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Supplemental Information

Neonatal Apex Resection Triggers Cardiomyocyte Proliferation, Neovascularization and Functional Recovery Despite Local Fibrosis

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Fig.S1

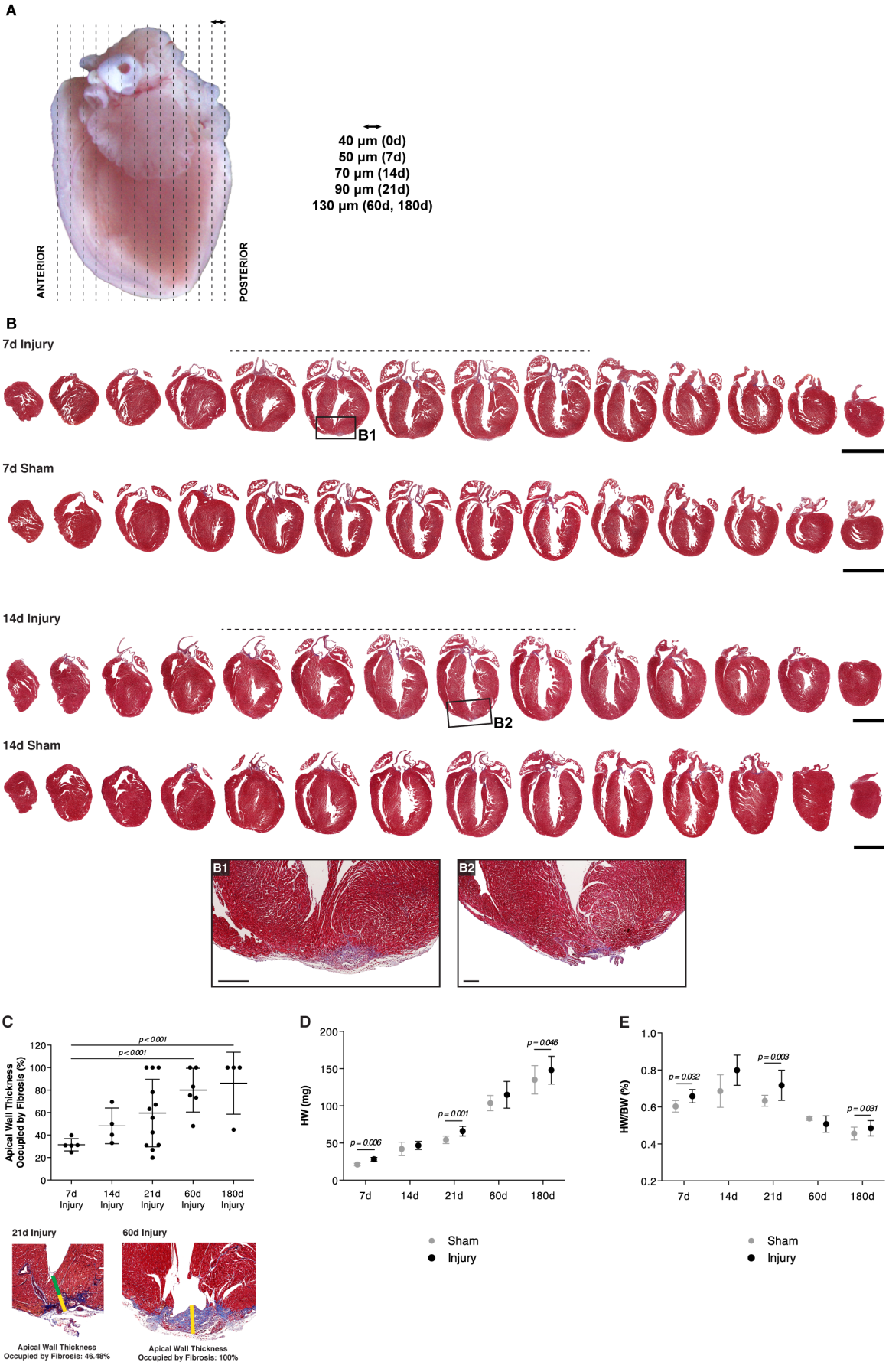
A
ANTERIOR
POSTERIOR
40 μm (0d)
50 μm (7d)
70 μm (14d)
90 μm (21d)
130 μm (60d, 180d)
B
7d Injury
B1
7d Sham
14d Injury
B2
14d Sham
B1
B2
C
Apical Wall Thickness Occupied by Fibrosis (%)
p < 0.001
p < 0.001
7d Injury
14d Injury
21d Injury
60d Injury
180d Injury
21d Injury
60d Injury
Apical Wall Thickness Occupied by Fibrosis: 46.48%
Apical Wall Thickness Occupied by Fibrosis: 100%
D
HW (mg)
p = 0.006
p = 0.001
p = 0.046
7d
14d
21d
60d
180d
Sham
Injury
E
HW/BW (%)
p = 0.032
p = 0.003
p = 0.031
7d
14d
21d
60d
180d
Sham
Injury

Figure S1 – Schematic Representation of Heart Sampling (Related to Experimental Procedures) & Cardiac Remodeling After Neonatal Apex Resection (Related to Figure 1)

(A) Representative sectioning was adjusted to the heart size (i.e. 40µm, 50µm, 70µm, 90µm and 130µm, between each section for 0d, 7d, 14d, 21d and 60/180d post resection, respectively), yielding approximately the same number of sections. **(B)** Representative MT stained sections of apex resected and surgical controls 7 and 14d following surgery. Sections exhibiting myocardial disruption and/or cardiac fibrosis are highlighted by a dashed line (scale bars, 2mm). High magnification images of the injury site (B1: 7d, B2: 14d) show collagen (blue staining) (scale bars, 250µm). **(C)** Percentage of left ventricle wall occupied by fibrosis in apex region was determined at 7d (n=5), 14d (n=4), 21d (n = 12), 60d (n = 6) and 180d (n = 4). **(D and E)** Heart weight (from left to right, n =4, 7, 3, 4, 8, 10, 5, 7, 19, 17) and heart to body weight ratio (from left to right, n = 4, 7, 3, 4, 8, 12, 3, 7, 19, 17). All values are presented as means ± SD.

Fig.S2

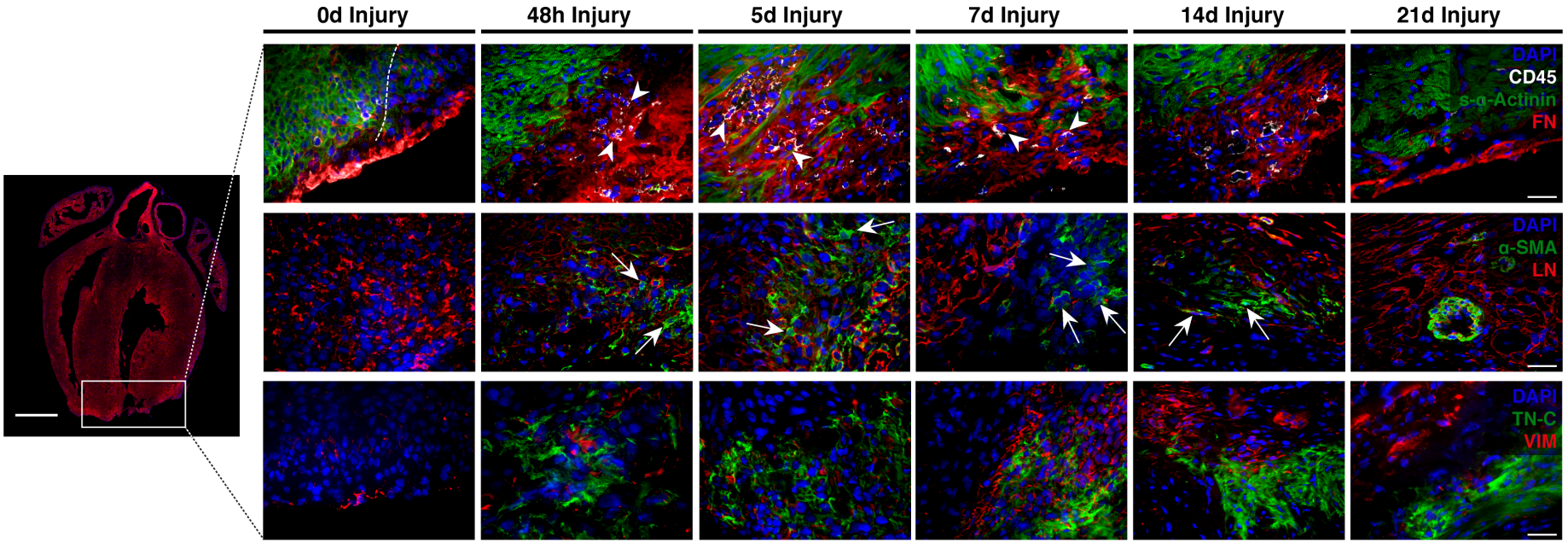

Figure S2 – Time-specific Deposition of Extracellular Matrix Correlates with Cellular Dynamics (Related to Figure 2)

Arrowheads: $CD45^+$ hematopoietic cells; arrows: αSMA^+ myofibroblasts; Low magnification image (scale bar, 500μm), High magnification images (scale bars, 30μm) (n = 4 for each time point).

Fig.S3

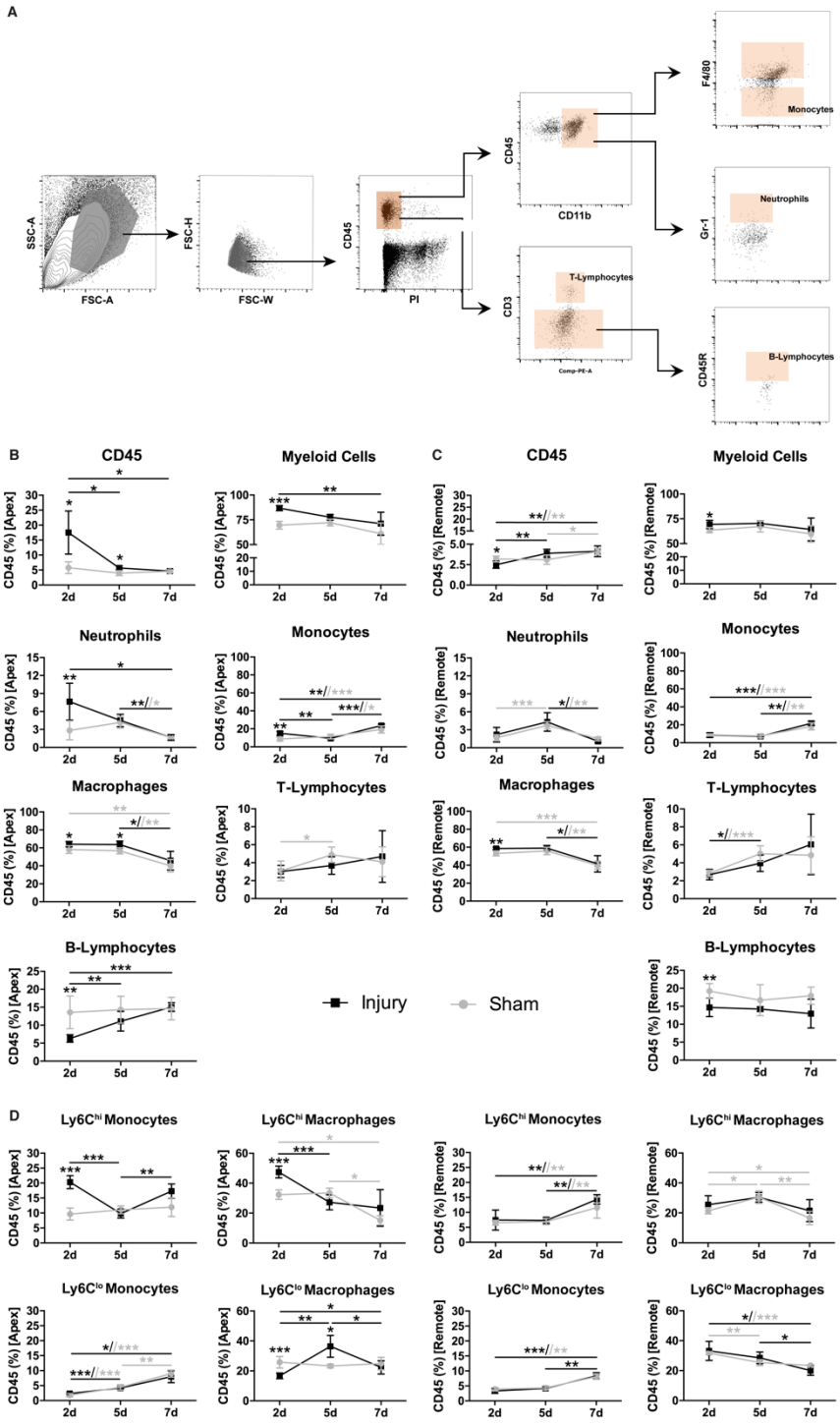

Figure S3 – Characterization of the Inflammatory Infiltrate After Injury

(A) Representative plots showing the discrimination of hematopoietic (CD45+) subsets after surgery. (B) Relative percentage of immune cell populations in the apex of apex-resected and sham-operated animals at 2d, 5d and 7d post-surgery. (C) Relative percentage of immune cell populations in the remote myocardium of apex-resected and sham-operated animals at 2d, 5d and 7d post-surgery. (D) Relative percentage of monocytes and macrophages according to Ly6C expression in the apex and remote myocardium at 2d, 5d and 7d post-surgery. (n = 7, 5, 4, 4, 3, 3; 2d sham, 2d injury, 5d sham, 5d injury, 7d sham, 7d injury). (*p<0.05; **p<0.01; ***p<0.001). All values are presented as means ± SD.

Fig.S4

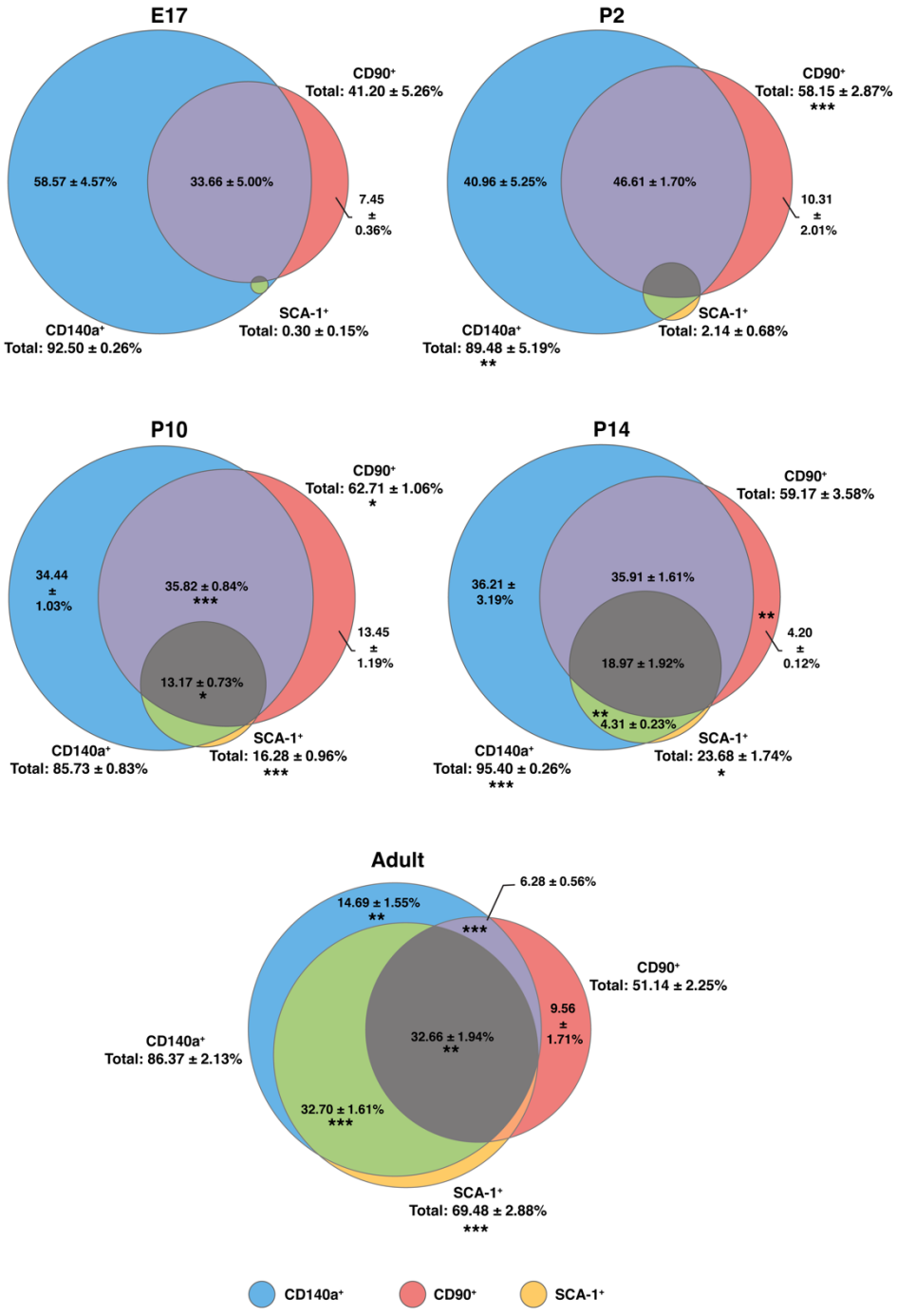

Figure S4 – Phenotypic Characterization of Cardiac Fibroblasts Throughout Ontogeny (Related to Figure 3)

Discrimination of fibroblasts based on the combined expression of CD140a, CD90 and SCA-1 at E17 (n = 2 [2 pools of 3 hearts]), P2 (n = 3), P10 (n = 3); P14 (n = 5) and adulthood (n = 4). Statistical differences (*p<0.05; **p<0.01; ***p<0.001) of each population between consecutive time points are shown. All values are presented as means ± SEM.

Fig.S5

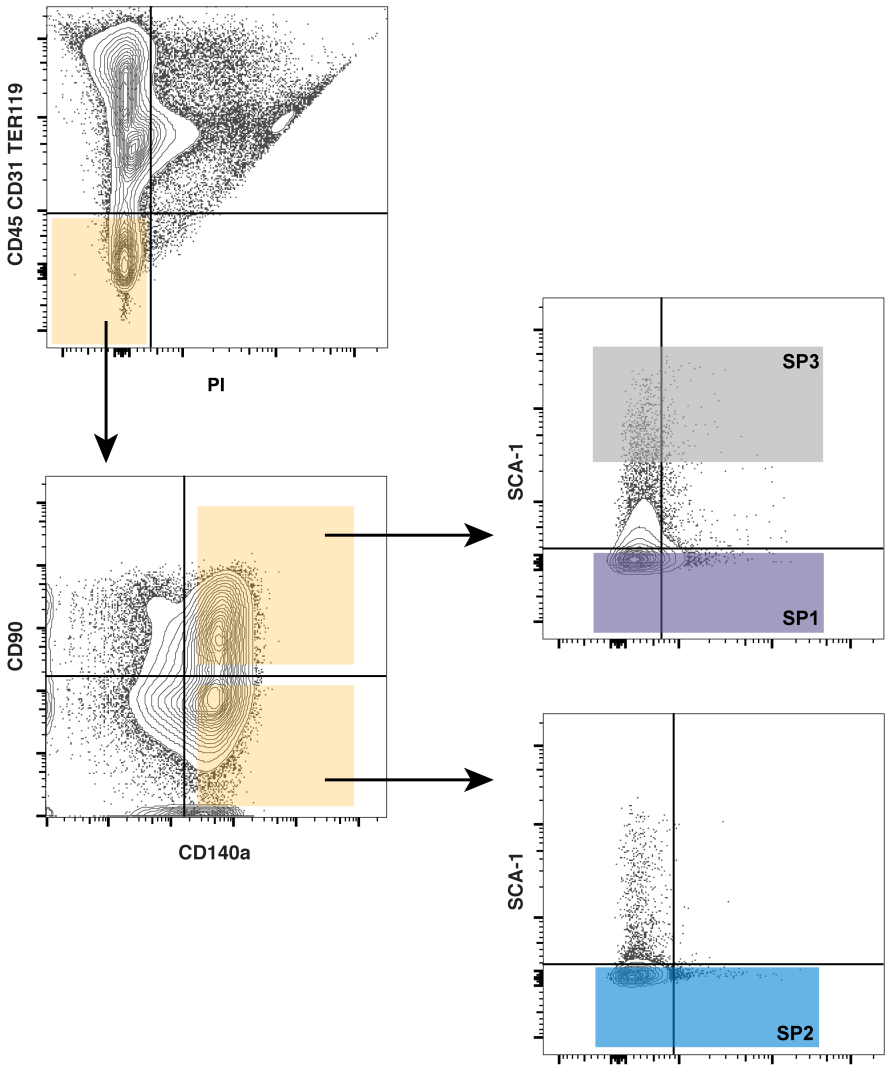

Figure S5 – Sorting Strategy to Isolate Activated Cardiac Fibroblast Populations (Related to Figure 3)

SP1, SP2 and SP3 subsets were sorted following removal of non-viable cells (PI^+), hematopoietic cells ($CD45^+$), endothelial cells ($CD31^+$) and erythrocytes ($TER119^+$) (n = 5, 4; 7d sham, 7d injury).

Fig.S6

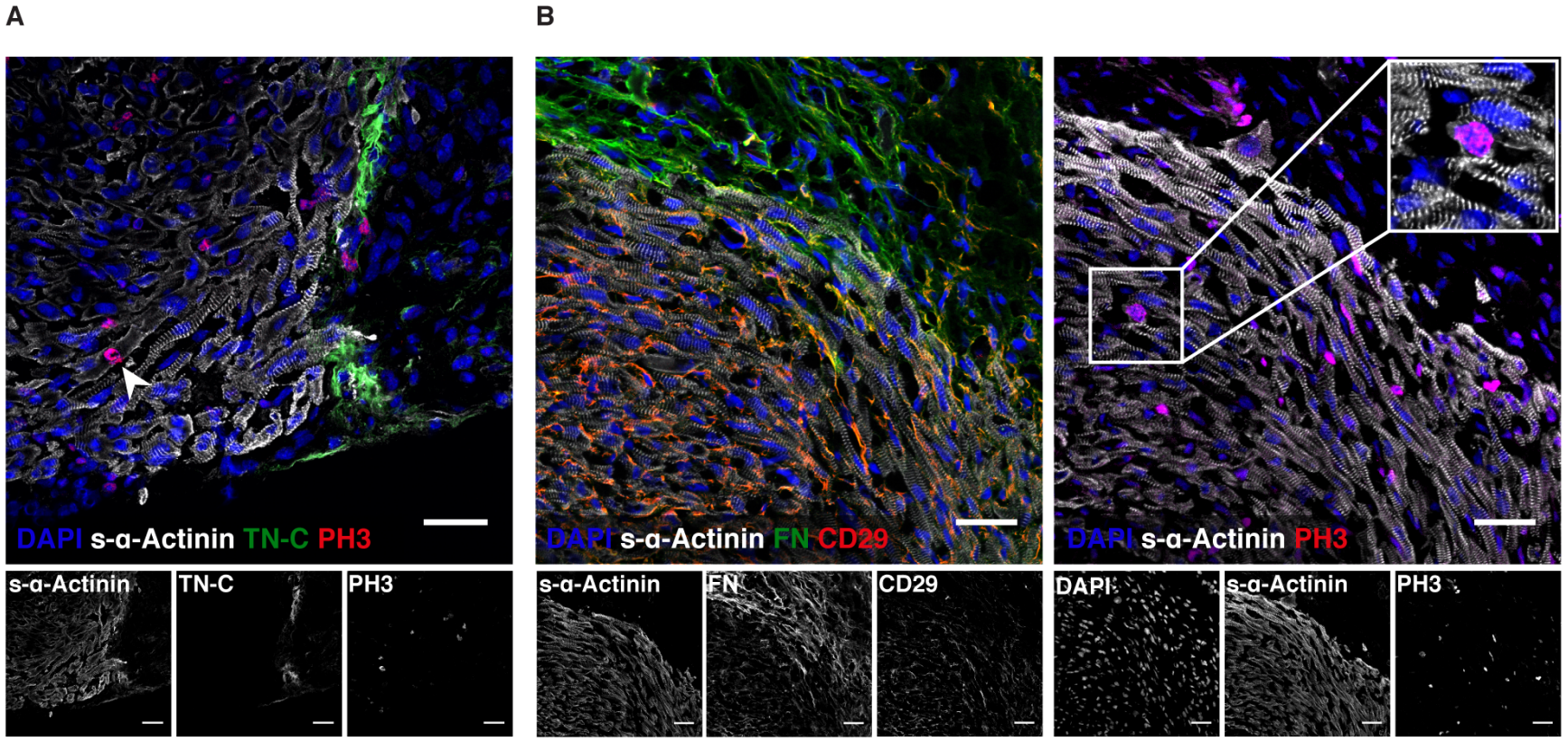

Figure S6 – CM Proliferation and ECM Production at 7d After Apex Resection (Related to Figures 3 and 4)

Representative images of consecutive sections stained for s-α-Actinin/FN/CD29 and s-α-Actinin/PH3 (n=10) at 7d post-surgery. High magnification image highlights a proliferating CMs located in a region marked by the deposition of FN and by the expression of CD29 in CMs (Scale bars, 30μm).

Table S1

Table S1 – Lead-II Electrocardiographic parameters at 180d post-surgery (Related to Figure 2)

Variables	180d Sham (Mean ± SEM) n=10	180d Injury (Mean ± SEM) n=6	P-Value
RR Interval (s)	0,1275 ± 0,0031	0,1265 ± 0,0012	0,875 (Mann-Whitney U Test)
Heart Rate (bpm)	474,01 ± 11,34	475,02 ± 4,67	1,000 (Mann-Whitney U Test)
PR Interval (s)	0,0407 ± 0,0064	0,0442 ± 0,0020	0,065 (T-Test)
P Duration (s)	0,0143 ± 0,0008	0,0153 ± 0,0010	0,447 (T-Test)
QRS Interval (s)	0,0095 ± 0,0003	0,0093 ± 0,0034	0,928 (T-Test)
QT Interval (s)	0,0131 ± 0,0004	0,0142 ± 0,0006	0,141 (T-Test)
QTc (s)[a]	0,0367 ± 0,0014	0,0399 ± 0,0018	0,189 (T-Test)
JT Interval (s)	0,0033 ± 0,0003	0,0040 ± 0,0006	0,285 (T-Test)
Tpeak Tend Interval (s)	0,0021 ± 0,0003	0,0026 ± 0,0005	0,391 (T-Test)
P Amplitude (mV)	0,0697 ± 0,2179	0,0634 ± 0,0263	0,607 (Mann-Whitney U Test)
Q Amplitude (mV)	0,0078 ± 0,0102	-0,0552 ± 0,0238	0,056 (Mann-Whitney U Test)
R Amplitude (mV)	1,1664 ± 0,1572	1,1284 ± 0,1848	0,881(T-Test)
S Amplitude (mV)	-0,8133 ± 0,0700	-0,7484 ± 0,0863	0,374 (Mann-Whitney U Test)
ST Height (mV)	-0,0363 ± 0,0291	-0,0390 ± 0,0224	0,950 (T-Test)
T Amplitude (mV)	0,1860 ± 0,0327	0,1100 ± 0,0399	0,179 (T-Test)

[a] QT interval was adjusted to heart rate (QTc) using Bazett's formula.

s – seconds
bpm – beats per minute
mV - millivolt

Supplemental Experimental Procedures

Neonatal Apex Resection and Sham-Surgery

The neonatal injury model consists of the apex surgical resection of post-natal day (P)1 C57BL/6 mice. Animals were anesthetized by hypothermia during three minutes (min) to cease cardiorespiratory movements. Animals were laid in lateral decubitus position, exposing the left side of the flank. After immobilization with sealing tape, the skin was cut and muscle fibers disrupted until the ribs were observed. The thoracic cavity was opened in the 4th intercostal space and the left ventricle apex cut with fine scissors until chamber exposure. Thorax and skin were closed by 7-0 absorbable suture (Coated Vicryl Ethicon). Animals recovered from anesthesia under an infrared lamp and were subjected to stimulation by tightening the paw, when necessary. The surgical procedure was completed when animals regained regular breathing. Neonates were returned to the progenitor cage immediately after all the litter was intervened. During all the procedure, except throughout surgery, neonates were warmed by warming pads and infrared light. Sham mice underwent the exact same procedure with the exception of apex resection (any kind of heart manipulation whatsoever).

Histological Assessment

Hearts were harvested at 0, 7, 14, 21, 60 and 180 days post-surgery (d injury/sham). Hearts were submersed in phosphate buffer saline (PBS) and fixed in 10% formalin neutral buffer (VWR BDH & Prolabo) during 16-24 hours at room temperature (RT). For paraffin embedding, hearts were processed during a total time of 12 hours in an automated system through successive PBS washes, increasing series of alcohols (Aga), Clear Rite 3® (Richard-Allan Scientific) and Shandon Histoplast (Thermo Scientific) at 56ºC. Hearts were included in paraffin and sectioned (microtome RM2255, Leica) longitudinally (3 μm sections) according to Figure S1. Sections were dewaxed and rehydrated prior to modified Masson's Trichrome (MT) staining. The MT was performed as in the Trichrome (Masson) Stain kit (HT15-1KT, Sigma-Aldrich), with the following modifications: nuclei were pre-stained with Celestine Blue and with Gill's Hematoxylin, followed by an incubation in aqueous Bouin solution for 1 hour to promote uniform staining. Sections were diafanized in xylene and mounted in DPX Mountant for histology (06522, Sigma-Aldrich®). Images were acquired in the Hamamatsu Nanozoomer 2.0-HT digital slide scanner and subsequent editing was performed in NDP view 2 software (Hamammatsu).

Morphometric Analysis

Resorting to MT stained sections and with assistance of FIJI v2.0.0-rc-54/.51h software, morphometric analysis was performed as follows:

1. Ventricular Surface Area: measurement of the whole ventricle cross-sectional area (i.e excluding great vessels, atria and tissue adherences in the apex region);
2. Heart Length/BW: the distance between the base of the heart (region corresponding to the insertion of the great vessels in the myocardium) and the apex was normalized by the body-weight.
3. Injury (%): the percentage of sections collected from a single heart demonstrating myocardial disruption and/or fibrosis.
4. Ventricle/Scar Volume: ventricle and scarring area multiplied by the distance separating adjacent sections.
5. Apical Wall Thickness Occupied by Fibrosis (%): percentage of the left ventricle wall thickness at the apical region occupied by fibrosis.
6. Cardiomyocyte cross-sectional area: the area of transversally cut cardiomyocytes was determined in at least 30 cardiomyocytes of the apical and remote myocardium per section (at least 8 sections were analyzed per heart).

Functional Characterization

Animals at 21, 60, 120 and 180d post-surgery were subjected to echocardiography using the Vevo2100 system and a 40MHz probe (Visualsonics). Anesthesia was induced in a chamber filled with 5% isoflurane (IsoVet, Braun) and verified by loss of body posture and the paw withdrawal reflex. The animal was transferred to a heated support and the state of anesthesia was maintained through a face mask (1.5% isoflurane). Animal fur was shaved and mice paws were placed over sensors and in contact with an electric-conductive gel to monitor heart and respiratory rates. Body temperature was assessed using a rectal probe. Two-dimensional images of the heart were acquired focusing both short-axis (SAX) and parasternal long-axis (PSLAX) views. Motion-mode (M-Mode) was conducted in the SAX view to determine the thickness of LV walls as well as chamber diameter during systole and diastole. Ejection fraction (EF) was determined by Simpson's method: a longitudinal segment and three transverse segments were traced in the distal, medial and proximal region of the heart in PSLAX view, which assisted on the determination of heart volume during diastole and systole.

$$Vol\text{d,s} = \frac{(\pi\left(\frac{[distal\text{d,s}]}{2}\right)^2 + \pi\left(\frac{[medial]\text{d,s}}{2}\right)^2 + \pi\left(\frac{[proximal\text{d,s}]}{2}\right)^2) \times Heart\ Length\text{d,s}}{3}$$

Stroke volume (SV) was determined by the difference between diastolic and systolic volume. Conversely, ejection fraction was determined as follows:

$$EF = \frac{SV}{Vold} \times 100$$

Cardiac output (CO) was calculated by multiplying SV by heart rate (HR).
Eccentricity index was determined by dividing left ventricle diameter (at the level of papillary muscles) by the longitudinal heart length (distance from the apex to great vessel insertion in the ventricle), both obtained in PSLAX during diastole.
Diastolic function was evaluated by the ratio between early (E) to late (A) filling velocities of the left ventricle.
Myocardial performance index (also known as Tei index) incorporates both systolic (IVCT - isovolumetric contraction time) and diastolic (IVRT – isovolumetric relaxation time) time intervals:

$$MPI = \frac{IVCT + IVRT}{Aortic\ Ejection\ Time}$$

By comparing these factors on sham operated and injured hearts the degree of functional restoration was ascertained.
Electrophysiological parameters were also assessed at 180d post-surgery. Anesthesia was induced and confirmed as described for echocardiographic evaluation. Animals were placed in supine position and intradermal electrodes were placed in a lead II-like configuration. ECG signals were obtained with a data acquisition hardware (PowerLab 8/35, ADInstruments) coupled to a signal amplifier (Animal Bio Amp, ADInstruments), with support of LabChart 8 software (ADInstruments).
Using an ECG analysis module RR Interval, HR, PR Interval, P Duration, QRS Interval, QT Interval, JT Interval, T-peak to T-end interval, the amplitudes of P, Q, R, S and T waves and the ST segment height were determined. Furthermore, QT corrected for the HR (QTc) was calculated through the Bazett's formula:

$$QTc = \frac{QT}{\sqrt{RR\ interval}}$$

Immunofluorescence
Both cryo and paraffin sections as well as cytospins were used to evaluate protein expression. Three µm paraffin sections were dewaxed and rehydrated by three changes in xylene, followed by sequential alcohol gradients and rinsing in deionized water. If antigen retrieval was necessary, heat-induced epitope retrieval (HIER) was applied. For HIER, sections were incubated for 35 min at 98ºC in a water bath, in 10mM Tris 1mM EDTA (Tris-EDTA), pH 9.0 and allowed to cool for 20 min at room temperature.
Tissue processing for cryosectioning involves 3 consecutive submersions (each during 24 hours at 4ºC) in different solutions: 0,2% Paraformaldehyde (PFA) in 0,12M phosphate buffer (PB); 4% sucrose in 0,12M PB and 15% sucrose in 0,12M PB. A fourth incubation is performed in 15% sucrose and 7,5% gelatin in 0,12M PB during 1 hour at 37ºC. Hearts were transferred to molds containing the last solution (warmed) and placed on top of dry-ice-chilled 2-methylbutane (GPR Rectapur, VWR). Frozen hearts were stored at -80ºC before being cut from one end to the other into 5µm sections using a cryomicrotome (Microtom HM 550, Thermo Scientific).
When targeting epitopes that required cell membrane permeabilization, sections were treated with 0.2% Triton X-100 (for intracytoplasmic motifs) or with 1% Triton X-100 (for nuclear motifs) in PBS. Tissue sections were blocked for 1 hour in 4% FBS and 1% bovine serum albumine (BSA) or in 5% BSA in PBS. If the primary antibody was produced in mouse, the M.O.M.™ Immunodetection Kit (Vector Lab) was applied to enable blocking of endogenous Fc receptors that could be recognized by the secondary antibody. Incubation with primary antibody was performed overnight at 4ºC in a humidified chamber. Following the primary antibody incubation, sections were washed in PBS and then incubated with the secondary antibody (RT) during 1 hour. In order to amplify the fluorescence intensity of several antibodies, two streptavidin conjugated fluorophores were used: Streptavidin conjugated with Alexa 555 (S32355, Invitrogen), at 1:500 dilution and Streptavidin conjugated with Allophycocyanin (APC) (SA1005, Life Technologies), at 1:100 dilution. After washing, sections were mounted with Fluoroshield™ (F6182, Sigma-Aldrich), Vectashield mounting media with DAPI (H-1200, Vector) and observed/photographed either in a Zeiss Axiovert 200M inverted fluorescence microscope, in a Nikon eclipse 90i

fluorescence microscope, in a Leica TCS SP5 laser scanning confocal microscope or in GE IN Cell Analyzer 2000 High-Content Screening microscope.
Cytospin immunofluorescence was performed following heart digestion (see neonatal cardiomyocyte isolation section). Isolated cardiac cells were plated onto glass slides using cytospin (Shandon Cytospin® 4, Thermo Scientific) and spun for 5 min at 130g. Cells were permeabilized with 0.5% Triton for 7 min and washed three times in PBS with 0.2%Tween. Incubation with the primary antibody was carried out for 2 hours and the secondary antibody incubation for 30min, in the dark. Finally, nuclei were stained with DAPI for 10 min and slides mounted in a PBS glycerol solution (1:9) with 2.5% n-propyl-gallate (Sigma-Aldrich) without DAPI.

List of primary antibodies used and specification of the working dilution.

Antibody	Dilution	Reference
Sarcomeric-αActinin (Mouse IgG)	1:400	A7811, Sigma
Vimentin (Mouse IgG1/K)	1:50	MS-129-P, Thermo
CD31 (Goat IgG)	1:250 (requires HIER with Tris-EDTA, pH 9.0)	sc-1506, Santa Cruz Biotechnology
CD45 (Goat IgG)	1:100	AF114, R&D
Alpha-Smooth Muscle Actin (Mouse IgG)	1:400	A5228, Sigma
PH3 (Rabbit IgG)	1:800	#3377, Cell Signaling
Fibronectin (Rabbit IgG)	1:400	F-3648, Sigma
Laminin (Rabbit IgG)	1:400	L9393, Sigma-Aldrich
Tenascin-C (Rat IgG)	1:100	LAT-2, gift A. Sonnenberg
CD29 (Rat IgG)	1:50	14-0292, eBioscience
Collagen IV (Goat IgG)	1:100	AB769, Chemicon
Aurora-B (Mouse IgG1)	1:50 (requires HIER with Sodium Citrate acid buffer)	611082, BD Transduction Laboratories
Cardiac Troponin I (Rabbit IgG)	1:200 (requires HIER with Sodium Citrate acid buffer)	ab47003, Abcam
PCM-1 (Rabbit Polyclonal)	1:500	HPA023370, Sigma

List of secondary antibodies used and specification of the working dilution

Antibody	Dilution	Reference
Alexa Fluor 488 Donkey anti Mouse IgG	1:1000	A-21202, Invitrogen
Alexa Fluor 594 Donkey anti Mouse IgG	1:1000	A-21203, Invitrogen
Alexa Fluor 488 Goat anti Mouse IgG	1:1000	A11017, Molecular Probes
Cy3 Goat anti Mouse IgG	1:200	115-165-003, Jackson
Alexa Fluor 488 Goat anti Rabbit	1:200	111-545-003, Jackson
Alexa Fluor 488 Donkey anti Rabbit IgG	1:1000	A-11055, Invitrogen
Alexa Fluor 568 Donkey anti Rabbit IgG	1:1000	A-10042, Invitrogen
Alexa Fluor 633 Goat anti Rabbit IgG	1:1000	A21070, Molecular Probes
Biotinylated Donkey anti Rabbit IgG	1:250	A16033, Life Technologies
Alexa Fluor 568 Donkey anti Goat IgG	1:1000	A-11057, Invitrogen
Biotinylated Donkey anti Goat IgG	1:250	A16009, Life Technologies
Alexa Fluor 488 Donkey anti Rat IgG	1:1000	A-21208, Invitrogen
Alexa Fluor 568 Goat anti Rat IgG	1:1000	A11077, Molecular Probes
M.O.M. Biotinylated anti mouse Ig	1:250	MKB-2225, Vector Laboratories
Biotinylated Goat anti Rat IgG	1:200	BA-9400, Vector Laboratories

High Content Screening (HCS)
The proliferative response of neonatal murine hearts at 7d injury and (neo)vascularization at 60d injury were evaluated using IN Cell Analyzer 2000 (GE Health Care Life Sciences) with assistance of IN Cell Developer software (GE Health Care Life Sciences). This microscope allowed a complete scan of the sample and its software was used to quantify, through overlapping regions of interest (ROIs), the total number of proliferating cardiomyocytes (colocalization of DAPI, PH3 and sarcomeric-α-Actinin expression) or the total number of

endothelial cells (colocalization of DAPI and CD31 expression) per area (quantified in advance by the assembly of individual images in FIJI v2.0.0-rc-54/.51h.).

Flow Cytometric profile of Cardiac Populations and Fluorescence Activated Cell Sorting (FACS)
Flow cytometry was performed to characterize cardiac stroma, namely fibroblast populations, from E17 to adulthood and inflammatory populations 2d, 5d and 7d post-surgery. Cardiac cells were isolated by digestion of cardiac tissue fragments with crude collagenase (C2139, Sigma-Aldrich®) at 200 µg/ml concentration and DNase (A3778, VWR) at 60U/ml in Hank's balanced salt solution (HBSS) (H9269, Sigma-Aldrich®). Collagenase digestions were performed during 15 min at 37ºC until no tissue was observed by visual inspection. After each digestion, the suspension was decanted, the medium collected (cellular portion) and a new collagenase/DNase solution was added to the remaining tissue fragments. The collected cell suspension was mixed with HBSS with 10% FBS to neutralize enzymatic activity and was kept on ice. The collected cellular fraction of each digestion was combined and washed in FACS medium (0.01% sodium azide and 3% FBS in PBS). Cells were evenly distributed for each staining in a round bottom multiwell plate. Fc receptors were blocked using anti-mouse CD16/CD32 at 0.5 µg/µl in FACS medium during 20 min. After two washes in FACS media, cells were incubated during 30 min with the antibody cocktail on ice and in the dark. Cells were washed twice in FACS media and transferred to FACS tubes. In order to exclude nonviable cells from the analysis, 0.5% of propidium iodide (PI) (P4170, Sigma-Aldrich) was added to the cell suspension 1-2 min prior to analysis. Fifty thousand events (of appropriate size and complexity) per staining were acquired in the cytometer FACS Canto II (BD Biosciences). For characterization of inflammatory cells, after antibody incubation, cells were fixed in 1% PFA in FACS medium during 15 min and acquired afterwards. Subsequent analysis and graphing were performed in FlowJo VX software.

List of antibodies and isotype controls used in flow cytometry and specifications of the working dilution.

Antibody	Dilution	Reference
CD31-PeCy7 (Rat IgG2a)	1:100	25-0311-81, eBioscience
CD45-PeCy7 (Rat IgG2b)	1:100	25-0451-81, eBioscience
TER119-PeCy7 (Rat IgG2b)	1:100	116222, Biolegend
CD90.2-FITC (Rat IgG2b)	1:100	105306, Biolegend
CD90.2 PE (Rat IgG2a)	1:100	553930, BD Pharmingen
CD140a-APC (FL4) (Rat IgG2a)	1:100	135907, Biolegend
SCA-1 FITC (Rat IgG2a)	1:100	11-5981-81, eBioscience
SCA-1-PE (FL1) (Rat IgG2a)	1:100	12-5981-81, eBioscience
CD44-APC/Cy7 (Rat IgG2b)	1:50	103028, Biolegend
CD45-PE(Rat IgG2b)	1:100	12-0451-82, eBioscience
CD11b-APC (Rat / IgG2b)	1:100	17-0112-82, eBioscience
F4/80-FITC (Rat / IgG2a)	1:100	11-4801-82, eBioscience
Ly6C-PeCy7 (Rat / IgG2c)	1:100	25-5932-82, eBioscience
Gr-1-FITC (Rat IgG2b)	1:100	11-5931-82, eBioscience
CD3e-PacBlue (S. Hamster IgG2)	1:100	558214, BD Biosciences
CD45R-PeCy7 (Rat IgG2a)	1:100	25-0452-82, eBioscience
Isotype Control PeCy7 (Rat IgG2a)	1:100	400521, Biolegend
Isotype Control PeCy7 (Rat IgG2b)	1:100	400617, Biolegend
Isotype Control FITC (Rat IgG2a)	1:100	400505, Biolegend
Isotype Control FITC (Rat IgG2b)	1:100	IC013F, R&D
Isotype Control PE (Rat IgG2a)	1:100	400508, Biolegend
Isotype Control APC (Rat IgG2a)	1:100	IC006A, R&D
Isotype Control APCCy7 (Rat IgG2b)	1:100	400623, Biolegend

Real-time Polymerase Chain Reaction (qRT-PCR)

Primer sequences for qRT-PCR

Gene	Primer Sequence (5' to 3')	PCR Product Length	Annealing Temperature (°C)
Gapdh	FW: CGTCCCGTAGACAAAATGGT RV: TTGATGGCAACAATCTCCAC	110 bp	60
Col1a1	FW: GCTCCTCTTAGGGGCCACT RV: CCACGTCTCACCATTGGGG	247 bp	60
Col3a1	FW: CCTGGCTCAAATGGCTCAC RV: GACCTCGTGTTCCGGGTAT	214 bp	60
Fn1	FW: GCTCAGCAAATCGTGCAGC RV: CTAGGTAGGTCCGTTCCCACT	117 bp	60
Fn1-EDA	FW: ACTCGAGCCCTGAGGATG RV: CTGAGGCCCTGCAGCTCT	82 bp	60
Tcf21	FW: CGCTCACTTAAGGCAGATCC RV: CTGTAGTTCCACACAAGCGG	149 bp	60
Tbx20	FW: AAACCCCTGGAACAATTTGTGG RV: CATCTCTTCGCTGGGGATGAT	171 bp	60
Tgfb1	FW: CTTCAATACGTCAGACATTCGGG RV: GTAACGCCAGGAATTGTTGCTA	142 bp	60
Tgfb3	FW: GCAAGAATCTGCCCACAAGG RV: CCATTGGGCTGAAAGGTGTG	145 bp	60
Postn	FW: TGGTATCAAGGTGCTATCTGCG RV: AATGCCCAGCGTGCCATAA	135 bp	60
Igf1	FW: CACACCTCTTCTACCTGGCG RV: GTACTTCCTTCTGAGTCTTGGGC	321 bp	60
Igf2	FW: GGGAAGTCGATGTTGGTGCT RV: AAGCAGCACTCTTCCACGAT	200 bp	60
Fstl1	FW: TAATGGCGACTCTCACCTGG RV: ATGAGGGCGTCAACACAGAG	135 bp	60
Vegfa	FW: CCACGACAGAAGGAGAGCAG RV: CACTCCAGGGCTTCATCGTT	196 bp	60

Neonatal Cardiomyocyte Isolation

Seven days post-surgery neonatal hearts were harvested and cut into small fragments (approximately 2mm), before being flash frozen in liquid nitrogen until further use. Then, fragments were thawed at RT and fixed with 4% PFA in PBS for 2 hours with stirring (100 rpm). Following fixation, fragments were digested with collagenase type II (Worthington, CLS-2) (3 mg/ml in HBSS) overnight at 37ºC (100 rpm) and mechanically dissociated until no fragments were detected (additional digestions were sometimes required, depending on the original size of the fragment). In order to inactivate collagenase, equal volume of HBSS with 10% FBS was added. At this stage, cells can be stored at 4ºC or, alternatively, processed for cytospin immunofluorescence or for imaging flow cytometry (see Imaging Flow Cytometry). This methodology yields ~70% purity of CMs.

Imaging Flow Cytometry

Aiming at performing a morphometric characterization of cardiomyocytes, cell suspensions were centrifuged at 300g for 10min and the cellular portion was then resuspended in FACS medium (0.01% sodium azide and 3% FBS in PBS) and permeabilized with 1X BD Perm/Wash™ Buffer during 15 min at RT. Incubation with primary and secondary antibodies lasted for 2 hours and 30 min on ice, respectively. Both antibodies were diluted in 1X BD Perm/Wash™ Buffer and incubations were separated by two washes with 1X BD Perm/Wash™ Buffer at 800g. Finally, they were ressuspended in PBS and kept at 4ºC until acquisition. Immediately before acquiring in Imagestream®X, cells were filtered (100µm cell strainer) and their nuclei were stained with 200 µM of DRAQ5 (5 mM, Biostatus).

Cardiomyocyte morphometric analysis was performed using IDEAS® software. This software calculates several features of CMs (area, major axis, minor axis and aspect ratio) and allows mask creation (definition of a specific area), which allows the assessment of nuclear number. Unfocused cells were promptly removed by eliminating cells with Gradient RMS smaller than 40 and debris and duplets were excluded by removing objects with a small area and high aspect ratio. Whenever the aspect ratio was smaller than 0.55, cardiomyocytes were classified as "Rod" and above this value were considered "Round".

Adult Cardiomyocyte Isolation

Adult cardiomyocytes were isolated by reperfusion of the heart in a Langendorff system with liberase TM (05401127001, Roche) at 13.3 μg/ml concentration and trypsin (27250018, Gibco) at 13.8 μg/ml concentration in a heated aqueous buffer containing: NaCl (113mM), KCl (4.7mM), $MgSO_4$ (1.2mM), Na_2HPO_4 (0.6mM), KH2PO4 (0.6mM), NaHCO3 (12mM), KHCO3 (10mM) and Taurine (30mM). Perfusion was performed under physiological pressure (± 80 mmHg) and rate (3ml/min) and lasted for 25 to 30 min. At this point the heart appears swollen, pale and flaccid and is cut into small pieces, which are further dissociated by a plastic pipette. Digestion is halted by adding ice-chilled FBS to the buffer (final concentration 10%) and the cell suspension is filtered using a 300 μm mesh. Then CMs are fixed for 30 min with 4% PFA in PBS and stored at 4ºC in PBS with 0,1% sodium azide.

The area of adult CMs, isolated from 60d and 180d post-surgery hearts, was evaluated by immunofluorescence in cytospins.

The volume of adult CMs, isolated from 60d post-surgery hearts, was evaluated with the assistance of IMARIS 8.4.1. The surface module allowed a semi-automatic segmentation of CMs and volume determination.