SUPPLEMENTAL MATERIAL

for

Young Bone Marrow Sca-1 Cells Rejuvenate the Aged Heart by Promoting Epithelial-to-Mesenchymal Transition

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Supplemental Methods

Animal procedures

The Animal Care Committee of the University Health Network approved all experimental procedures, which were carried out according to the Guide for the Care and Use of Laboratory Animals (NIH, revised 2011).

Sca-1$^+$ and Sca-1$^-$ BM cells from young (Y) GFP transgenic mice were used to reconstitute irradiated old (O) wild type recipient mice, generating Y(Sca1$^+$)-O and Y(Sca1$^-$)-O chimeras (Figure S1A). BM of C57BL/6-Tg-GFP mice aged 2–3 months was flushed from the tibias and femurs and mononuclear cells were separated by density gradient centrifugation, and then separated into Sca-1$^+$- and Sca-1$^-$-labeled fractions by immunomagnetic activated cell sorting following the manufacturer's instruction (Stem Cell Technology, Cat#: 18756). The purity of positive cells was confirmed by flow cytometry. Female C57BL/6 mice aged 20–22 months [old (O) recipients] were lethally irradiated (9.5 Gy) and immediately received an infusion (through the tail vein) of fresh Sca-1$^+$ or Sca-1$^-$ BM cells (2x10^6) from young (Y) donor mice, generating Y (Sca-1$^+$)-O chimeras and Y(Sca-1$^-$)-O chimeras. Male Sca-1 knockout (KO) mice (C57BL/6 background) and their WT littermates aged 2–3 months were used in this study. Sca-1 KO mice were kindly provided by Dr William L. Stanford [1] and reconstituted with WT BM Sca-1$^+$ cells as described above.

Epicardium-derived cell culture

EPDCs isolated from 20–22 month old mice were cultured as previously described [2, 3].Briefly, hearts were removed aseptically from mice, and washed with ice-cold nominally

calcium-free KH buffer. After rinsing, hearts were placed in 0.25% trypsin-EDTA (1 mM) at room temperature. After 20 min, the epicardial surface of the heart was gently scraped, and the resulting cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal calf serum. The medium was changed after 1 h to remove non-attached cells and subsequently every 3 days. The purity of EPDCs was confirmed by immunofluorescent staining of WT1 (wilms tumor 1, a specific marker for epicardial cells). Cells in passage 2 were used for the experiments.

Hypoxia conditions and co-culture studies

EPDCs were isolated and co-cultured with bone marrow (BM) Sca-1^+ cells or Sca-1^- cells under normoxia and hypoxia (0.1% O_2) conditions. To test cell proliferation, MTT [3-(4,-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide, Sigma, Cat#: M2128] assay and BrdU (5-bromo-2'-deoxyuridine) labeling were carried out. For MTT assay, co-culture was carried out under hypoxia condition for 1, 3, 5, 7 days respectively. For BrdU labeling, EPDCs were co-cultured with BM Sca-1^+ cells or Sca-1^- cells under normoxia and hypoxia conditions for 72 h with BrdU (10μM) for labeling of proliferative cells. Cell migration was evaluated by the transwell and wound-scratch assays after co-culture for 24 h. To test the changes in EMT related genes and proteins, co-culture was carried out for 72 h.

For the co-culture studies, two transwell systems were used. For the transwell system used to investigate EPDC migration, Young Sca1^+ or Sca1^- BM cells ($1x10^5/cm^2$) in serum-free DMEM medium were cultured in the lower compartment. EPDCs ($2x10^4/cm^2$) were plated in the transwell cell culture inserts (8-μm diameter pores). For all the other assays related to co-culture (MTT assay, BrdU labeling, RT-qPCR, immunostaining, Western blot), young BM Sca1^+ or

$Sca1^-$ cells ($1x10^5/cm^2$) in serum-free DMEM medium were plated in the transwell cell culture inserts (1-μm diameter pores). EPDCs ($2x10^4/cm^2$) were cultured in the lower compartment.

Cell proliferation assay

EPDCs isolated from 20–22 month old mice were seeded into 96-well plates ($4x10^3$/well), and co-cultured with young $Sca1^+$ or $Sca1^-$ BM cells ($2x10^4$/well) in serum-free DMEM medium under normoxia and hypoxia (0.1% O_2) conditions. An MTT assay was used to detect viable proliferating cells at 1, 3, 5, and 7 days after co-culture. The assay was normally carried out in half-area flat-bottomed microtitre trays. Each well contained the cells to be tested with the culture medium removed. Supernatant removal was accomplished by inverting, flicking and blotting the plate. To the cells in each well added 50ul of 1mg/ml solution of MTT in PBS. The tray was gently shaken and incubated for 3-4h at 37^0C. Then remove the untransformed MTT carefully, add propanol 50ul to each well. The optical density of each well was measured using an automatic plate reader with a 560 nm test and a 690 nm reference wavelength. For BrdU staining, the serum-free DMEM medium was supplemented with BrdU (10 μM/mL). After co-culture with young $Sca1^+$ or $Sca1^-$ BM cells under normoxia and hypoxia conditions for 72 h, the cells were fixed for immunofluorescent staining with BrdU (Abcam, Cat#: ab6326).

Cell migration assay

Wound-scratch and transwell assays were used to investigate EPDC migration. Briefly, EPDCs isolated from 20–22 month old mice were passed into the wells ($2x10^4/cm^2$). After a scratch was created with a p200 pipette tip, cell debris was removed. Young $Sca1^+$ or $Sca1^-$ BM cells ($1x10^5/cm^2$) in serum-free DMEM medium were added to the well. After 24 h in normoxia

and hypoxia conditions, images were obtained using a microscope (Nikon Eclipse Ti) after washing. The transwell system was also used to investigate EPDC migration. Young $Sca1^+$ or $Sca1^-$ BM cells ($1x10^5/cm^2$) in serum-free DMEM medium were cultured in the lower compartment. EPDCs ($2x10^4/cm^2$) were plated in the transwell cell culture inserts (8-μm diameter pores). After 24 h in normoxia and hypoxia conditions, cells on the upper layer of the insert membrane were completely removed by a cotton swab. EPDCs that migrated to the other side of the membrane were fixed and stained with 0.5% crystal violet.

Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated with TRIzol reagent (Invitrogen, Grand Island, NY) and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and random primers. Real-time polymerase chain reaction was conducted using SensiFAST SYBR Green PCR Master Mix (Bioline USA Inc., Taunton, MA) with the following parameters: 95^o 2 min; [95^o 5 s; 60^o 30 s for 40 cycles]. The oligonucleotide primer sequences are shown in Table S1.

In vitro EMT induction

Young $Sca1^+$ or $Sca1^-$ BM cells ($1x10^5/cm^2$) in serum-free DMEM medium were co-cultured with EPDCs isolated from 20–22 month old mice ($2x10^4/cm^2$) under normoxia and hypoxia conditions to induce EMT. After 72h, the mesenchymal and epithelial markers of EPDCs were detected. The medium was supplemented with growth factors TGF-β1 (5 ng/ml, R&D system, Cat#: 7754-BH) or TGF-β1 blocker MAB 240 (1 μg/mL) [R&D Systems Inc., Minneapolis, MN] in the experiments indicated in the Results section.

Immunofluorescent staining

Hearts were fixed in 2% paraformaldehyde (PFA) for 24 h after being well perfused with PFA and were then stored in 0.5M sucrose at 4 ^{o}C overnight. Hearts were then embedded with OCT, and 5-μm-thick frozen sections were prepared. Slides were incubated with one of the following primary antibodies: Alexa488 conjugated anti-GFP (Invitrogen, Cat#: A21311, 1:400) or Goat anti-GFP (Abcam, Cat#: ab6673, 1:400), anti-α-SMA (Sigma, Cat#: A-2547, 1:400), anti-Calponin (Abcam, Cat#: ab46794, 1:100), anti-ZO1 (Abcam, Cat#:59720, 1:100), anti-Vimentin (Abcam, Cat#:11256, 1:100), anti-WT1 (Santa Cruz, Cat#: SC-192, 1:100), anti-BrdU (1:80; Abcam, Cat#: ab6326) at room temperature for 2 h. Incubation with respective Alexa 488 or 568 or 647 conjugated secondary antibodies (Invitrogen, all 1:400) was carried out at room temperature with light protection for 1 h. The nuclei were identified with DAPI. The number of positive cells in 6 randomly selected high-power fields per section was determined and quantified as % out of total cells ($DAPI^{+}$) in the corresponding high-power fields, then averaged for 6 sections (6 mouse hearts) with a Nikon fluorescent microscope. An Olympus Fluoview 2000 laser scanning confocal microscope was used to confirm the co-localization of fluorescent signals. To detect cell proliferation after MI, BrdU (50 mg/kg, Sigma) was administered to mice by intraperitoneal injection for 3 consecutive days. Coronary artery ligation was performed 1 day later.

Flow Cytometry Analysis

BM of C57BL/6-Tg-GFP mice aged 2–3 months was flushed from the tibias and femurs and mononuclear cells were separated by density gradient centrifugation, and then separated into $Sca\text{-}1^{+}$ and $Sca\text{-}1^{-}$ fractions by immunomagnetic activated cell sorting following the

manufacturer's instruction (Stem Cell Technology, Cat#: 18756). The cells were resuspended in PBS supplemented with 2% FBS and 0.1% sodium azide. One million cells were taken for antibody staining (all 1:100 dilution) of APC anti-mouse/human CD11b (Biolegend, Cat#:10211), APC anti-mouse CD31 (Biolegend, Cat#:102509), APC anti-mouse CD45 (Biolegend, Cat#:103112), APC anti-mouse/human CD44 (Biolegend, Cat#:103011), Alexa647 anti-mouse CD34 (Biolegend, Cat#:152205), Rb pAb to CD105 (Abcam, Cat#:ab107595), Purified Anti-Mouse CD90.2 (eBioscience, Cat#:14-0902-81), c-Kit (M-14) goat plyclonal IgG (Santa Cruz, Cat#: SC-1494), PDGFRβ (958) rabbit polyclonal IgG (Santa Cruz, Cat#: SC-432), CD90.1 PE-Cy5 (eBioscience, Cat#:15-0900-82), anti-Flk-1 affinity purified goat IgG (R&D, Cat#: AF644), mAb anti-Integrin beta1/CD29 (Abcam, Cat#: ab179471). All antibody incubation was carried out for 30 min at 4^{o}C in the dark. Alexa647-conjugated donkey anti-rabbit, or chicken anti-goat (Invitrogen, all 1:400) was added for staining with mouse cKit, Flk-1, CD29, CD105, PDGFRβ respectively. Isotype-identical IgG served as controls (Becton Dickinson). Cells were analyzed using a Becton Dickinson LSRII flow cytometer. The fluorescence intensity of 10,000 cells for each sample was quantified.

Western blotting

For Western blotting, 50 μg of lysate was fractionated through a 4% stacking and 10% running SDS-PAGE gel, and the fractionated proteins were transferred to a PVDF membrane. Blots were blocked for 1 h at room temperature with blocking buffer. The antibodies (α-SMA, calponin, and vimentin, all 1:1000) reacted with the blots overnight at 4°C. After washing (3 x 5 min in 1xTBS-0.1% Tween 20), the blots were incubated with horseradish peroxidase-conjugated secondary antibody at 1:2000 dilution for 1 h at room temperature. Visualization was performed

with enhanced chemiluminescence. For quantification, densitometry of the target bands was divided by the corresponding densitometry of the GAPDH (Millipore, Cat#: mab374, 1:5000) band using AlphaImager 2200 software.

Enzyme-linked Immunosorbent Assay (ELISA)

Young $Sca1^+$ or $Sca1^-$ BM cells were cultured in serum-free DMEM medium under normoxia and hypoxia conditions for 72 h. The supernatant and cells were collected and protein concentration was determined using a Bio-Rad DC protein assay kit. The level of TGF-β1 (R&D Systems, Cat#: mb100b) was determined using ELISA following the manufacturer's instruction.

Table S1. Oligonucleotide primer sequences

Gene name	Forward primer	Reverse primer
Snail	ACATCCGAAGCCACACG	GTCAGCAAAAGCACGGTTG
Slug	ACACATTAGAACTCACACTGGG	TGGAGAAGGTTTTGGAGCAG
Krt14	CAAAGACTACAGCCCCTACTTC	TCTGCTCCGTCTCAAACTTG
BVES	GAACAGTATCACCAGCTCCAG	CTTCTACCCCTTCCTCGATTG
Calponin	GAAGGTCAATGAGTCAACTCAGAA	CCATACTTGGTAATGGCTTTGA
Cdh6	GATCCGATTATCAGTACGTGGG	TGTATGTCGCCTGTGTTCTC
Col7a1	GCCTAGCACCTCTTATCAAGTC	CAGGTGATGCTGACAGATGAG
WT1	ATGACCTCCCAGCTTGAATG	GTTCTCACTCTCATACCCTGTG
Tbx18	GGAGTCATACGCATTCTGGAG	CAGGTGAGGATGTGTAGCAG
TGF-β1	CCTGAGTGGCTGTCTTTTGA	CGTGGAGTTTGTTATCTTTGCTG
Pdgfa	TTAACCATGTGCCCGAGAAG	ATCAGGAAGTTGGCCGATG
Pdgfb	CACCGAAAGTTTAAGCACACC	CATTATCACTCCAAGGACCCC
Fgf1	TGGGACAAGGGACAGGAG	TCCTCATTTGGTGTCTGCG
Fgf2	TCTACTGCAAGAACGGCG	CTCCCTTGATAGACACAACTCC
Vegfa	GGCAGCTTGAGTTAAACGAAC	TGGTGACATGGTTAATCGGTC
Vegfc	CAGCCCACCCTCAATACC	CTCCTTCCCCACATCTATACAC
Igf1	CCCCACTGAAGCCTACAAAAG	TCACCTTTCCTTCTCCTTTGC
SCF	TCAAGAGGTGTAATTGTGGACG	GGGTAGCAAGAACAGGTAAGG

Supplemental Figure Legends

Figure S1. Overall experimental design. (A) Schematic diagram of bone marrow (BM) reconstitution. Sca-1^+ and Sca-1^- BM cells from young (Y) GFP (green fluorescent protein) transgenic mice were used to reconstitute irradiated old (O) wild type mice, generating Y(Sca1^+)-O and Y(Sca1^-)-O chimeras, respectively. **(B)** Schematic diagram of experimental design. To detect cell proliferation after myocardial infarction (MI), BrdU (5-bromo-2'-deoxyuridine, 50 mg/kg) was administered to mice by intraperitoneal injection for 3 consecutive days. Coronary artery ligation was performed 1 day later. BM cell homing, epicardial cell activation, and cell proliferation were measured 3 and 7 days post-MI. The epithelial-to-mesenchymal transition (EMT) process of epicardial cells was evaluated 3 days post-MI.

Figure S2. Characterization of Sca-1^+ and Sca-1^- bone marrow cells. Bone marrow (BM) of C57BL/6-Tg-GFP mice aged 2–3 months was separated into Sca-1^+ and Sca-1^- fractions by immunomagnetic activated cell sorting. The GFP$^+$Sca-1^+ **(A)** and the GFP$^+$ Sca-1^- **(B)** BM cells were characterized with the hematopoietic lineage markers (CD45, CD44, CD11b, cKit, CD34, CD90.1), mesenchymal lineage markers (CD105, CD29, CD90.2), endothelial progenitor markers (Flk-1), endothelial marker (CD31) and pericyte marker (PDGFRβ). The number of cKit$^+$ or CD90.1^+ cells in the GFP$^+$Sca-1^+ fraction was significantly higher than that of the GFP$^+$ Sca-1^- fraction. The number of Flk-1^+ or CD31^+ or PDGFRβ^+ in the GFP$^+$Sca-1^+ fraction was significantly higher than that of the GFP$^+$ Sca-1^- fraction. N=6/group, Mean±SD; $p<0.05$.

Figure S3: Total number of WT1^+ Cells in the infarct hearts. Sca-1^+ and Sca-1^- bone marrow (BM, $2X10^6$) cells from young (Y) GFP (green fluorescent protein) transgenic mice were used to reconstitute irradiated old (O, 9.5 Gy) wild type mice, generating Y(Sca1^+)-O and Y(Sca1^-)-O

chimeras, respectively. Twelve weeks after BM reconstitution, coronary occlusion was performed to induce myocardial infarction (MI). Quantification of $WT1^+$ (a specific marker for epicardial cells) cells in the infarct region (from all three layers of heart in the infarct zone) of the Y($Sca1^+$)-O and the Y($Sca1^-$)-O chimeric hearts at 3 and 7 days post-MI. The total number of $WT1^+$ cells in the infarct region of the Y($Sca1^+$)-O chimeric hearts was significantly higher than that in Y($Sca1^-$)-O chimeric hearts at 3 days post-MI. n= 6/group, Mean ±SD; **P<0.01. WT1: wilms tumor 1.

Figure S4: BM Sca-1^+ cells activated EMT of epicardial cells after MI. Bone marrow (BM, $2X10^6$) Sca-1^+ and Sca-1^- cells from young (Y) GFP (green fluorescent protein) transgenic mice were used to reconstitute irradiated old (O, 9.5 Gy) wild type mice, generating Y($Sca1^+$)-O and Y ($Sca1^-$)-O chimeras, respectively. Twelve weeks after BM reconstitution, coronary occlusion was performed to induce myocardial infarction (MI). **(A)** Immunofluorescent staining of smooth muscle actin (SMA), Vimentin and Calponin at 3 days post-MI. Yellow arrows indicate GFP, WT1, and SMA or Vimentin or Calponin triple-positive cells (Donor-derived epicardial cell obtained the mesenchymal phenotype). White arrows indicate WT1 and SMA or Vimentin or Calponin double-positive cells (host-derived epicardial cell obtained the mesenchymal phenotype). **(B)** Quantification of WT1 and SMA or Vimentin or Calponin double-positive cells (host-derived epicardial cell obtained the mesenchymal phenotype) and GFP, WT1, and SMA or Vimentin or Calponin triple-positive cells (Donor-derived epicardial cell obtained the mesenchymal phenotype) in the infarct region of the hearts. n=6/group, Mean ±SD; **P<0.01. EMT: epithelial-to-mesenchymal transition.

Figure S5. Differential gene expression in bone marrow Sca-1$^+$ cells and Sca-1$^-$ cells under normoxia and hypoxia conditions. Bone marrow (BM) Sca-1$^+$ and Sca-1$^-$ cells were isolated from young (Y) GFP (green fluorescent protein) transgenic mice and subjected to normoxia and hypoxia (0.1% O_2) conditions for 72 h. Differential gene expression were determined by RT-qPCR. n=6/group, Mean±SD; *P<0.05,**P<0.01.

Figure S6: Reconstitution of Sca-1 KO mice with WT BM Sca-1$^+$ cells restored EMT response after MI. Sca-1 knock-out (KO) mice were reconstituted with wild type (WT) bone marrow (BM) Sca-1$^+$ cells [2X10^6, (Sca-1$^+$)-KO] for 3 months, then underwent myocardial infarction (MI). **(A)** Immunofluorescent staining of smooth muscle actin (SMA), Vimentin, and Calponin at the infarct region of WT, Sca-1 KO and (Sca-1$^+$)-KO hearts at 3 days post-MI. **(B)** Quantification of WT1$^+$SMA$^+$, WT1$^+$Vimentin$^+$ and WT1$^+$Calponin$^+$ cell out of total cell number (DAPI$^+$) in the infarct region of WT, Sca-1 KO and (Sca-1$^+$)-KO hearts at 3 days post-MI. n=6/group; Mean±SD; * vs WT, P<0.01, # vs KO P<0.05. EMT: epithelial-to-mesenchymal transition.

Supplemental Figures

Figure S1

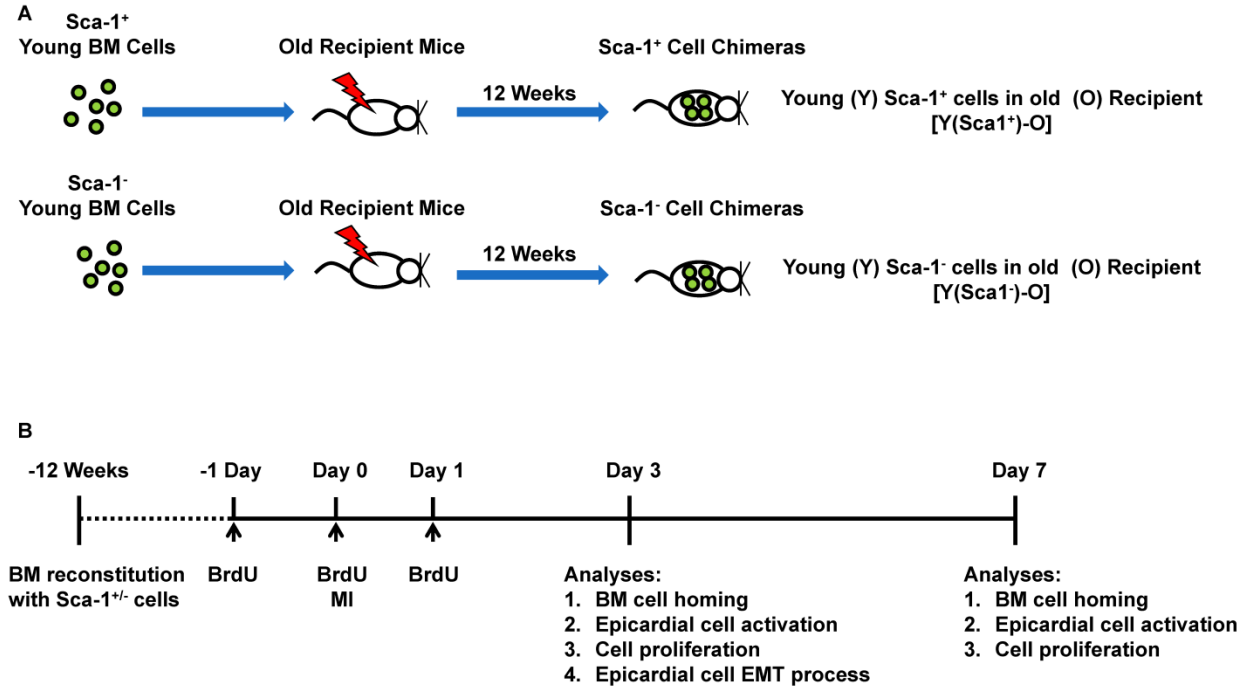
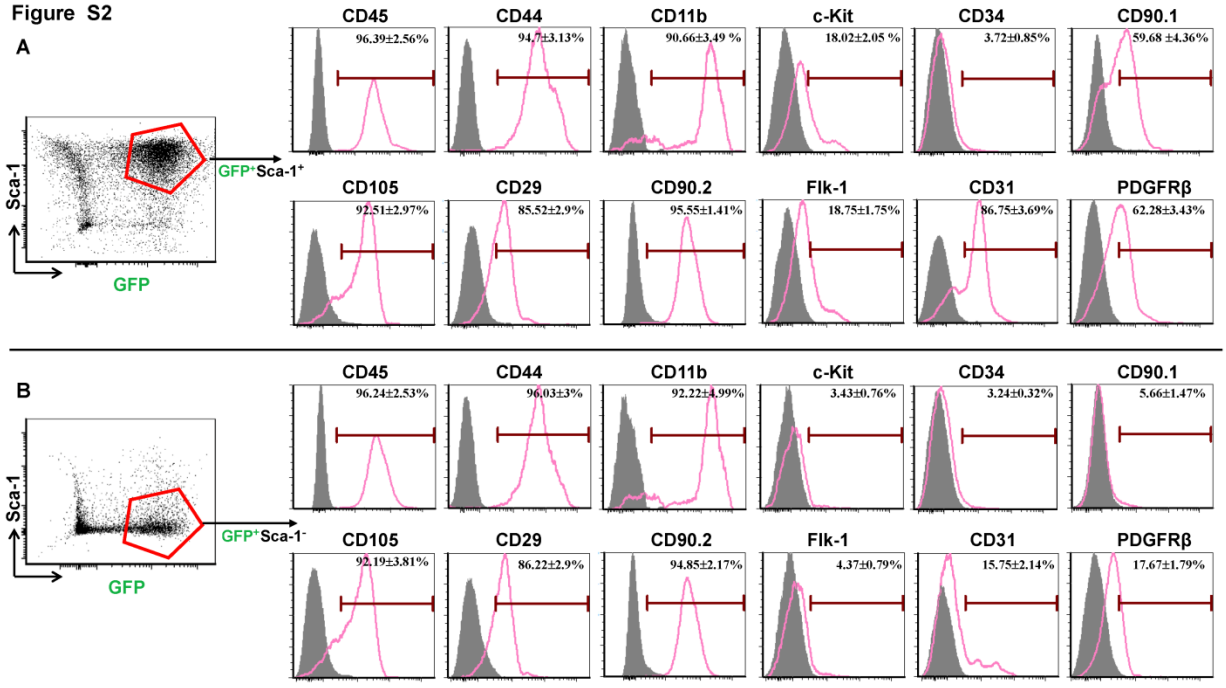


Figure S2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CD44
94.7±3.13%
CD11b
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c-Kit
18.02±2.05 %
CD34
3.72±0.85%
CD90.1
59.68 ±4.36%
CD105
92.51±2.97%
CD29
85.52±2.9%
CD90.2
95.55±1.41%
Flk-1
18.75±1.75%
CD31
86.75±3.69%
PDGFRβ
62.28±3.43%
B
Sca-1
GFP
GFP+Sca-1-
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CD44
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CD11b
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Figure S3

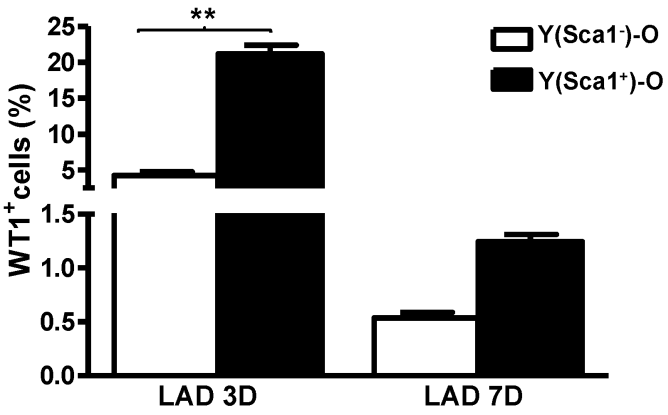
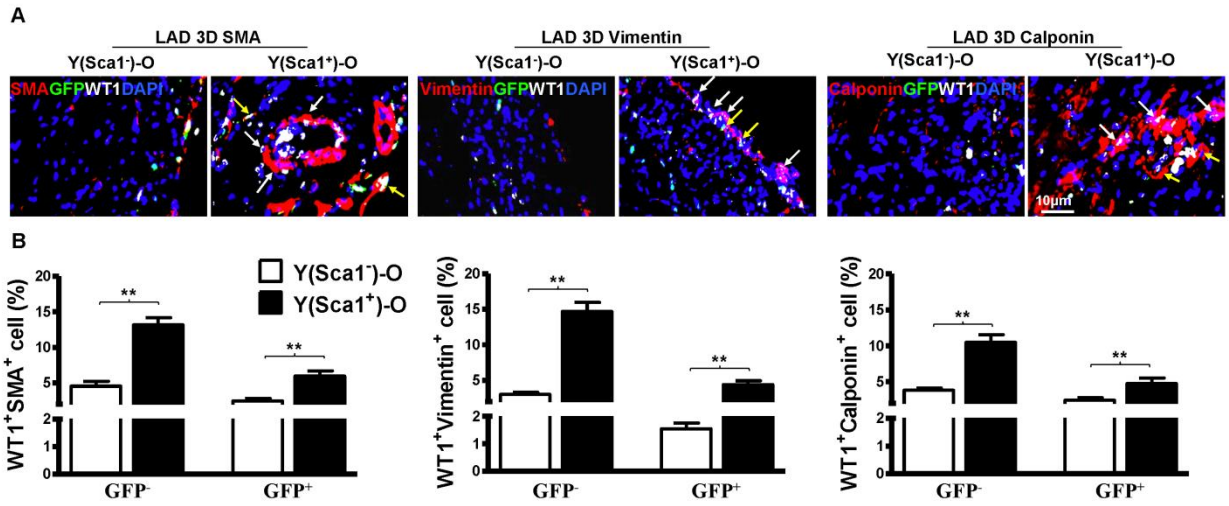


Figure S4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LAD 3D Calponin
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Y(Sca1+)-O
CalponinGFPWT1DAPI
10μm
B
Y(Sca1-)-O
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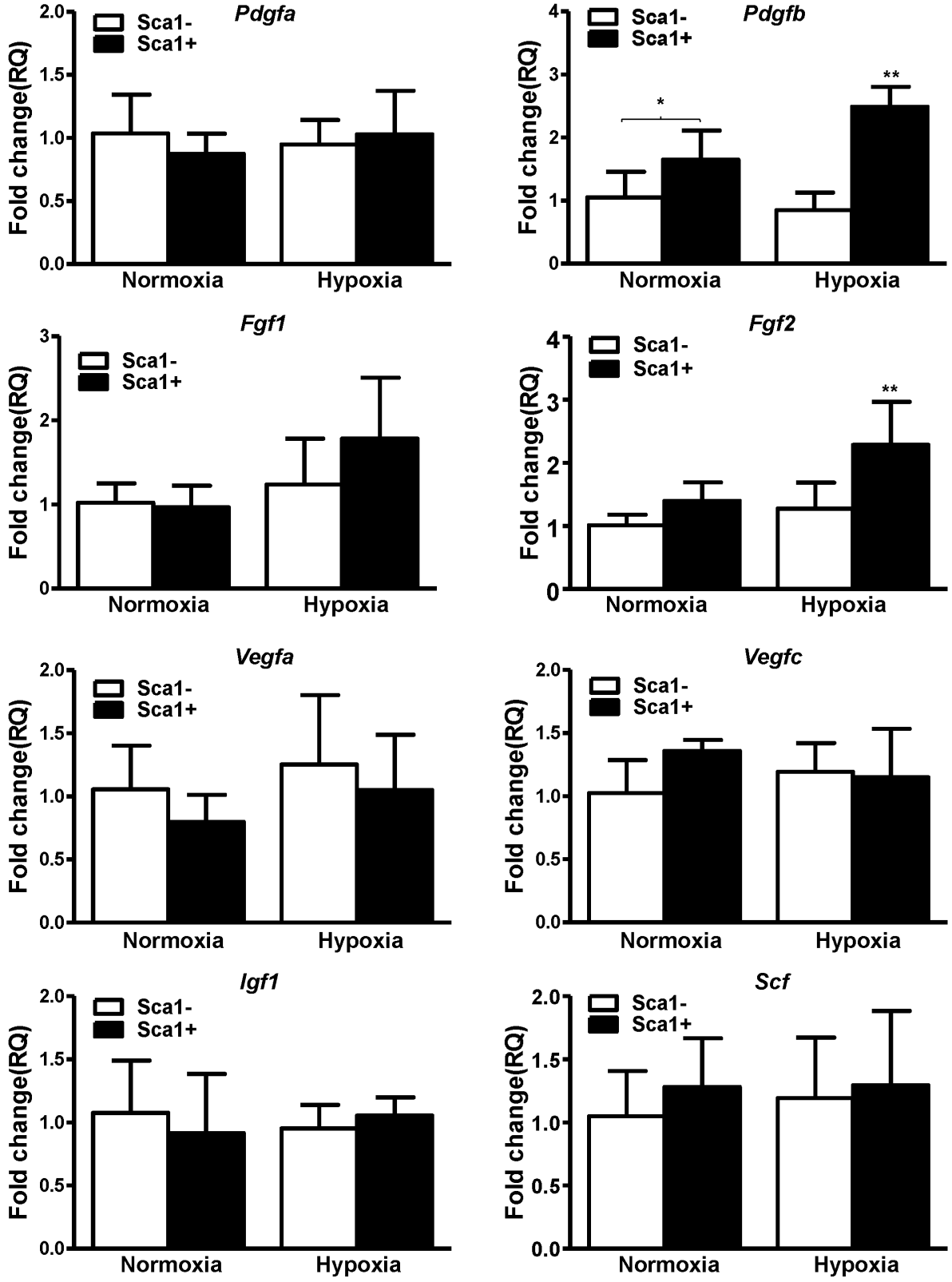
WT1+Calponin+ cell (%)
GFP-
GFP+
**

Figure S5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Sca1-
Sca1+
Fold change(RQ)
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3
2
1
0
*
**
Normoxia
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Fgf1
Sca1-
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Fgf2
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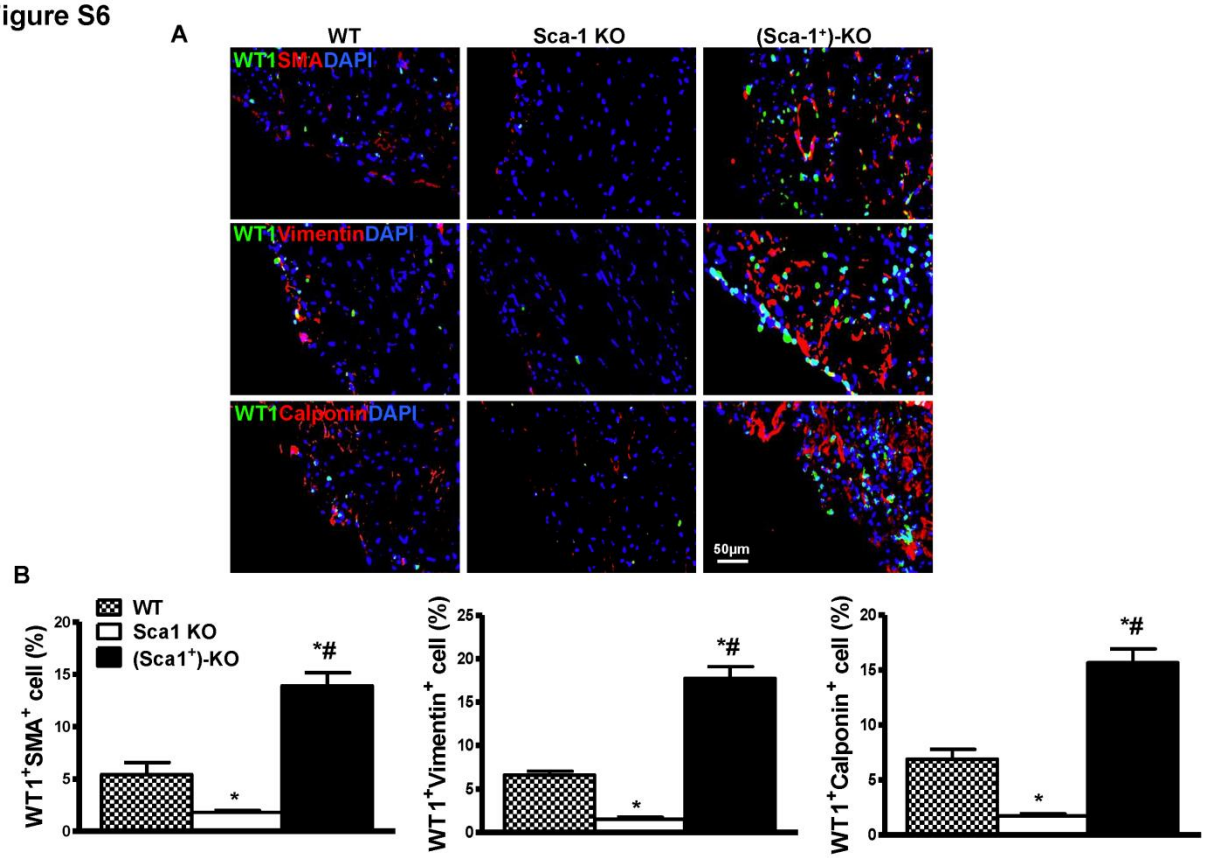
Hypoxia

Figure S6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WT1VimentinDAPI
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50μm
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WT
Sca1 KO
(Sca1+)-KO
WT1+SMA+ cell (%)
WT1+Vimentin+ cell (%)
WT1+Calponin+ cell (%)
*
*#

Supplemental References

1. Ito CY, Li CYJ, Bernstein A, Dick JE, Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood. 2003 Jan 15;101(2):517–23.

2. Winter EM, Grauss RW, Hogers B, van Tuyn J, van der Geest R, Lie-Venema H, et al. Preservation of left ventricular function and attenuation of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart. Circulation. 2007 Aug 21;116(8):917–27.

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