Supplemental Material

Detailed methods:

Myocardial infarction.

MIs were generated using male mice at 8-10 weeks of age by surgical ligation of the left anterior descending coronary artery (LAD). Sham operations were performed with similar procedures without occlusion of LAD. Mice were observed for up to five weeks after MI induction. The survival rate was calculated as the ratio of mice alive to the original number of mice. Cardiac functions at base line and post-MI were measured by echocardiograph at indicated time points using Vevo 2100. For determination of infarct size, histological sections at the remote level, suture level, and three levels below the suture at intervals of 500 μm were stained with Masson's trichrome and photographed. Infarct size was calculated as the ratio of the infarction length to the perimeter of the left ventricle in each section following the method previously described[1].

To determine the area at risk, at one day post-MI, mice were given 500 μl of Heparin (100 units/ml) and allowed to sit for 15 min. Upon sacrifice mouse hearts were immediately perfused with approximately 0.35 ml of 1% Evans blue solution via injection into the left ventricle from the apex of the heart. Hearts were then harvested, fixed with 10% formalin in PBS, and sliced into 1-mm thick sections. Pictures of each section on both sides were taken, and each section was weighed. The percentage of the area that is not perfused with the dye (pale color) to the total area was defined as area at risk for that section. The area at risk of the whole left ventricle was then calculated following the methods described previously[2].

To study the effect of various drugs on post-MI LV remodeling, mice were anesthetized with isofluorane, and arginase inhibitor BEC (S-(2-boronoethyl)-L-cysteine) (Cayman Chemical; 2.3 mg / kg) or NOS inhibitor L-NAME (N5-[imino(nitroamino)methyl]L-ornithine,

methyl ester, monohydrochloride) (Cayman Chemical; 10 mg/kg) were given by iv injection through the tail vein. Mice were given inhibitors on the day before, the day of the surgery, and the day after the surgery.

Heart tissue preparation.

Mice were given Heparin (500 μl of 100 units/ml) and allowed to sit for 15 min before sacrifice. The chest was opened and the right atrium was cut. The left ventricle was perfused with ~ 5 ml ice cold PBS containing 10 units/ml heparin to remove the blood. After removing the atrium and right ventricle, the left ventricle was separated into three parts (the infarct area, the border area, and the remote area). Different parts of the heart were snap frozen with liquid nitrogen and saved at -80°C for downstream applications. For histological analysis, the whole hearts were taken out after perfusion and were either embedded with OCT tissue embedding medium for frozen section preparation or fixed with 4% paraformaldehyde at room temperature for 48 hr for paraffin embedding and sectioning.

Immunocytochemistry and Western blot analysis.

Immunohistochemistry staining was performed on frozen sections of MI hearts with anti-Ly6G antibody (eBioscience) following the manufacturer's protocol. Immunofluorescence was performed on paraffin-embedded sections with anti-Arg1 antibody[3]. Western blot analysis was performed according to standard protocols using rabbit anti-Arg1 antibody and control anti-GAPDH antibody (Santa Cruz).

Heart cell isolation and FACS analysis of non-cardiomyocytes (CM).

Hearts from male mice of 8-10 weeks old were isolated and immediately retrograde perfused with Krebs-Ringer (K-R) solution (35 mM NaCl; 4.75 mM KCl; 1.19 mM KH_2PO4; 16 mM Na_2HPO_4; 134 mM sucrose; 25 mM $NaHCO_3$; 10 mM glucose; and 10 mM HEPES, pH 7.4) for 5 min (2 ml/min). The hearts were then perfused for another 15 min with K-R solution

containing 0.8 mg/ml type II collagenase (Worthington). The left ventricle was removed and minced in KB solution (20 mM taurine; 100 mM glutamic acid; 25 mM KCl; 10 mM KH_2PO_4; 10 mM glucose; 5 mM HEPES, 1 mM $MgSO_4$, and 0.5 mM EGTA, pH 7.2). After trituration and filtration, CMs were precipitated by brief centrifugation and non-CMs were then pelleted from supernatant. For FACS analysis, non-CMs were blocked with 5% BSA-PBS for 20 min on ice and stained with PE-conjugated anti-Ly6G antibodies (BD 561104) (1:100 in 1% BSA-PBS) for 30 min on ice. After staining, cells were washed twice with 1% BSA-PBS and centrifuged at 3,000x rpm for 5 min. Finally, cells were resuspended in 1% BSA-PBS containing 0.5% PFA and subjected to flow cytometry assay. All centrifuge steps were performed at 4^{o}C.

RNA, Microarray, and real-time polymerase chain reaction (RT-PCR).

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the protocol of the manufacturer. RNA (5 μg) was used to generate cDNA using Superscript III (Invitrogen). qRT-PCR was performed using SYBR Green (BioRad) with gene-specific primers listed in Supplemental Table 1. Microarrays were performed on the Illumina platform by the UT Southwestern microarray core.

Cell culture, transfection, reporter, and adhesion assays, measurement of NO.

293A and U937 cells were grown in DMEM supplemented with 10%FBS. For reporter assays, 293A cells were transfected with Arg1-luc reporter[4] and plasmids as indicated using Fugene6 (Roche). pCMV-lacZ was co-transfected as the internal control. Luciferase was measured using the luciferase assay kit (Promega) and normalized against co-transfected β-galactosidase activity. Human endothelial cells (HAECs) were gifts from Dr. Chieko Minno (UTSW) and cultured in EGM-2 bullet medium (Lonza). Adhesion assays with monocyte U937 cells were performed following a published protocol[5].Control, FoxO4, and Arg1 siRNAs were purchased from Sigma and transfected using RNAimax (Invitrogen). HAECs were transfected

with control, FoxO4, or Arg1 siRNA for about 60 hr and treated with or without 50 ng/ml of human TNFα (R&D systems) for 18 hr. HAECs were washed with complete RPMI 1640 once and U937 cell suspension ($1x10^6$) was added to each well of the 6-well plate. The plate was then incubated at room temperature for 20 min with gentle shaking at 75 rpm. U937 cells were removed and the HAEC monolayer was washed gently with 1xPBS and fixed with 1% PRA in PBS for 5 min. Finally PFA was removed and pictures were taken immediately. In rescue experiments, siRNA-transfected HAECs were transduced with lentiviruses expressing GFP, Flag-Arg1, or FoxO4 before adhesion assays. For NO measurement, 4-amino-5-methylamino-29,79-difluororescein diacetate (DAF-FM DA, 2.5 μM, Sigma), a fluorescent dye that specifically binds NO[6], was incubated with HAECs under ischemic conditions[7] for 60 min. Cells were washed, fixed, and photographed under fluorescence microscopy.

Electrophoretic mobility-shift assay.

Electrophoretic mobility-shift assay was performed using double-stranded oligonucleotides containing the Arg1 Foxo-binding sequences (Supplemental Table 1). Protein lysates from 293T cells transfected with Flag-FoxO4, Flag-FoxO1, Flag-FoxO3, or Flag-pcDNA3.1 were incubated with ^{32}P-labeled oligonucleotide probes in the presence of 1 μl of poly(dI-dC) (1.0 μg/μl), with/without 100-fold unlabeled oligonucleotide probes for 20 min at room temperature. Supershift formation was detected by adding anti-Flag M2 antibody (Sigma).

Arginase assay

Arginase activity assay was performed according to the instructions of the QuantiChrom Arginase Assay Kit (DARG-200, BioAssay Systems). Heart tissue samples were homogenized with 10 mM Tris-HCl (pH 7.4) lysis buffer containing 1 μM pepstatin A, 1 μM leupeptin, and 0.4% (w/v) Triton X-100. Lysates were centrifuged at 14,000 g for 10 min and the clear

supernatant was used for the assay. Arginase activity was normalized to protein concentration and presented as units per gram protein.

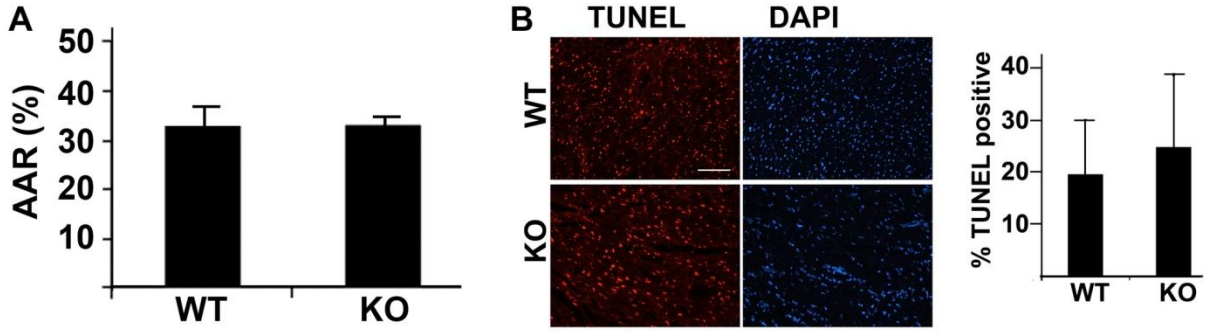
Animal care, *FoxO4* KO, *FoxO4* cKO, *FoxO4* ecKO, αMHC-cre, and Tie2-Cre mice.

All animal usage in this study was approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center. The *FoxO4*$^{-/-}$ and *FoxO4*$^{f/f}$ mouse lines were provided by Dr. R.A. DePinho[8] and maintained on FVB background. *Tie2*-cre[9] and *Tie2*-cre;*R26RT*$^{+/-}$ mice were generated by backcrossing male *Tie2*-cre;PDGFβ$^{f/+}$;*R26RT*$^{+/-}$(on C57BL6xSJLF background, from Dr. Michelle D. Tallquist)[10] with female WT FVB mice. The resulting male *Tie2*-cre or *Tie2*-cre;*R26RT*$^{+/-}$ mice were then crossed with female *FoxO4*$^{f/f}$ to generate *Tie2*-cre;*FoxO4*$^{f/f}$ (*FoxO4* ecKO) or *Tie2*-cre;*R26RT*$^{+/-}$;*FoxO4*$^{f/f}$ mice, respectively. *Tie2*-cre;*R26RT*$^{+/-}$;*FoxO4*$^{f/f}$ mice were used to confirm successful deletion of *FoxO4* in *Tie2*-cre targeted cells (Online Figure VII, C & D). αMHC-cre (C57BL6) and CAG-cre mice (C57BL6) were from Dr. Eric Olson (UTSW). αMHC-cre;*FoxO4*$^{f/f}$ (*FoxO4* cKO) mice were generated by crossing male αMHC-cre with female *FoxO4*$^{f/f}$ mice. Only male off-springs were used for MI surgery. Successful deletion of *FoxO4* in cardiomyocyte was confirmed by RT-qPCR in isolated cardiomyocytes (Online Figure VII, A & B).

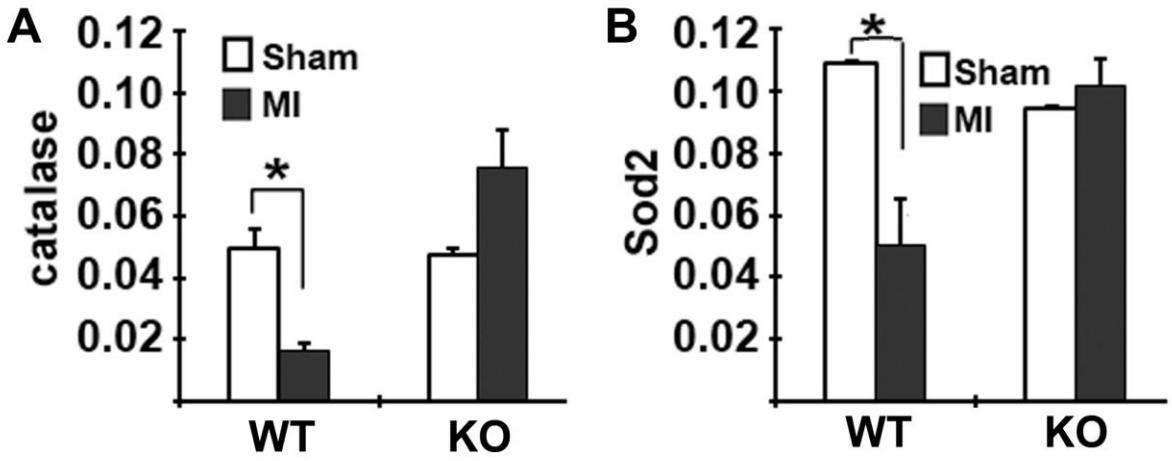
Statistical analysis.

Data are presented as means ± SEM unless otherwise stated. All statistical analysis was performed using GraphPad Prism software (San Diego, CA). The two-tailed *t*-test was used for comparisons between experimental groups. Differences were considered statistically significant as $p < 0.05$.

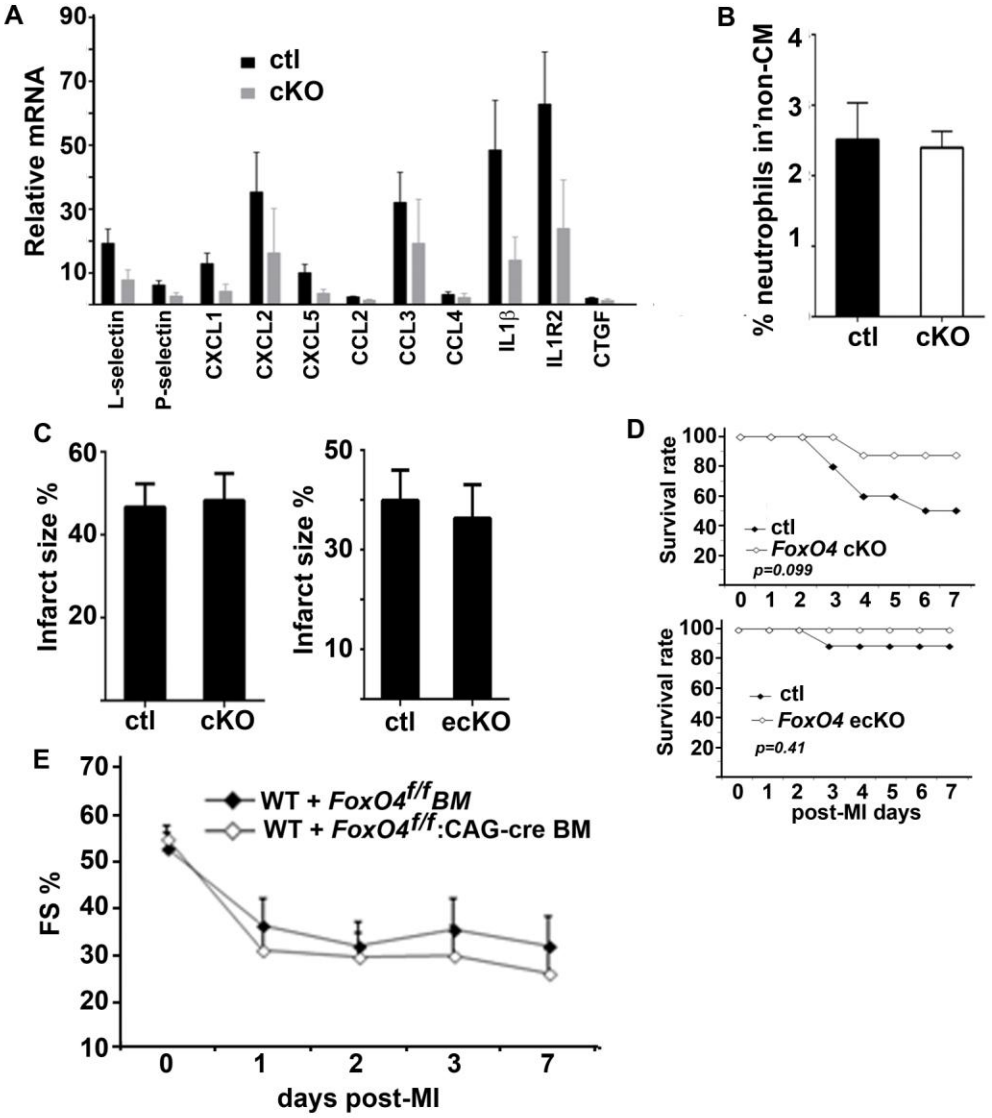
Online Figures.



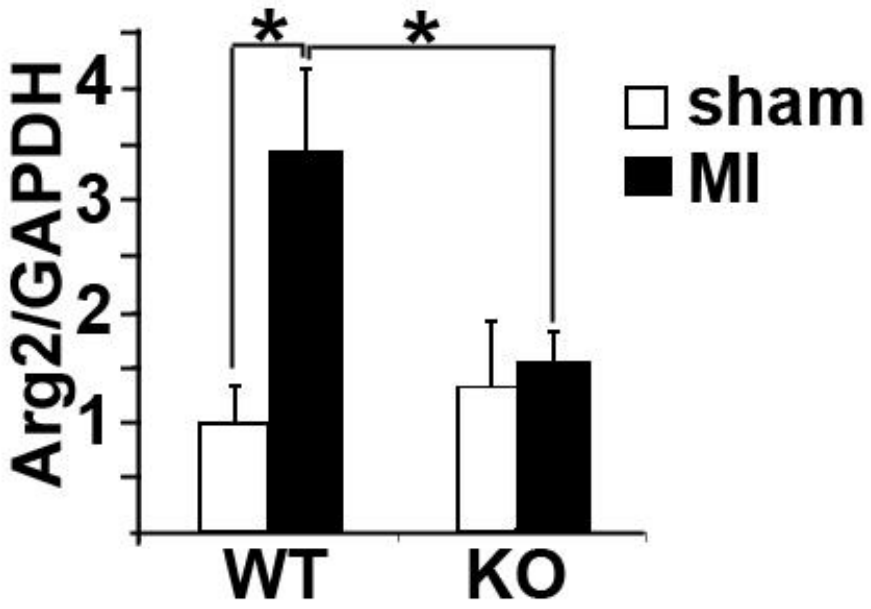
Online Figure I. WT and *FoxO4* KO mice have similar area at risk (AAR) and levels of apoptosis. (A) AAR of WT and *FoxO4* KO mice one day post-MI (n=5). (B) Frozen sections from one-day post-MI hearts were stained with TUNEL reaction solution following the manufacturers' protocol. For each tissue sample, 5 sections were stained and one picture was taken for each section. Scale bar=100 μm. Apoptotic cells (red) and total cells (DAPI) in each section were counted using Image J (NIH), and the percentage of apoptotic cells was averaged over all 5 sections. No significant differences in apoptotic cells were observed between infarcted WT and FoxO4 KO mouse hearts (n=3).



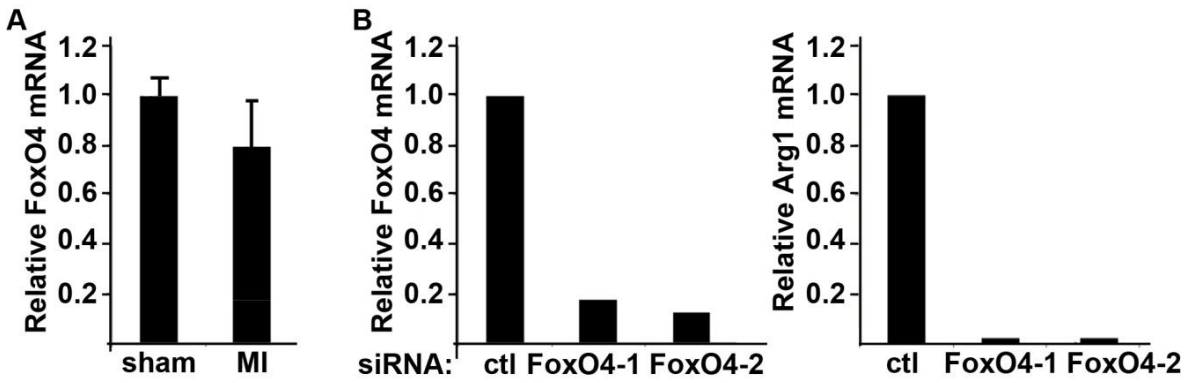
Online Figure II. Expression of Catalase and Sod2 in post-MI WT and FoxO4 KO mouse hearts. qRT-PCR of Catalase (A) and Sod2 (B) mRNAs in WT and *FoxO4* KO hearts one day after Sham and MI injury. mRNA was normalized against GAPDH. No significant changes in catalase and Sod2 were observed in *FoxO4* KO mice after MI whereas their expression in WT mice was significantly downregulated. n=3 ± SEM, *, $p<0.05$.



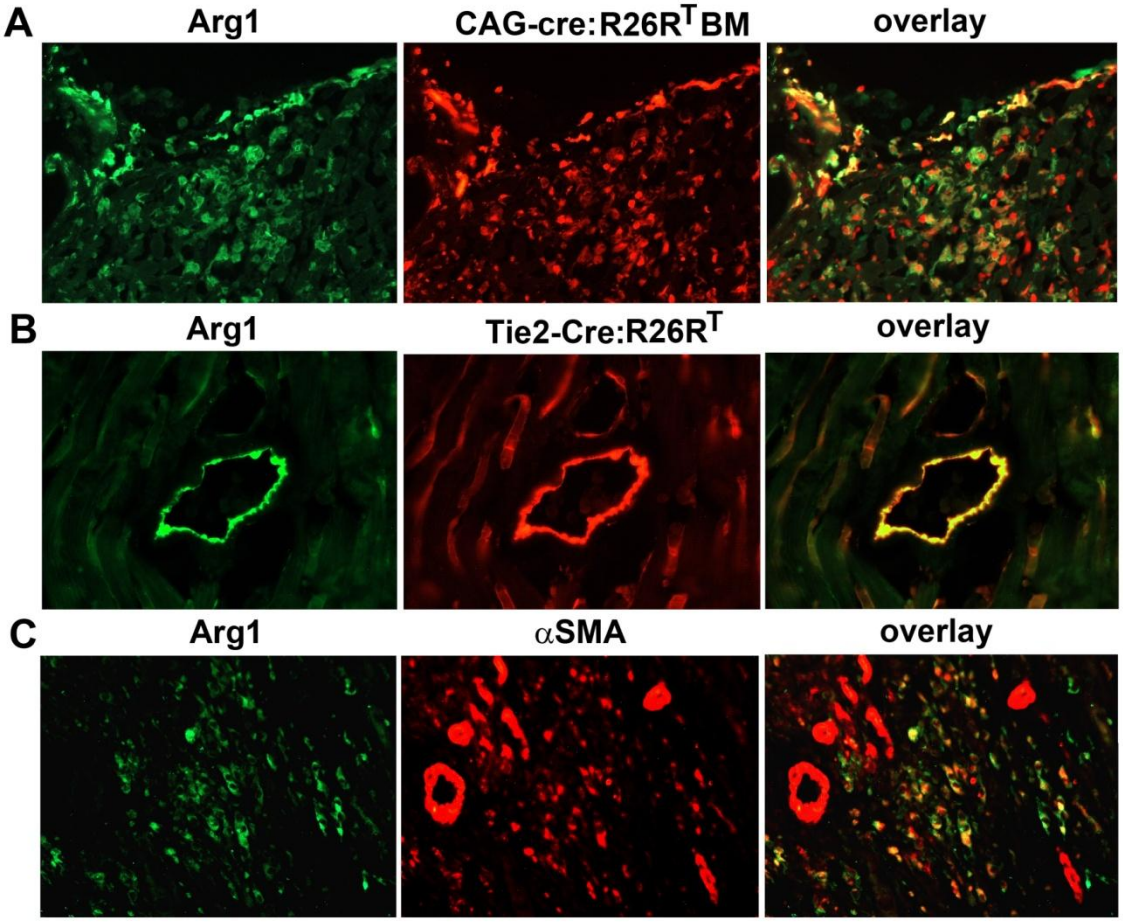
Online Figure III. Phenotypes of conditional deletion of *FoxO4* in cardiomyocytes, endothelial cells, or bone marrow. (A) qRT-PCR of relative mRNA of selective cytokines/chemokines of control (ctl) and *FoxO4* cKO mouse hearts post-MI 1 day (n=5). (B) Percent of neutrophils in non-cardiomyocyte fraction of control and *FoxO4* cKO mouse hearts post-MI 2 days (n=3). (C) Infarct sizes of *FoxO4* cKO and ecKO, and their respective control littermates at post-MI 7 days (n=3-6). (D) Survival rates of *FoxO4* cKO and ecKO (n=6-10). (E) Fraction shortening of WT mice with *FoxO4*-null bone marrow (WT+*FoxO4*$^{f/f}$;CAG-cre BM) or with control mouse bone marrow (WT+*FoxO4*$^{f/f}$ BM) at indicated post-MI days (n=4-5).



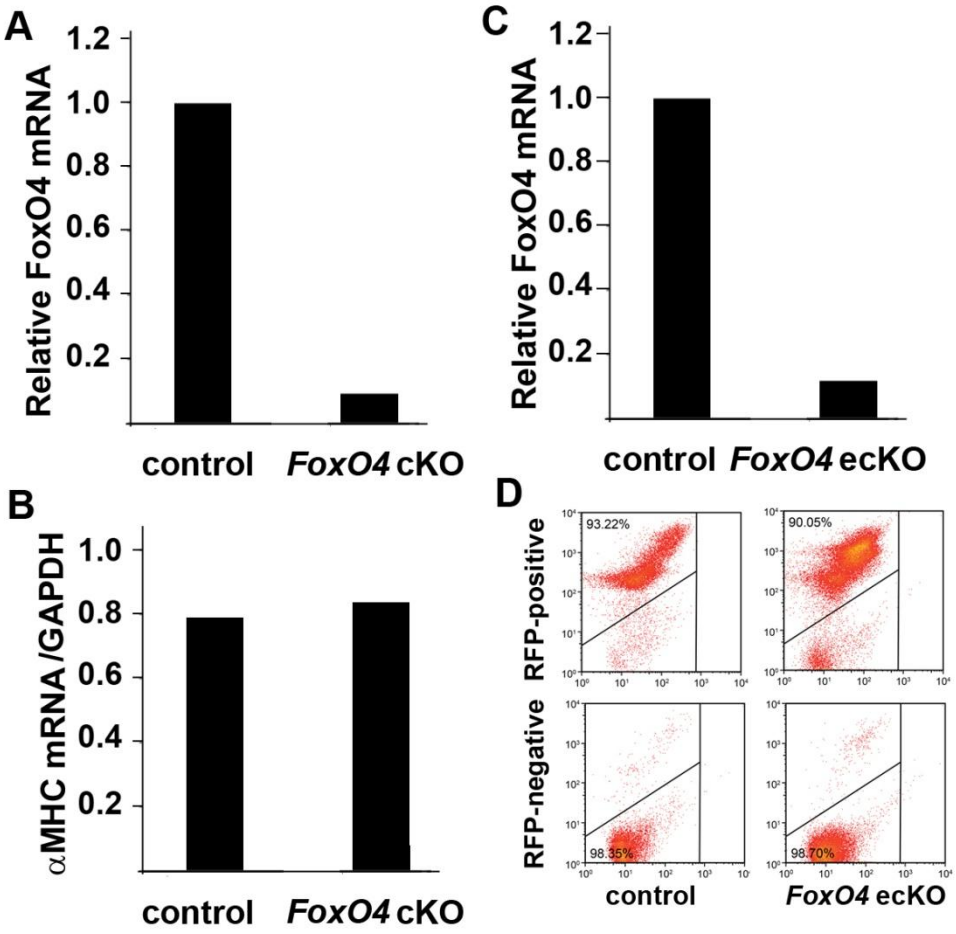
Online Figure IV. Arg2 expression in WT and *FoxO4* KO mice that underwent sham and MI surgery. mRNA was measured by qRT-PCR, normalized against internal GAPDH, and expressed relative to WT-Sham animals (n=8). Error bars are SEM, *, $p<0.05$.



Online Figure V. (A) Relative mRNA of FoxO4 in sham and MI hearts at post-MI day 1 (n=5-6). (B) Relative FoxO4 and Arg1 mRNA in HAECs transfected with two independent FoxO4 siRNAs. mRNA was normalized against internal GAPDH and expressed relative to sham (in A) or control (ctl) siRNA transfected cells (in B).



Online Figure VI. Arg1 is expressed ubiquitously in non-cardiomyocytes of the infarct heart. (A) Immunofluorescence of infarct mouse hearts stained with antibodies against Arg1 (green). WT mice were transplanted with bone marrow from *ROSA26R*^{tdTomato} (*R26R*^{T}) mice[11] that contain CAG-cre. Arg1 is expressed in the immune cells marked by *R26R*^{T} fluorescence (red) (yellow, overlap). (B) Immunofluorescence of Arg1 (green) in the infarct heart of *R26R*^{T} mice that contain Tie2-cre. Arg1 is expressed in endothelial cells of blood vessel marked by *R26R*^{T} fluorescence (red) (yellow, overlay). (C) Immunofluorescence of Arg1 (green) and αSMA (red) of infarct mouse hearts. Arg1 is expressed in myofibroblasts marked by αSMA (yellow, overlay).



Online Figure VII. Deletion of *FoxO4* in cardiomyocyte of *FoxO4* cKO (A, B) and Tie2-marked cells of *FoxO4* ecKO (C, D) mice. (A) Cardiomyocytes from *FoxO4* cKO (*αMHC-cre;FoxO4*$^{f/f}$) and control littermates (*FoxO4*$^{f/f}$) were isolated by Langendorff retrograde perfusion**.** About 90% of FoxO4 was deleted in myocyte prep of *FoxO4* cKO mice (A). The remaining FoxO4-expressing cells may be due to the impurity of the preparation. (B) αMHC mRNA in cardiomyocytes from control and *FoxO4* cKO mice. (C & D) Tie2-labeled cells from *Tie2-cre;R26R*^{T}*;FoxO4*$^{f/f}$ and control littermates *Tie2-cre;R26R*^{T} were isolated by Langendorff retrograde perfusion followed by red-fluorescence protein (RFP) FACS sorting (D). Approximately 85% of *FoxO4* was deleted (C).

Online Table I.

	Oligo name	Oligo sequence (5' to 3')
	mArg1_F	cctgaaggaactgaaaggaaag
	mArg1_R	ttggcagatatgcagggagt
	mL-selectin_F	ggtcatctccagagccaatc
	mL-selectin_R	tccatggtacccaactcagg
	mP-selectin_F	atgcctggctactggacact
	mP-selectin_R	gactgagcataggggcaca
	mCXCL1_F	agactccagccacactccaa
	mCXCL1_R	tgacagcgcagctcattg
	mCXCL2_F	aaaatcatccaaaagatactgaacaa
	mCXCL2_R	ctttggttcttccgttgagg
	mCXCL5_F	cctggtccgggatcttgt
	mCXCL5_R	catgaatggcgagatggaa
	mCCL2_F	catccacgtgttggctca
	mCCL2_R	gatcatcttgctggtgaatgagt
	mCCL3_F	tgcccttgctgttcttctct
	mCCL3_R	gtggaatcttccggctgtag
	mCCL4_F	gccctctctctcctcttgct
	mCCL4_R	gagggtcagagcccattg
	mIL1B_F	CAACCAACAAGTGATATTCTCCATG
	mIL1B_R	GATCCACACTCTCCAGCTGCA
	mIL1R2_F	cccatccctgtgatcatttc
	mIL1R2_R	gcacgggactatcagtcttga
	mCTGF_F	tgacctggaggaaaacattaaga
	mCTGF_R	agccctgtatgtcttcacactg
	mGAPDH_F	GGGTGCAGCGAACTTTATTGA
	mGAPDH_R	ATGGCCTCCAAGGAGTAAGAA
	mTBP_F	GTGAAGGGTACAAGGGGGTG
	mTBP_R	ACATCTCAGCAACCCACACA
	mArg2-F	tatggtccagctgccattc
	mArg2-R	ccaaagtcttttaggtggcatc
Cloning primers	Arg1-xho1-F(-178bp)	aaaaactcgagGTTGACTCTGTCATTCTTCCAT
	Arg1-xho1-F(-128bp)	aaaaactcgagTAAACAGATCCAACCTGATTAT
	Arg1-BglII-R	AAAAAagatctgctctctctgggttccagc

References

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