Supplemental Materials

Supplemental Methods

Animal Treatment and MI Surgery.

All experiments involving mice were in compliance with protocols approved by the Institutional Animal Care and Use Committee of Peking University. Postn-MCM$^{+/+}$ mice[1] were obtained from Dr. Molkentin Laboratories and crossed with Rosa26-lsl-tdTomato$^{+/+}$ mice[2] for lineage tracing of cardiac myofibroblast after myocardial injury. Myh6 promoter-driven mCherry transgenic mice (Myh6-mCherry) [3] specifically expressed mCherry in cardiomyocytes, and were a gift from Dr. Kotlikoff. Tnni3-Dre[4], R26-iCre and IR1[5] mice were obtained from Bin Zhou Laboratories. For reprogrammed cardiac myofibroblast lineage tracing *in vivo*, Tnni3-Dre, Postn-MCM, and IR1 mice were crossed to obtain triple transgenic mice. For reprogrammed cardiac non-myocyte *in vivo* lineage tracing, Tnni3-Dre mice were crossed with R26-iCre and IR1 mice. Myocardial infarction (MI) surgery was induced by permanent ligation of the left anterior descending artery (LAD) as described[6]. Briefly, 8–9-week-old mice were anaesthetized with 2.5% isoflurane/97.5% oxygen and placed in a vertical support, suspended by upper incisors. Animals were intubated with a 24 G stump needle and placed in a supine position. Thereafter, the mice were ventilated with 1.5% isoflurane/98.5% oxygen using a VentElite mouse ventilator (Harvard Apparatus; stroke volume, 220 mL; respiratory rate, 120 breaths per minute). MI was induced by permanent ligation of the LAD using a

6-0 nylon suture. The occlusion was confirmed through color change (increased pallor) of the anterior wall of the left ventricle.

FACS analyses and sorting

For quiescent fibroblast and activated fibroblast separation, isolation from Postn-MCM$^{+/-}$/Rosa26-lsl-tdTomato$^{+/-}$ transgenic mice was performed 7 days post-MI. Adherent cells were harvested from culture dishes and washed with FACS buffer (10% FBS in PBS, filtered through a 0.22μm filtration membrane). Cells were incubated with CD140a (PDGFRA) (Invitrogen, 17-1401-81) and CD68 antibodies (Invitrogen, 53-0681-82) for 15 min at room temperature in FACS buffer. Thereafter, cells were washed with FACS buffer and filtered through a 40μm cell strainer. Cells were subsequently sorted and analyzed by FACS (BD FACS Aria™ III) and FlowJo software. A wild-type mouse cardiac fibroblast population was used as control to establish the gating strategy.

For single cell RNA-seq, cardiac cells were isolated from healthy and MI-D3 mice hearts. Briefly, hearts were minced and digested as previously described prior to filtering through 40μm filters. Cells were resuspended in red blood cell lysis buffer, followed by dead cell removal through use of a Dead Cell Removal Kit (Miltenyi Biotec, 130-090-101), with 7-AAD employed for further enrichment of active cells. For MI heart, to reduce contamination by endothelial cells and immune cells, cardiac cells were immunostained for 15 min at room temperature with fluorophore-conjugated CD31 (Biolegend, 102405) and CD68 (Biolegend, 137007) antibodies. The cells were washed two times with FACS

buffer before acquisition, and CD31-CD68- cells were sorted apart from the 7-AAD-negative live single cells using a BD Aria™ III cell sorter.

Two weeks post-induction, iCMs were digested in an enzyme mixture. First, cells were incubated in 0.25% Trypsin/EDTA at 37°C for 5–7 mins, then collagenase I/II/IV (2 mg/mL) and DispaseⅡ (2 mg/mL) were added to wells and incubated for another 30–40 mins. Cells were washed in FACS buffer and passed through 40μm cell filters. Reprogrammed cells expressing mCherry were analyzed using CytoFlex (Beckman Coulter) and FlowJo software.

Molecular cloning and viral transfection

All cardiac reprogramming transcription factors were cloned into Fu-tet-O lentiviral vectors (Addgene, #19778)[7]. The polycistronic pMX-MGT was provided by Dr. Qian[8]. Briefly, CDS sequences of *Gata4*, *Mef2c*, and *Tbx5* or the polycistronic cassette MGT from pMX vectors were amplified by PCR and cloned into the Fu-tet-O vector by Gibson assembly according to the manufacturer's instructions (TransGene). TetO-Myocd was a gift from John Gearhart (Addgene plasmid # 46033)[9]. TetO-Sall4 was described previously[10]. All shRNAs were constructed in pLKO.1 HIV-based lentiviral vector and were purchased from Sigma-Aldrich. Lentiviral vector (15μg) together with pMDLg/pRRE, RSV/Rev, and VSV-G (5μg each), were co-transfected into 293T cells together with 75μL PEI Max (1mg/mL, PolySciences) and 800μL Opti-MEM (Gibco) in 10-cm dishes following the manufacturer's instructions. After 12 hours of incubation, the medium was changed. Viral supernatant was

harvested after an additional 36 hours of incubation and filtered through 0.45μm filters (Millipore). Viruses were stored at -80°C for future use. For lentiviral gene transfer, lentiviral-containing supernatants were collected, filtered through 0.45μm filters, and concentrated 500-fold via ultra-centrifugation (120000 x g for 2h at 4℃) before being re-suspended in PBS containing polybrene (8μg/mL).

Human iPSCs culture

All pluripotent and iPSC differentiation cells were maintained at 37 °C, with 5% CO2 and atmospheric (21%) O2. iPS-B1 (CA4025106, CELLapy) were routinely cultured in PGM1 medium (CELLAPY) on the growth factor–reduced Matrigel-coated (Corning) plate. iPSCs were passaged every 4 days using Versene (Gibco) when cell confluency reached ~70%. 3-5 μM Rock inhibiter, Y27632 (Selleck Chemicals), dissolved in dimethyl sulfoxide (DMSO) was added for the first 12-24h after passage. The iPSCs were seeded in 6-well plates.

Cardiac fibroblasts differentiation from iPSCs

iPSCs were split into E8 medium (Cauliscell Inc.) at a ratio of 1:10 for the last passage before beginning differentiation. Cells differentiated in 24-well plates or 96-well plates. When they reached ~85% confluence, the medium was changed to RPMI + B27- medium, consisting of RPMI 1640 (RPMI, Gibco), 2% B27 without insulin (Gibco), and 1% Penicillin-Streptomycin (Life Technologies). From day 0 to day 2 (from iPSC to mesoderm), iPSCs were initially treated with

~8 μM CHIR99021 (CHIR, GSK3 inhibitor, Selleck Chemicals). CHIR concentration and duration may fluctuate depending on the cell passage number or other details. After CHIR treatment, the medium was replaced with RPMI + B27- medium until 72h (day 3). From day 4 to day 6, the RPMI + B27- medium was renewed and supplemented with 5 μM IWR1 (Wnt inhibitor, Selleck Chemicals) for 48 hours. Cardiac progenitor cells (CPCs) could be induced on day 6. On day 6, CPCs were digested by Accutase (Gibco) in a 37 °C, 5% CO2 incubator for 4 min, centrifuged at 200 x g for 3 min, and passaged onto a Matrigel-coated 6-well plate at a 1:10 split ratio; the medium was changed to Advanced DMEM/GlutaMAX medium (Advanced DMEM/F12 with 1% GlutaMAX and 1% Penicillin-Streptomycin, both from Gibco) containing 5 μM CHIR, 2 μM retinoid acid (Selleck), 5 μM of Y27632, and 1% FBS. On day 7, the medium was changed to DMEM/GlutaMAX medium containing 5 μM CHIR, and 2 μM retinoid acid. On day 9, medium was changed to DMEM/GlutaMAX medium. On day 11, cells were digested by Accutase in a 37°C, 5% CO2 incubator for 4 min, centrifuged at 200 x g for 3 min, and passaged onto a Matrigel-coated 6-well plate at a 1:3 split ratio; the medium was changed to Advanced DMEM/GlutaMAX medium containing 2 μM SB431542 (a TGFβ inhibitor, Selleck). Epicardial cells (EPCs) were obtained on day 14. Once EPCs were confluent on day 14, they were digested by Accutase in a 37°C, 5% CO2 incubator for 4 min, centrifuged at 200 x g for 3 min, and passaged onto a Matrigel-coated 6-well plate at a 1:3 split ratio; the

medium was changed to Fibroblast Growth Medium (Macgene) containing 1% Penicillin-Streptomycin, 20ng/mL of FGF2 and 10 μM of SB431542, to induce cardiac fibroblasts (CFs). Medium was changed every 2 days. On day 18, CFs were digested by Accutase in a 37 °C, 5% CO2 incubator for 4 min, centrifuged at 200 x g for 3 min, and passaged onto a 48-well plate at 80,000 cells/well. To induce MFs, the replated iPSC-CFs were treated with 5ng/mL TGF-β1 for 48h, and these iPSC-MFs were used for iCM reprogramming.

Immunofluorescence staining and microscopy

Cells were washed with PBS 3 times, and fixed in 4% paraformaldehyde for 15 min. Membranes of the cells were permeabilized with PBS containing 0.2% Triton X-100 (Biotopped, T6200) for 10 min and blocked with 3% normal donkey serum (Jackson, 017-000-121) for 1 h at room temperature. The primary antibody was then added and incubated at 4°C overnight. Primary antibodies were used at the following dilutions in blocking solution: mouse anti-cTnT (Thermo Fisher Scientific, MA5-12960, 1:200), goat anti-cTnI (Abcam, ab56357, 1:400), rabbit anti-α-Actinin (Abcam, ab137346, 1:500). On the second day, the primary antibody was removed, and the cells were washed with PBS. Alexa fluorogenic secondary antibodies (Invitrogen) were used at a dilution of 1:1000 in PBS containing 0.1% BSA (GPC BIOTECH, AA904-100G) at 37°C for 1 hour. Hoechst (YEASEN, 40732ES03, 1:1000) was used to locate the nuclei of cells. Images were captured using Zeiss inverted fluorescence microscopes (AXIO Vert.A1).

Hearts were collected and washed in cold PBS to remove excess blood before being fixed in 4% PFA at room temperature for 30 min. After washing in PBS several times, heart tissues were dehydrated in 30% sucrose at 4 °C overnight. Tissues were embedded in OCT (Sakura). 10μm cryosections were collected on positively charged slides. Sections were washed in TBST supplemented with 0.3% Triton X-100 for 10 min, and blocked with TBST supplemented with 0.3% Triton X-100 and 10% normal donkey serum for 30 min at room temperature, followed by primary antibody incubation at 4 °C overnight. Signals were developed using Alexa fluorescence-conjugated secondary antibodies (Invitrogen, 1:1000) for 30 min at room temperature. After washing with TBST three times, sections were counterstained using mounting medium containing 4′6-diamidino-2-phenylindole (DAPI, Southern Biotech, 0100-20). Fluorescence images were acquired using stereomicroscope (Zeiss AXIO Zoom. V16), confocal laser scanning microscope (Nikon A1R), or fluorescence microscope (Zeiss Vert. A1). The image data were analyzed using ImageJ (NIH) software.

Quantitative RT-PCR

Cells were lysed using TRIzol reagent (Invitrogen). Total RNA was extracted by Direct-zol™ RNA Microprep kit (ZYMO) and reverse-transcribed into cDNA using HiScript III All-in-one RT SuperMix (Vazyme). Quantitative RT-PCR was performed using a q225 Real-Time PCR system (Kubo Tech) and ChamQ SYBR qPCR Master Mix (Vazyme, Q321) according to the manufacturer's

instructions. The primer sequences are provided in Supplemental Information.

Single-cell library preparation and sequencing

Single cell capture, barcoding, and library preparation were performed according to the commercially available method, 10x Genomics Chromium System. The number of cells loaded into the system was calculated based upon the desired number of captured cells following manufacturer's instructions. scRNA-seq libraries were generated following capture. Single-cell libraries were sequenced using a Novaseq 6000 (Illumina).

Single-cell RNA-seq data analysis.

Raw scRNA-seq data was processed using 10x Genomics Cell Ranger software. Reads were filtered, barcodes counted, peaks called, and GEX molecules counted using cellranger-arc count software. Fastq files were aligned to the mm10 reference genome. The Seurat[11] software package version 4.1.1 was used in R version 4.1.3 to analyze processed scRNA-Seq data. R scripts containing data processing and clustering capabilities are available in Source code 1. 5006 cells from healthy hearts and 2752 cells from MI-D3 hearts remained after filtering of cells in which < 200 genes were detected; < 3 cells included features detected. Data were integrated using the Seurat function "FindIntegrationAnchors" with default parameters. Read counts were normalized by log transformation. Variable genes were detected using the Seurat function "FindVariableGenes" with default parameters. Dimensionality

reduction was used to explore transcriptional heterogeneity and clustering. Specifically, PCA was used to assess dimensionality reduction. The first 24 principal components (PCs) were used as inputs for a graph-based approach for clustering of cells by type using resolution 0.5 and as an input to UMAP for reduction to two dimensions to facilitate visualization.

Average expression of all genes at each time-point and each cluster were obtained. Two Seurat functions were employed to identify marker genes: “FindAllMarkers()” to compare each cluster with all others, and “FindMarkers()” for pairwise comparisons among clusters or sub-clusters. For each marker gene, the average fold change (avg_logFC), percentage of expression in each specific cluster (pct.1), percentage of expression in all the other clusters (pct.2), and adjusted p-value (p_val_adj) were calculated. Avg-logFC was chosen to represent cluster-specific markers.

mRNA sequencing and data analysis.

Cell samples were lysed using TRIzol (Invitrogen) and total RNA was extracted by an RNA purification kit (ZYMO RESEARCH) according to the manufacturer’s protocol. Sequencing was performed using the Illumina NovaSeq 6000. Index of the reference genome was built using Hisat2 (v2.0.5) and paired-end clean reads were aligned to the mm10 reference genome using Hisat2 (v2.0.5). To calculate differentially expressed genes (DEGs), a raw counts matrix consisting of 2 conditions was fed into DESeq2[12] after filtering out genes that were not

expressed across any replicates. Fold change>2 and qval<0.05 were used as thresholds. For Gene Ontology and KEGG pathway analysis, DEGs in different groups were used as input in the clusterProfiler software package[13]. Gene set enrichment analysis was applied to DEGs between conditions to find the enrichment of senescence genes using GSEA software[14]. Network analysis of Meox1 top 30 correlated genes was calculated by Spearman correlation using combined scRNA-seq data. The correlation score was larger than 0.36.

Cardiac echocardiography

Mouse cardiac function was evaluated by echocardiography using a VisualSonics Vevo2100 imaging system. Fractional shortening (FS) and ejection fraction (EF) were examined as indices of cardiac contractile function. M-mode tracings were used to measure left ventricular internal diameter at end of diastole (LVIDd) and end of systole (LVIDs). FS and EF were calculated according to the following formula: FS=[(LVIDd – LVIDs)/LVIDd]*100%; EF=[(LVEDV 2 LVESV)/LVEDV]*100%. LVESV, left ventricular end systolic volume; LVEDV, end diastolic volume. All measurements were performed double-blinded.