PNAS
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Supplementary Information for

Profiling proliferative cells and their progeny in damaged murine hearts

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SI Materials and Methods

Surgical Procedures. For sham, myocardial infarction (MI) and ischaemia/reperfusion (I/R) surgeries, 7- to 10-week old mice were anesthetised with mixture of ketamine and xylazine by intra-peritoneal (IP) injection. The fur was removed from the ventral surface of the neck and thorax with Nair hair removal cream (Church & Dwight). Mice were then artificially ventilated via a tracheal tube connected to a ventilator. After cleaning the surgical site with iodine and 70% ethanol, the skin was incised left of the midline with allowing access to the third intercostal space. Next, the pectoral muscles were retracted, the intercostal muscles were cut caudal to the third rib and a rib spreader was applied to allow access to the heart. The pericardium was incised longitudinally and the left anterior descending coronary artery (LAD) was identified.

For the sham control surgery, the rib cage was then closed with 5-0 silk suture without placing a ligature around the LAD and the skin was closed with tissue adhesive.

For the MI injury, a 7-0 silk suture was placed beneath the LAD to permanently occlude the artery. The rib cage was closed with 5-0 silk suture and the skin was closed with tissue adhesive.

For the I/R injury, a 2–3 mm piece of PE 10 tubing was placed over the LAD and the ligature secured around the LAD and PE tubing. Following 1-hour ischaemic period, the PE tubing was removed and the ligature was cut to allow for reperfusion via the LAD. The rib cage was closed with 5-0 silk suture and the skin was closed with tissue adhesive.

After these surgical procedures, the mice were disconnected from the ventilator, the tracheal tube was removed and the animal was placed unrestrained on a nose cone with 100% oxygen in a warm recovery cage until fully ambulatory, at which point the oxygen was turned off. To alleviate pain or distress Buprenorphine was injected subcutaneously as analgesic (0.05–0.1 $mg \cdot kg^{-1}$ bodyweight), given once at completion of surgery. Mice were killed at time points indicated below or in the main text.

Genetic Lineage-Tracing Experiments. For genetic lineage tracing of *Mki67*-expressing cells in neonatal hearts, 1-week old *Mki67*$^{IRES\text{-}CreERT2}$ × LSL-tdTomato mice were treated once with 150 µg tamoxifen (Sigma-Aldrich) dissolved in sunflower oil via the IP route. Mice were killed 1 day, 7 days and 2 months after tamoxifen injection and tissue was collected for histological analysis.

For genetic lineage tracing of *Mki67*-expressing cells in adult homoeostatic hearts, 8-week old *Mki67*$^{IRES\text{-}CreERT2}$ × LSL-tdTomato mice were treated either once or five consecutive times with 5 mg tamoxifen dissolved in sunflower oil via IP injection. Mice were killed (1 day after tamoxifen injection or at 1.3–1.5 years of age) and tissue was collected for histological analysis.

For genetic lineage tracing of *Mki67*-expressing cells in upon myocardial infarction surgery, 8-week old *Mki67*$^{IRES\text{-}CreERT2}$ × LSL-tdTomato mice that had undergone sham or MI surgery were IP injected with 5 mg tamoxifen dissolved in sunflower oil every other day for 3 doses in total. Mice were killed 7 and 28 days following surgery and tissue was collected for histological analysis.

For genetic lineage tracing of *Fstl1*-expressing cells in upon myocardial infarction surgery, 7-week old *Fstl1*$^{eGFP\text{-}IRES\text{-}CreERT2}$ × LSL-tdTomato were IP injected once with 5 mg

tamoxifen dissolved in sunflower oil. One week later, MI surgery was performed. Mice were killed 2 weeks following MI surgery and tissue was collected for histological analysis.

Cardiac Cell Isolation. Mouse hearts were dissected, the atria were removed and, when suitable, hearts were divided up into remote and apex (*i.e.* for MI or I/R surgery, borderzone and infarcted zone). Whole ventricles were prepped from dissected hearts of neonatal mice. Dissected tissue was minced into small pieces (<1 mm) and incubated for 15 minutes at 37 °C (water bath) in 0.5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBSO). Incubated pieces were disrupted using pre-wetted glass Pasteur pipettes by repeatedly pipetting up and down (>10 times). Pieces were centrifuged at 100×*g* for 5 minutes at 4 °C and the supernatant was carefully aspirated. Tissue pieces were washed in PBS and again centrifuged at 100×*g* for 5 minutes at 4 °C. After supernatant removal, tissue pieces were resuspended in dissociation buffer: Advanced DMEM/F12 containing collagenase II (1 $mg \cdot mL^{-1}$; Thermo Scientific), DNase I (10 $U \cdot mL^{-1}$; Sigma-Aldrich) and hyaluronidase (10 $\mu g \cdot mL^{-1}$; Sigma-Aldrich). Samples were incubated for 20-30 minutes at 37 °C on a rocker and then further disrupted using pre-wetted glass Pasteur pipettes by repeatedly pipetting up and down (>10 times). To inactivate enzymatic activity, foetal calf serum (FCS) was added to the cell suspension, which was then centrifuged at 300×*g* for 5 minutes at 4 °C. After supernatant removal, the cell pellet was incubated in 1× red blood cell (RBC) lysis solution (freshly prepared from a 10× stock solution containing 1.55M NH_4Cl, 100 mM $KHCO_3$ and 0.1 mM EDTA dissolved in MilliQ water) for 5 minutes at room temperature. Upon RBC lysis, cells were centrifuged at 300×*g* for 5 minutes at 4 °C and washed in PBS, followed by another centrifugation at 300×*g* for 5 minutes at 4 °C. Cells were stained with MitoTracker Deep Red (Cell Signaling) for 10 minutes at 37 °C on a shaker. Cells were resuspended in cold flow cytometry buffer (PBS, 5% FCS, 2 mM EDTA) and filtered through a 70-µm cell strainer (Greiner) before proceeding with flow sorting. Cardiac cell isolation from some wildtype (*Mki67*$^{wt/wt}$) mice was performed as recently described (1).

Messenger RNA Sequencing. Samples were lysed and RNA from each bulk sort sample or single cell was barcoded and processed using the CEL-Seq2 technique (2-4). Briefly, for bulk sequencing, samples sorted into TRIzol were extracted following the manufacturer's instruction, except, total RNA was precipitated with 2 µg GlycoBlue (Ambion) overnight at –80 °C. RNA pellets were dissolved in reverse transcription reaction mix (Invitrogen) containing UMI barcode primers and dNTPs (Promega) and incubated at 70 °C for 2 minutes. For single-cell mRNA-sequencing, cells were directly lysed at 65 °C for 5 minutes, followed by first and second strand synthesis (Invitrogen). Sequencing samples were pooled into single library prior to *in vitro* transcription (Ambion). Amplified RNA was used as template to generate complementary DNA (cDNA) libraries using Illumina TruSeq primers. Libraries were sequenced on an Illumina NextSeq500 using 75-bp pair-end sequencing. Bioinformatics analysis of sequenced libraries was performed as described below.

Histology. Tissues were fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 2 hours. After fixation, the hearts were incubated in cryoprotective solution (30% sucrose in PBS) at 4 °C overnight, then frozen blocks were prepared in tissue freezing

medium (Leica) and stored at −80 °C. Ten μm thick cryosections were cut and mounted on cover glass. Dried sections were fixed using 4% paraformaldehyde for 15 minutes, permeabilised with 0.5% Triton X-100 in PBS for 5 min and blocked for 2 hours in PBS containing 10% FCS, 3% bovine serum albumin and 0.5% fish skin gelatin (Sigma-Aldrich). Next, sections were incubated overnight with primary antibody in blocking solution, washed and incubated with secondary antibody for 2 hours at room temperature. Finally, sections were washed and mounted in ProLong Gold Antifade Mounting Medium with DAPI (Life Technologies). For paraffin blocks, hearts were fixed using 4% PFA, dehydrated using ethanol and embedded in paraffin. Five μm-sections were processed using standard methods and antigen retrieval was performed using pH 6 citrate buffer. Dewaxed sections were incubated with primary antibody overnight at 4 °C and then incubated for 1 hour at room temperature with BrightVision poly-HRT anti-rabbit (Immunologic). Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB).

***In Situ* Hybridisation.** *In situ* hybridisation (ISH) was performed on 8 μm paraffin sections of mouse hearts, as described (5). Sections were deparaffinised, rehydrated in a series of alcohol and incubated for 15 minutes in 20 μg/mL proteinase K in PBS for 15 minutes at 37 °C. Proteinase K was inactivated using 0.2% glycine in PBS, after which the sections were washed in PBS, fixed in 4% PFA and 0.2% glutaraldehyde in PBS. Sections were incubated overnight with the following probes in hybridization mix: *Col1a1* (0.5 $ng \cdot \mu L^{-1}$) and *Col3a1* (0.5 $ng \cdot \mu L^{-1}$) After hybridisation, the sections were washed in 2× SSC (150mM NaCl, 15mM Na-citrate, pH 7), 50% formamide in 2× SSC pH 7 and TNT (150 mM NaCl, 100 mM Tris-HCl, 0.05% Tween-20, pH 7.5). Blocking was done in MABT (150 mM NaCl, 100 mM maleic acid, 0.1% Tween-20, pH 7.4) plus 2% Block Reagent (Roche 1096176), followed by 2 hours incubation with anti-DIG-alkaline in MABT-Block 2% (1:1500), washed in TNT and NTM (100 mM NaCl, 100 mM Tris-HCl, 50 mM $MgCl_2$, pH9.2), and subsequently followed by incubation with NBT/BCIP (1:50). After colour development the sections were rinsed with double distilled water, dehydrated in a graded series of ethanol, washed in xylene and mounted in Entellan.

Bioinformatics Analysis. DNA library sequencing, mapping to the mouse reference genome and quantification of transcript abundance were performed as described elsewhere (4). Bulk sequencing libraries were analysed using the DESeq2 package (6). Cell clusters and gene expression levels across clusters were generated using the RaceID2 algorithm (3) from single-cell libraries. For initial cell type analysis, ERCC92 spike-ins and mitochondrial genes and cell transcriptomes with less than 1,000 uniquely expressed transcripts were removed from the single-cell dataset. Single-cell sequencing libraries were normalised by downsampling to a minimum number of 1,000 transcripts. After first running RaceID2, 2,144 cells making the 1,000-transcript threshold separated into 14 cell clusters. Subsequently, cell clusters enriched for *Kcnq1ot1* (cluster 5) and *Rn45s* (cluster 13), markers of necrotic cells with low quality RNA, were removed followed by running RaceID2 again to generate the cell clusters presented in this paper (7, 8). For refined analysis of cardiac cell subpopulations, cells assigned to the five main cardiac cell types were extracted and, subsequently, cells with more than 2,000 transcript counts assigned to the lineages of cardiomyocytes and cardiac fibroblasts were used for further analysis. To increase robustness, clusters containing less than 10 cells were removed from further

analysis (8): this applied to the initial subclustering of cardiomyocytes, where clusters 5, 7 and 9 (of a total of 11 clusters) were not considered for the subsequent differentially gene-expression analysis. After removal of these clusters, RaceID2 was run again, which resulted in 6 clusters. All bioinformatics analysis was performed using R version 3.4.0 (R Foundation, https://www.r-project.org) and RStudio version 1.0.143 (https://www.rstudio.com).

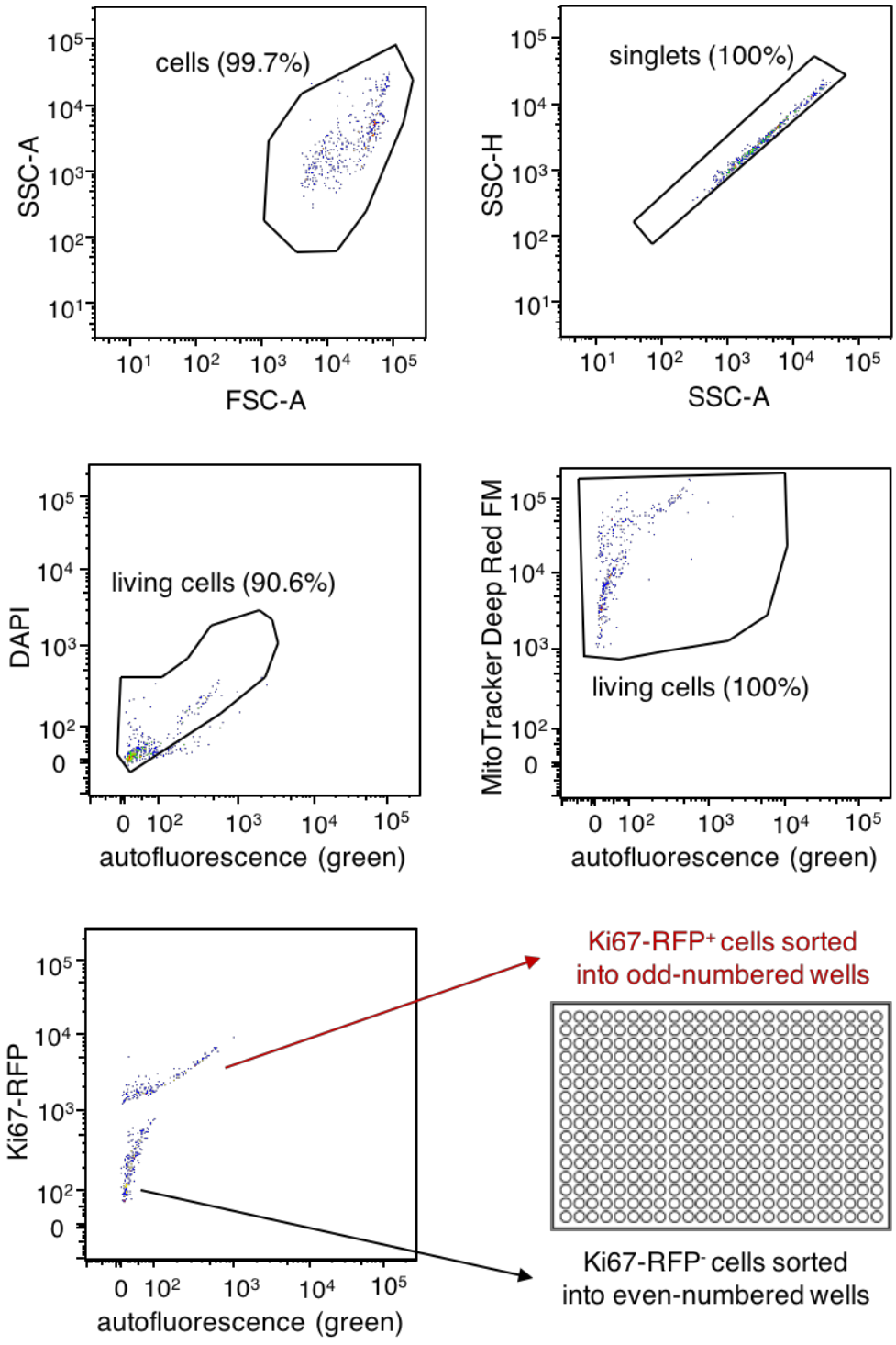

Fig. S1. Gating and sorting strategy for the purification of Ki67-RFP^{+} cells. Representative dot plots showing the gating and sorting strategy (from left to right, from top to bottom). Area scaling, A; forward scatter, FSC; height scaling, H; side scatter, SSC.

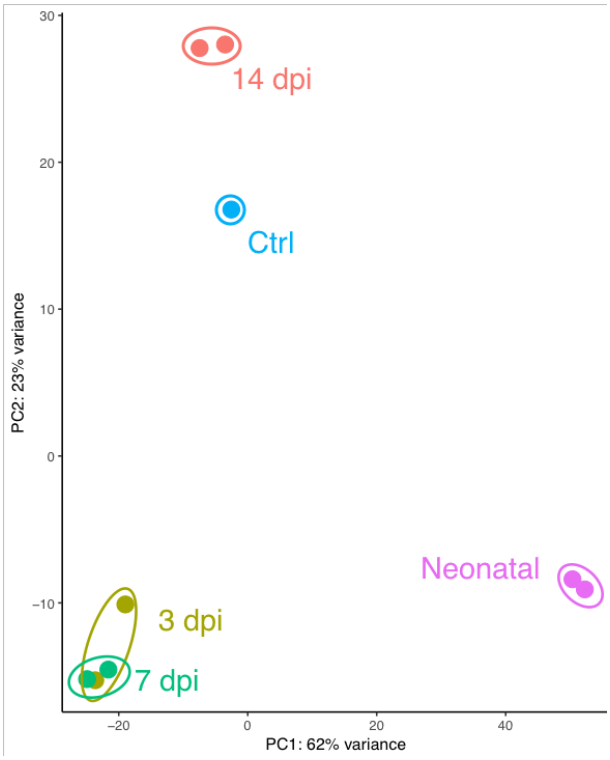

Fig. S2. Principal component analysis (PCA) of bulk mRNA-sequencing of Ki67-RFP^{+} cells. Principal component analysis (PCA) plot of mRNA expression profiles of Ki67-RFP^{+} cells isolated at 7 days of age (Neonatal), 3, 7 and 14 days after myocardial infarction (dpi) and or 14 days after sham surgery (Ctrl).

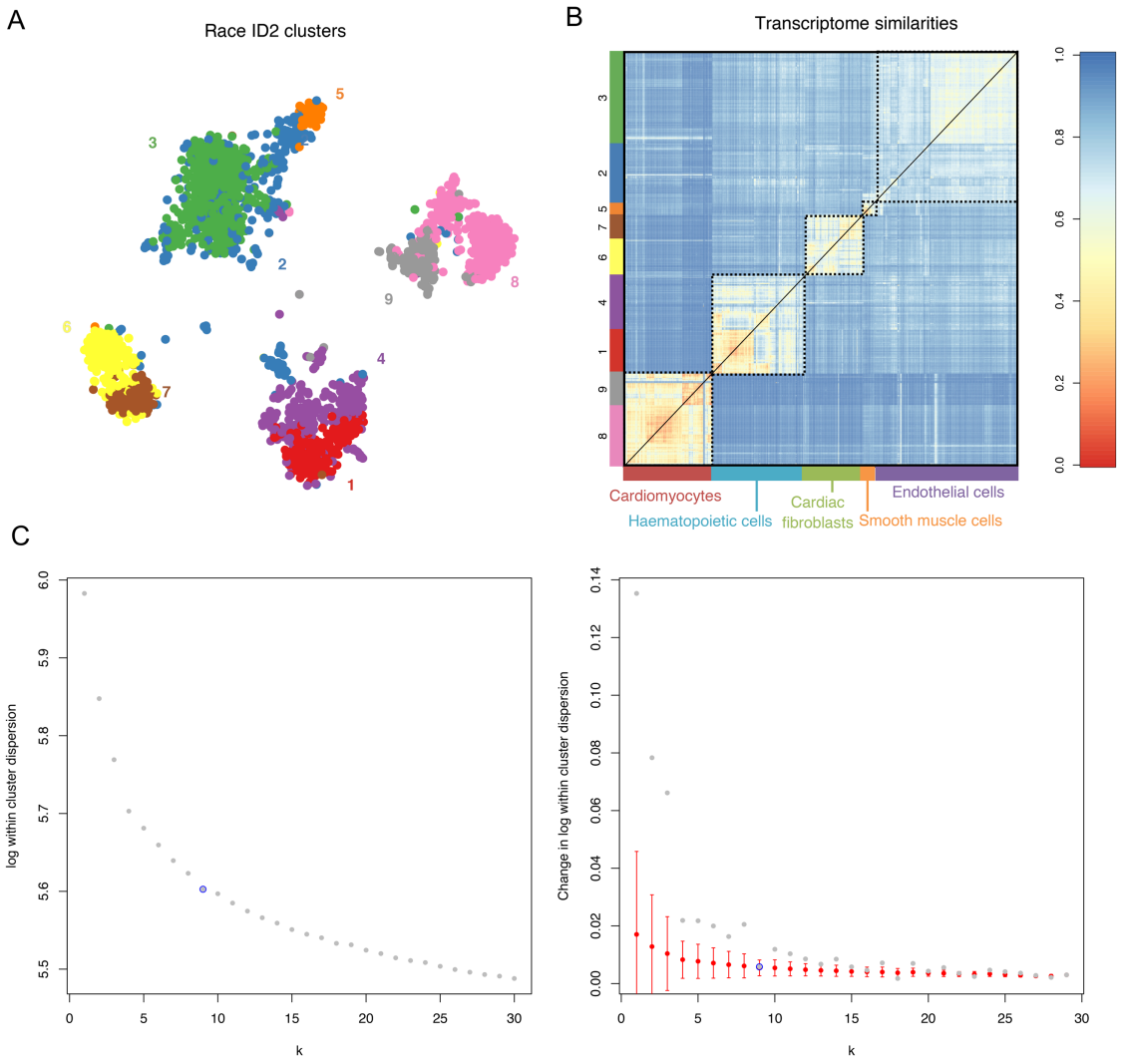

Fig. S3. Cell clustering. (A) Clustering of cardiac cells and cell-to-cell distances visualized by t-distributed stochastic neighbour-embedding (t-SNE) map, highlighting 9 identified clusters. (B) Heatmap representation of the transcriptome similarities between 1,939 single cells from the 9 identified clusters. (C) *k*-medoids clustering. If the change of the within-cluster dispersion upon increasing the cluster number ($k_{i+1} = k_i + 1$) is within the error of the average change upon further increase (k_{i+2}, …, k_{max}), k_i is chosen as input. The average change across cluster numbers k_{i+2}, …, k_{max} and its error is computed from a linear regression. Within-cluster dispersion (see Figure below, left) and change of the within-cluster dispersion (see Figure below, right) as a function of the number of clusters. The average change at cluster number k upon further increase is shown in red. In both panels the selected cluster number is circled in blue.

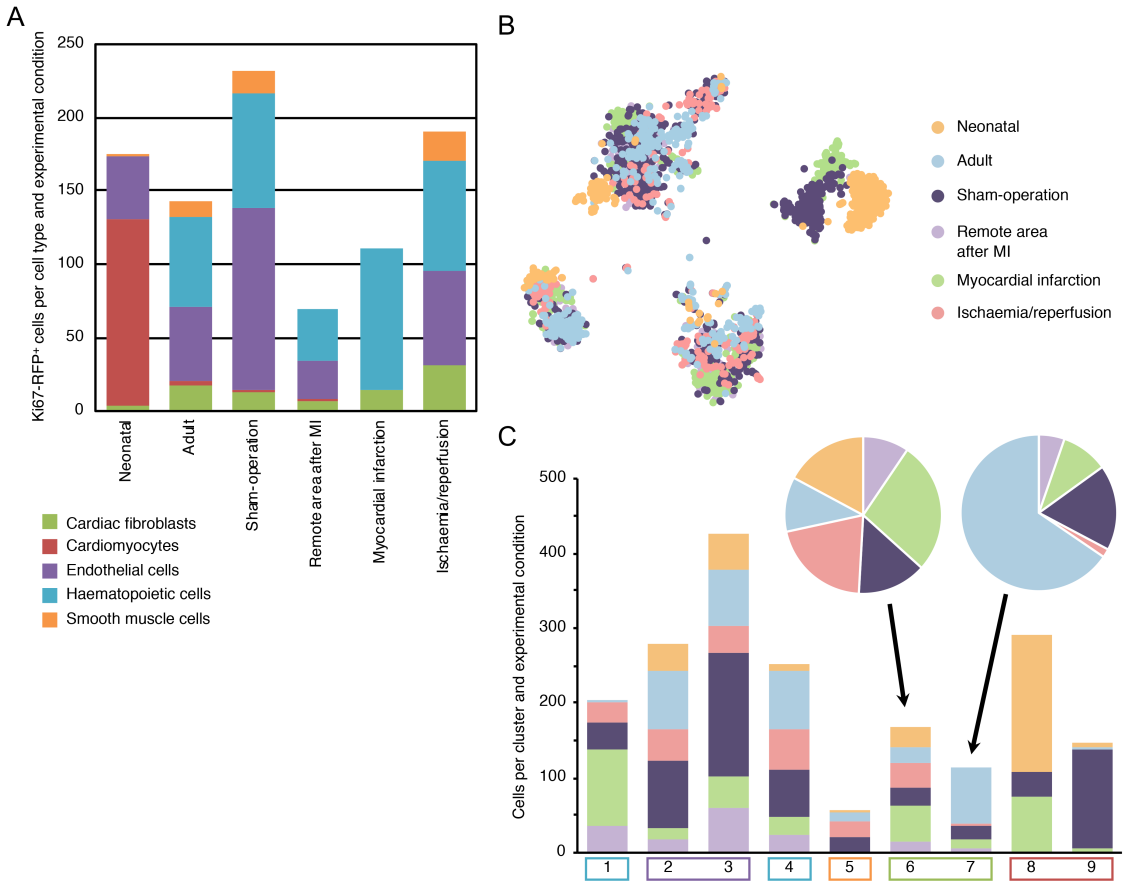

Fig. S4. Expression patterns of cardiac lineage genes in sequenced cardiac single cells. (A) Ki67-RFP^{+} cells per experimental group assigned to cell types based on marker genes enriched in each major cell cluster. (B) t-SNE map representation of the experimental condition for each sequenced cell. (C) Quantification of the conditions of origin shown on B within each cell cluster (colour of the frame around the cell cluster number corresponds to the cell types shown in panel A).

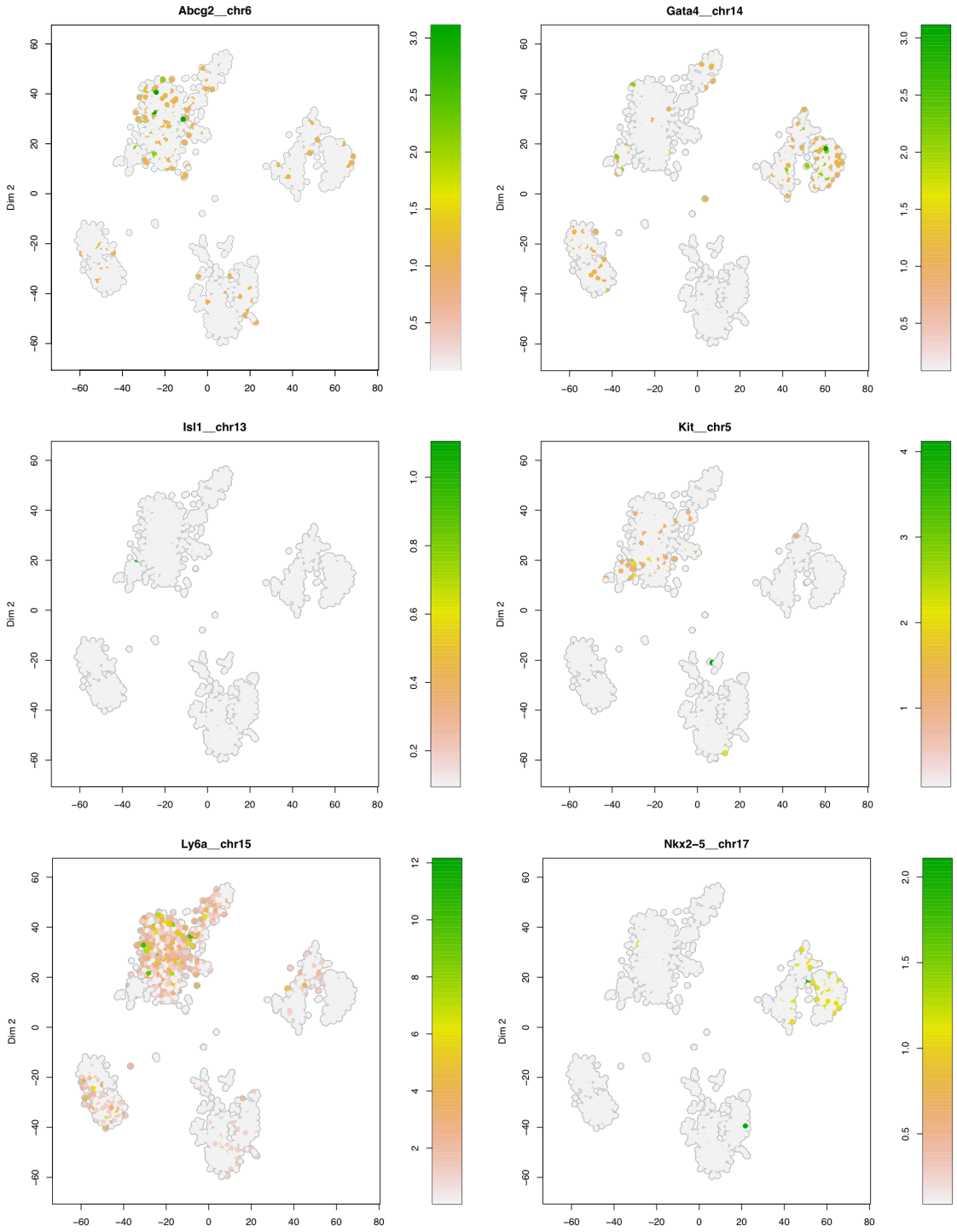

Fig. S5. Expression (normalised transcript counts) of putative cardiac stem cell markers in the dataset. mRNA transcript counts of *Abcg2, Gata4, Isl1*, *Kit* (c-KIT), *Ly6a* (SCA-1) and *Nkx2-5*.

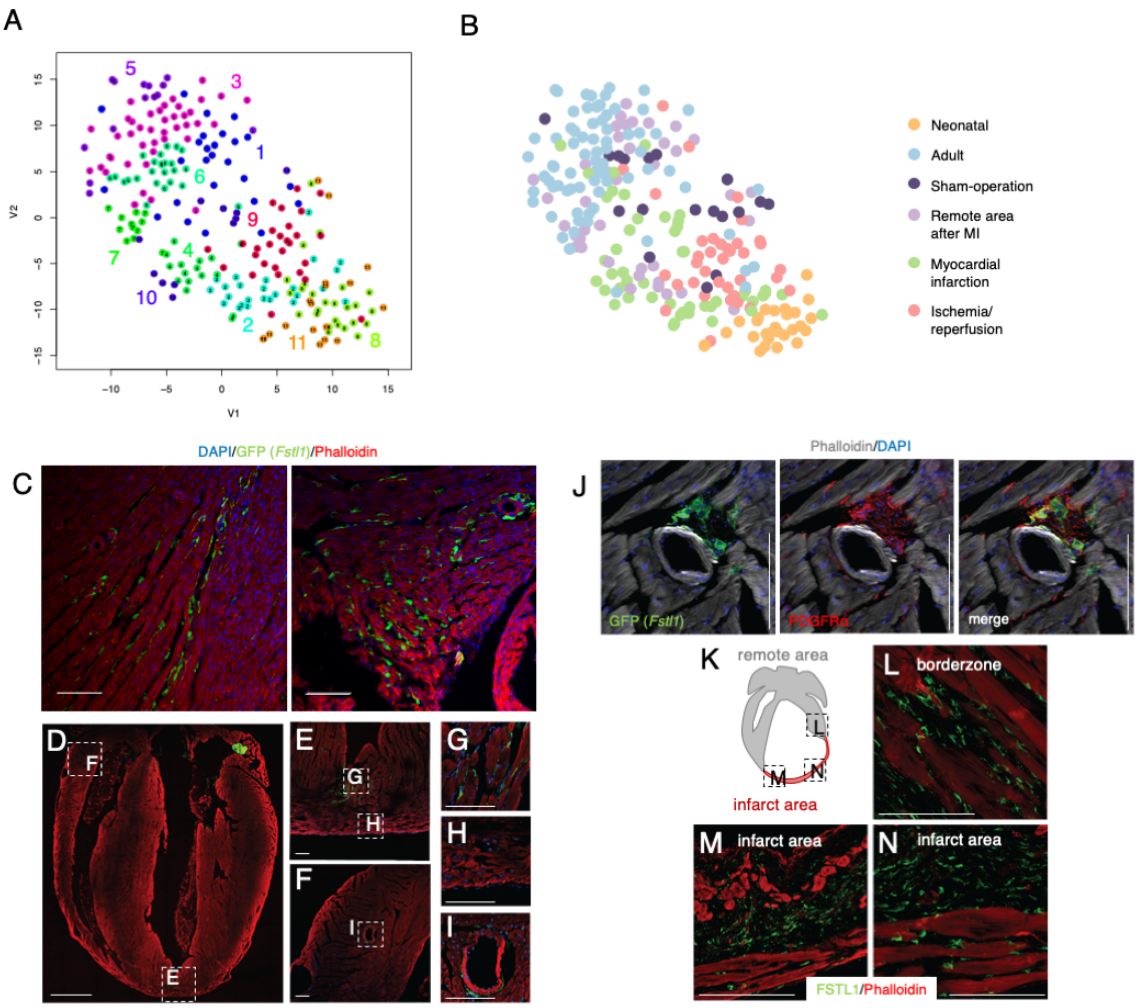

Fig. S6. *Fstl1* is highly expressed in fibroblasts from neonatal mice and after MI. (A) Clustering of cardiac fibroblasts and cell-to-cell distances visualised by t-distributed stochastic neighbour-embedding (t-SNE) map, highlighting 11 identified clusters. (B) Projection of the experimental conditions over the fibroblast sub-clusters. (C-J) eGFP^{+} cells (green) in hearts of neonatal (C) and homoeostatic (D-J) *Fstl1*$^{\text{eGFP-IRES-CreERT2}}$ mice overlap with fibroblast marker PDGFRα (J, red) and no eGFP^{+} were found in the epicardium (H). Nuclei were counterstained with DAPI (blue) and cell borders visualized by Phalloidin (as indicated, either red or white). (K-N) Validation of FSTL1 expression in the borderzone and in the infarcted region of wildtype mice two after weeks after MI using a specific anti-FSTL1 antibody (green) and cell border visualised by Phalloidin (red). Scale bars: 100 μm (C, E-J, K-N) and 1 mm (D).

References for SI reference citations

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3. Grün D*, et al.* (2016) De Novo Prediction of Stem Cell Identity using Single-Cell Transcriptome Data. *Cell Stem Cell* 19(2):266-277.
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5. Sylva M*, et al.* (2011) The BMP antagonist follistatin-like 1 is required for skeletal and lung organogenesis. *PLoS One* 6(8):e22616.
6. Love MI, Huber W, & Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15(12):550.
7. Scheele CL*, et al.* (2017) Identity and dynamics of mammary stem cells during branching morphogenesis. *Nature* 542(7641):313-317.
8. Grün D*, et al.* (2015) Single-cell messenger RNA sequencing reveals rare intestinal cell types. *Nature* 525(7568):251-255.

Other Supporting Information Files

Dataset S1. Differentially expressed genes of the initial cardiac cell clustering.

Dataset S2. Differentially expressed genes of the cardiomyocyte subclustering.

Dataset S3. TOP 100 differentially expressed genes in neonatal cardiomyocytes, adult injured-associated cardiomyocytes and adult homeostatic cardiomyocytes.

Dataset S4. Differentially expressed genes of the cardiac fibroblast subclustering.