression profile of SCA1⁻/CD31⁻ CSP cells with SCA1⁺/CD31⁻ (which express genes associated with cardiomyocyte and endothelial early development) and SCA1⁺/CD31⁺ CSP cells (which express endothelial early commitment genes) [14, 15]. Our data showed that SCA1⁻/CD31⁻ CSP cells expressed CD133, a gene associated with endothelial progenitors. They did not express genes associated with endothelial cell differentiation (VEGFR1, VEGFR2 and Tie2) nor an early cardiac commitment gene (Nkx2.5). In contrast, SCA1⁺/CD31⁻ cells expressed Nkx2.5 and CD133, but not VEGFR1, VEGFR2 and Tie2, further confirming their roles as progenitors for both cardiac and endothelial cells [14]. SCA1⁺/CD31⁺ CSP cells expressed CD133, VEGFR1, VEGFR2 and Tie2, consistent with our previous study [15]. Notably, these three sub-population cells strongly expressed ABCG2, consistent with their shared dye-extruding phenotype. A summary for the gene expression patterns of SCA1⁺/CD31⁺, SCA1⁺/CD31⁺ and SCA1⁺/CD31⁺ CSP cells is shown in Table 1. **Importantly, SCA1⁻/CD31⁻ CSP cells were capable of DiI-Ac-LDL uptake, suggesting that they were endothelial progenitors rather than hematopoietic progenitors.** In summary, these results suggest that SCA1⁻/CD31⁻ CSP cells might serve as cardiac endothelial progenitor cells in the adult mouse heart.

We further found that SCA1⁻/CD31⁻ CSP cells displayed a much higher capacity for colony-forming and cellular renewal than SCA1⁻/CD31⁻ MP cells. This result is in

accordance with previous studies in SCA1⁺/CD31⁻, SCA1⁺/CD31⁺ CSP cells and SP cells from other tissues, showing that SP cells, in general, have a much higher colony forming capacity compared to non-SP cells [7, 15]. The SCA1⁻/CD31⁻ CSP cells were capable of differentiation into mature endothelial cells, forming vascular tube-like structures *in vivo* and *in vitro*.

Following MI, the majority of cardiomyocytes and blood vessels die by necrosis and apoptosis [20, 21]. Resident stem cells within the infarcted area probably suffer the same fate. Stem and progenitor cells migrating from healthy surrounding myocardium will therefore play an important role in endothelial reconstruction and repair of damaged myocardium after MI. In this study, we observed that transplanted SCA1⁻/CD31⁻ CSP cells were able to migrate from the non-ischemic area to the ischemic region, where they formed vascular structures to aid in repopulating vessels. This is similar to SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells [14, 15]. We also found that the expression of CXCR4 was markedly increased on SCA1⁻/CD31⁻ CSP cells after MI. SDF-1 α is up-regulated in the ischemic myocardium [14, 15, 22, 23]. Our *in vitro* assays demonstrated that SDF-1 α can induce migration of SCA1⁻/CD31⁻ CSP cells and that this migration could be blocked by antibody specific for CXCR4. Together these findings suggest that the SDF-1 α /CXCR4 pathway may play an important role in the homing of SCA1⁻/CD31⁻ CSP cells to the injured myocardium following MI.

E-cadherin, a major adhesion junction molecule, has a role in maintaining the adhesiveness and quiescent status of stem and progenitor cells, preventing them from

441 becoming motile within their niches [24, 25]. Consequently alteration in expression of
442 adherent junctions may affect the physical interaction between stem cells and their
443 supporting cells. When stem cells lose contact with the neighboring cells, they change
444 location more easily within the niche [24, 26]. In our study, expression of E-cadherin
445 was detected in all CSP cells. Interestingly, we found that expression of E-cadherin
446 was significantly decreased on all CSP cells after MI compared to CSP cells from
447 sham-operated hearts. These findings are consistent with previous studies that
448 suggested that down-regulation of E-cadherin might convert immobile cells into
449 migratory cells and thereby promote cell migration [19, 27, 28]. Together our results
450 suggest that decreased expression of E-cadherin might facilitate CXCR4-expressing
451 CSP cells out of their niches and toward a gradient of SDF-1 α arising from the
452 infarcted region. The role of adherent junction proteins in CSP cells migration will be
453 subjected to future study.

454 Our findings may have relevance to MI in humans. Emmert and colleagues have
455 recently demonstrated an increased number of human CSP cells in the ischemic
456 myocardium of patients following myocardial infarction [29]. The ability of CSP cells
457 to respond to endogenous stimuli, may offer great potential in cell therapy for
458 ischemic heart diseases [30].

459 Our previous studies demonstrated that lung SP cells were located in blood vessel
460 wall and mesenchyme of murine embryonic lung. Lung SP cells consisted of different
461 subpopulations [11]. Recently, SCA1⁺/CD31⁺ CSP cells were found to be distributed
462 within blood vessels in the adult mouse heart [15]. In the current study, we found that

these cells predominantly resided in the mesenchyme of myocardium. Based upon their surface expression of SCA1 and CD31, gene expression profiles and location of CSP cells, we speculate that the CSP is composed of three subpopulations of cells: SCA1⁺/CD31⁻, SCA1⁺/CD31⁺ and SCA1⁻/CD31⁻ CSP cells. This is reminiscent of SP cells isolated from murine embryonic lung, which likewise contains several subpopulations [11]. Taken together, our data may explain the capacities of: (1) SCA1⁺/CD31⁻ CSP cells to differentiate into cardiomyocytes and endothelial cells [7, 14]; (2) SCA1⁺/CD31⁺ CSP cells to differentiate into endothelial cells, but not cardiomyocytes [7, 15], and (3) SCA1⁻/CD31⁻ CSP cells to differentiate into endothelial cells. Lack of expression of Nkx2.5 indicated that SCA1⁻/CD31⁻ CSP cells might not be able to differentiate into cardiomyocytes.

SCA1⁻/CD31⁻ CSP cells are located close to SCA1⁺/CD31⁺ CSP cells in the myocardium and both populations express CD133. However the transcript profile of SCA1⁺/CD31⁺ CSP cells is close to that of endothelial cells suggesting they are more committed to the endothelial phenotype. SCA1⁻/CD31⁻ and SCA1⁺/CD31⁺ CSP cells could be part of the same lineage (different stages of commitment) or they may derive from distinct populations. This issue remains to be determined.

5. Conclusion

In this study, we have characterized the SCA1⁻/CD31⁻ CSP cells from the adult mouse heart. Based on their gene expression profile, location, and ability in self-renewal, proliferation, differentiation, cellular uptake DiI-AC-DLD,

vascularization and migration *in vitro* and *in vivo*, we suggest that SCA1⁺/CD31⁺ CSP cells may serve as a source of endothelial progenitor cells in the adult mouse heart. By understanding their function and finding ways to enhance their proliferation, differentiation and migration, these cells may offer potential for the development of novel therapeutic strategies to promote vessel regeneration and myocardial repair in ischemic heart disease.

Authorship contributions

X. Wang, R. Gao and P. Sun performed experiments and wrote the manuscript; S. Liu and Y. Xu carried out experiments; D. Z. Liang and L. Yin performed analysis and interpretation of data; W.D. Phillips analyzed data and edited the manuscript; S. X. Liang designed and organized the entire research, analyzed the data, and edited the manuscript. All the authors have read and proved the final revised version of manuscript to be submitted.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [31].

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Figure Legends

Figure 1. Isolation of CSP and MP cells from the adult mouse heart by FACS and comparison of SCA1⁻/CD31⁻, SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells for their

gene expression. (A) CSP cells (box at lower left) and MP cells (box to right) were identified based upon their intensity of Hoechst staining. Cardiac SP cells represented approximately 1.0% of the total heart cell population. This low-Hoechst staining subpopulation disappeared after pretreatment with verapamil, which blocks the ABCG2 transporter blocker (box at upper right). (B) Based on expression of SCA1 and CD31, CSP cells were further separated into SCA1⁻/CD31⁻, SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells representing approximately 22%, 10% and 67% of the total CSP cells respectively. Cardiac MP cells were separated into SCA1⁻/CD31⁻ cells by the same procedures (data not shown). (C) SCA1⁻/CD31⁻ CSP cells were enriched to 98% after sorting. (D) qRT-PCR analysis of SCA1⁻/CD31⁻, SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP cells to assess their expression of genes for stem cell (ABCG2), endothelial progenitor (CD133) and early cardiac commitment (Nkx2.5) phenotypes. Data represent means $\pm$ SD (n = 3 experiments). SP: side population. MP: main population. CSP: cardiac SP.

Figure 2. Colony-forming assay of CSP cells. (A) Representative colony formed by cardiac SCA1⁻/CD31⁻ CSP cells in a methylcellulose assay visualized by phase contrast microscopy. (B) Representative image of SCA1⁻/CD31⁻/Hoechst MP cells. (C) SCA1⁻/CD31⁻ CSP cells formed many more colonies than SCA1⁻/CD31⁻ MP cells ($p < 0.01$). (D) The expression of stem- (ABCG2) and endothelial progenitor- (CD133) cell specific genes analyzed by qRT-PCR. Colony-forming cells isolated from Methocult-media are compared with freshly isolated SCA1⁻/CD31⁻ CSP cells. (E)

Expression of SCA1 by colony-forming cells isolated from Methocult-media are compared with freshly isolated SCA1⁻/CD31⁻ and SCA1⁺/CD31⁻ CSP cells by qRT-PCR. Data represent means $\pm$ SD (n = 3 experiments). # $p > 0.05$.

Figure 3. *In vitro* differentiation SCA1⁻/CD31⁻ CSP cells. **(A)** Representative photomicrographs show SCA1⁻/CD31⁻ CSP cells forming vascular networks after 2 weeks in culture under differentiation-inducing conditions (see Methods). Scale bar is 35 μ m; **(B)** Quantitative RT-PCR results, compare relative expression of the ABCG2, CD133, vWF and Tie2 genes in freshly isolated SCA1⁻/CD31⁻ CSP (black bars) and SCA1⁻/CD31⁻ CSP cells after 2 weeks in culture (grey bars). Data represent means $\pm$ SD (n = 3 experiments).

Figure 4. Distribution of ABCG2⁺/SCA1⁻ (SCA1⁻/CD31⁻) CSP cells in adult mouse heart. **(A-D)** A representative microscope field from a section through the myocardium showing immunofluorescence for the stem cell marker, ABCG2 (A; red) and for the cardiomyocyte marker, α -actinin (B; green), nuclei counter-stained with DAPI (C; blue) and the merged fluorescence images (D). Arrows in D indicate ABCG2⁺ (CSP) cells. **(E-H)** A serial section stained for the stem cell specific marker ABCG2 (E; red) and SCA1 (F; green), nuclei (G; blue) and the merged images. Arrows in H indicate two ABCG2⁺ cells, one of which is a SCA1⁻ (SCA1⁻/CD31⁻) CSP cell. Scale bars are 50 μ m.

Figure 5. *In vivo* migration and differentiation of transplanted SCA1⁺/CD31⁺ CSP cells. Purified SCA1⁺/CD31⁺ CSP cells injected into the healthy myocardium of the right ventricle were visualized by PKH26 fluorescence 14 days post-MI. **(A-D)** Section through the myocardium 14 days after cell injection and artery ligation. **(A)** Immunofluorescence staining for the muscle marker, α -actinin (green), delineates the border between healthy myocardium and the infarcted region (dark). **(B)** PKH26 fluorescence labeling (red) reveals transplanted SCA1⁺/CD31⁺ CSP cells that had migrated from healthy myocardium into the infarcted area. **(C)** DAPI labeling (blue) reveals nuclei. **(D)** Merge of the three fluorescence channels. Transplanted SCA1⁺/CD31⁺ CSP cells formed a vascular tube-like structure (arrow) within the ischemic myocardium. Scale bar is 25 μ m. **(E-G)** Identification of the cell injection site. **(E)** Residual PKH26 dye (red) staining reveals the needle tract, demarking the cell injection site within the healthy myocardium in the right ventricle. **(F)** Immunofluorescence staining for α -actinin expression (green). **(G)** Merge of the two fluorescence channels confirms normal myocardium architecture at the injection site. The point of needle entry into the myocardium is indicated by an arrow. Scale bar is 25 μ m.

Figure 6. Endothelial differentiation of transplanted SCA1⁺/CD31⁺ CSP cells. **(A-D)** A section through the infarcted myocardium 14 days post-MI reveals endothelial differentiation and vascularization. **(A)** Immunofluorescence staining of vWF (green). **(B)** PKH26 dye (red) reveals transplanted SCA1⁺/CD31⁺ CSP cells. **(C)** Dapi (blue)

reveals nuclei. **(D)** Merge of the three fluorescence channels. Yellow merged fluorescence shows that the transplanted SCA1⁺/CD31⁺ CSP cells expressed vWF and formed vessel-like structure (arrow). Scale bar is 30 μ m.

Figure 7. Increased expression of CXCR4 on SCA1⁺/CD31⁺ CSP cells 3 days post-MI. Contaminating CD45⁺ cells were first depleted from the heart cell suspension before FACS analysis. **(A)** Cell preparation from normal/control heart (sham-operation, no LADCA ligation) showing gating for CSP cells. **(B)** Further gating of CSP cells from normal/control heart to isolate SCA1⁺/CD31⁺ CSP cells (22.1% of CSP cells). **(C)** Approximately 9% of SCA1⁺/CD31⁺ CSP cells from normal heart/control expressed CXCR4 (inset shows results with the IgG isotype control used to determine anti-CXCR4 gating). **(D)** Gating used to isolate CSP cells from heart cell suspensions 3 days post-MI. The CSP sub-population disappeared in the presence of verapamil (inset). **(E)** Further gating to isolate SCA1⁺/CD31⁺ CSP cells (21.9%) from the 3-day post-MI heart. **(F)** In 3-day post-MI heart 56.3% of SCA1⁺/CD31⁺ CSP cells expressed CXCR4. **(G)** The average intensity of CXCR4 labeling on SCA1⁺/CD31⁺ CSP cells was at least 6-fold higher for post-MI heart compared to SCA1⁺/CD31⁺ CSP cells of normal/control heart. **(H)** Expression of E-cadherin mRNA in CSP cells before and after MI (open and grey bars respectively). Expression of E-cadherin was significantly decreased after MI. Data represent means $\pm$ SD (n = 3 experiments).

Figure 8. Chemotactic response in SCA1⁺/CD31⁺ CSP cells to SDF-1 α . Cells were

705 plated on one side of a polycarbonate membrane and the number that migrated
706 through the membrane toward a compartment containing SDF-1 α (50 ng/ml, 100
707 ng/ml and 500 ng/ml, black columns) was measured. Bars show a dose-dependent
708 chemotactic response of SCA1⁺/CD31⁺ CSP cells to SDF-1 α compared with control
709 medium (no SDF-1 α , white column). The chemotactic response of SCA1⁺/CD31⁺ CSP
710 cells to 500 ng/ml SDF-1 α was blocked by a CXCR4 antibody (grey column). Data
711 represent means $\pm$ SD (n=3 experiments).

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731 Table1. Gene expression of SCA1⁻/CD31⁻, SCA1⁺/CD31⁻ and SCA1⁺/CD31⁺ CSP

732 cells

	SCA1 ⁻ /CD31 ⁻	SCA1 ⁺ /CD31 ⁻	SCA1 ⁺ /CD31 ⁺
ABCG2	+	+	+
CD133	+	+	+
Tie2	-	-	+
VEDFR1	-	-	+
VEDFR2	-	-	+
Nkx2.5	-	+	-

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