SUPPLEMENTAL MATERIAL

Supplemental Methods

Isolation of adult mouse myocytes

Adult mouse myocytes were isolated as previously described with minor modification [1]. Hearts were rapidly excised and cannulated via the aorta and connected to the perfusion apparatus. Hearts were perfused with calcium-free media containing Joklik-modified minimal essential medium (MEM), HEPES (10 mM), taurine (30 mM), DL-carnitine (2 mM), creatine (2 mM), 2,3 butanedione monoxime (BDM) (10 mM), and Liberase Blendzyme (Roche) and 20 μM calcium chloride at a rate of 3 ml/min for 15 min. After perfusion, atria were removed and ventricles cut into small pieces and triturated with a transfer pipette in Liberase Blendzyme solution. Myocytes were washed twice with heart medium containing 1% bovine serum albumin (BSA) and calcium chloride (20 μM). Calcium concentration were increased to 1.2 mM in 5-min intervals before the myocytes were plated on dishes coated with 10 μg/L laminin for 2 hrs.

Assessment of Cardiac Function

Hemodynamic function was performed in 15 animals per group (saline vs. doxorubicin) under non-stressed conditions at 8-12 weeks of age, 11 saline-injected (sedentary n=5, swimming n=6), and 13 doxorubicin-exposed mice (sedentary n=8, swimming n=5). Cardiac function was assessed *in vivo* in anesthetized mice by inserting a Millar microtip catheter into the carotid artery. Baseline functional measurements including heart rate (HR) and developed pressure (DP) were determined.

Microsphere Blood Flow Measurements

The measurement of blood flow has been described previously [2]. Briefly, 8 mice (saline n=4 and DOX n=4) were initially anesthetized with a mixture of ketamine (0.07 mg/g) and xylazine (8

μg/g). Anesthesia was maintained throughout the experiment with 0.5% isoflurane balanced with 1 L/min of oxygen. 18,000 fluorescent microspheres (10 μm size, Invitrogen) were injected into the left ventricle at a flow rate of 200 μl/min for 15 sec. At the same time of microsphere injection, a reference blood sample was withdrawn from the femoral artery at 0.2 ml/min. Blood and heart samples were collected and digested with 4N KOH for 48 hours according to the manufacture's protocol (Invitrogen). To elute the dye, the hydrolysate containing the digested tissue/microsphere was filtered with 3 μm membrane and placed in 1 ml of cellosolve acetate for 8 hours. Fluorescence in each sample was measured in a 96-well plate reader. Relative tissue blood flow rate was calculated using following formula:

$$F_i=(I_i)(R)/I_{ref}$$

Where F_i is blood flow in the sample, I_i and I_{ref} are the intensity of fluorescence in sample and reference blood, R is the withdrawal rate of reference blood sample.

Microfil Injection and Coronary Cast

The microfil injection was based on the protocol described by Kaneko et al. [3]. Corrosion casts were prepared and processed according to protocols in Batson's No. 17 plastic replica and corrosion kit (Polysciences Inc.). The hearts (saline n=3 and DOX n=3) were placed into PBS overnight and then dehydrated in serial ethanol and cleared in methyl salicylate.

Western Blotting

Hearts from 8 mice (saline n=4 and DOX n=4) were harvested at days 21, 40, and 60 after birth. Hearts were homogenized in ice-cold Lysis Buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and complete protease inhibitor cocktail (Roche). Lysates were incubated on ice for 30 min and then cleared by centrifugation at 20,000 x g for 20 min. The protein concentration of the supernatants were determined by the Coomassie Blue

binding assay (Pierce Chemical Co) with BSA standards. Equal amounts of proteins were loaded and separated on SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting using monoclonal antibodies against VEGF (Santa Cruz Biotech.) and tubulin (Sigma). VEGF was normalized against tubulin and data are presented as fold decrease compared to saline control.

ELISA

Blood was collected from mice upon sacrifice and then spun at 2500 x g for 20 min to obtain the serum. A sandwich ELISA was performed on the serum samples using Quantitative Mouse ELISA Kit (100-230-VEM, Alpha Diagnostic International, San Antonio, TX). The serum was incubated overnight in microtiter wells to allow binding to the immobilized VEGF antibody. A second VEGF antibody conjugated to biotin was added for 60 minutes followed by 4 washes. Next, a streptavidin horseradish peroxidase conjugate was added for 30 minutes followed by 5 washes. The levels of VEGF were measured by obtaining readings at $OD_{450\ nm}$. Values were plotted against a standard curve of purified recombinant mouse VEGF.

Histological Analysis and Immunostaining

Hearts were fixed for histological analysis in 4% formaldehyde, embedded in paraffin and sectioned. 4 μm thick sections were stained with hematoxylin and eosin (H & E) or Masson's trichrome according to standard protocols. For immunostaining, tissue sections were rehydrated and then microwaved on 50% power for 15 min in 10mM citrate buffer (pH 6.0) for antigen retrieval. After endogenous tissue peroxidase activity was quenched in 3% H_2O_2 for 20 min, samples were blocked with TN buffer (TNB) supplied in amplification kit (Perkin/Elmer) and incubated with primary antibodies overnight at 4° C (c-Kit: R&D; p16: Santa Cruz Biotechnology; Tropomyosin, Sigma; α-smooth muscle actin and CD31; Abcam: Flk1: Invitrogen; GATA4: Santa Cruz; MEF2C: Aviva).

Sections were washed 5 times for 5 min before incubation of secondary antibody diluted in TNB. Tyramide was used to amplify the c-kit signal according to the manufacturer's protocol. Endothelial cells stained with anti-CD31 were detected with Vectastain ABC kit peroxidase. Tissue sections and cells were observed through a Nikon TE300 fluorescence microscope (Nikon) equipped with a cooled CCD camera (Orca-ER, Hamamatsu). 7 fields per heart were assessed in every experiment, selecting epi/mid myocardium and endo/mid myocardium for 3 quadrants plus mid myocardium for septum. For MI experiments, 7 fields were assessed in the border zone of the infarct.

Transmission Electron Microscopy

Hearts were fixed *in vivo* in 4% paraformaldehyde + 1.5% glutaraldehyde in 0.1M cacodylate buffer. The hearts were removed, each sliced longitudinally into 2 pieces for continued immersion fixation in the same fixative for 6hrs on ice. The left and right ventricles were then sliced into long strips for further overnight fixation in 3% glutaraldehyde in 0.1M cacodylate overnight at 4°C. The tissue pieces were washed in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer and dehydrated in a graded ethanol series. The tissue was then treated with propylene oxide as the transition solvent before embedding in Epon/Araldite (Electron Microscopy Sciences (Hatfield PA). Thick sections were cut and stained with toluidine blue for general tissue assessment. Thin sections (60nm) were cut with a diamond knife (Diatome, Hatfield PA), mounted on copper slot grids coated with parlodion and subsequently stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI, Hillsbrough OR). Images were documented using a Megaview III ccd camera (Olympus Soft Imaging Solutions, Lakewood CO). Images were then handled in Adobe Photoshop.

MTT assay

An equal number of cells were plated in a 96 well plate one day prior to treatment with vehicle or doxorubicin. Proliferation was assessed by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoyltetrazolium bromide (MTT) to formazan by viable cells. Cells were incubated with MTT solution (5 mg/mL in PBS) for 4 h, the formazan (blue crystals) were solubilized in 10% SDS, 0.01 N HCl o/n, and MTT reduction was measured the next day by absorbance at 570 nm in a plate reader. Absorbance values that were lower than control cells indicate a reduction in the rate of proliferation.

TUNEL Staining and Cell Death Assay

TUNEL staining to label fragmented DNA in heart sections was performed using the In Situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. Nuclei were counterstained with Hoechst 33342 (Molecular Probes). TUNEL positive nuclei were counted in 10 randomly selected fields of each condition and expressed as a percentage of the total number of nuclei. Cells were treated with vehicle or doxorubicin for 72 h and cell death was measured by Trypan blue dye uptake using a bright-field microscopy. Living cells exclude the dye, whereas dead cells take up the blue dye. Each condition was set up in triplicate wells and a minimum of 300 cells/well were counted.

BrdU Pulse Chase Experiment

C-kit positive cells were isolated from 12 day old mice which had received injections of saline (n=4) or 1 mg/kg doxorubicin (n=4) at day 5 and 10. The cells were plated overnight and then labeled with10 μM BrdU (Sigma) for 6 h. Alternatively, cultured c-kit+ cells (passage 2-3) isolated from untreated mice were treated with saline or 100 nM doxorubicin for 24 h before pulse-labeling with BrdU. After 6 h, the cells were washed to remove unincorporated BrdU, and then fixed in cold 70% ethanol for 30 min. Cells were incubated in 2 N HCl for 20 min, washed in 0.1 M sodium borate (pH

8.5) for 2 min, followed by a wash in PBS for 5 min. Cells were stained with anti-BrdU (Sigma) and with a secondary Alexa 594 antibody (invitrogen). Nuclei were counterstained with Hoechst 33342.

Telomerase Activity Assay

Telomerase activity was measured in extracts prepared from cultured progenitor cells treated with 100 nM DOX for 72 h and 96 h. Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP) using the *Telo*TAGGG PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals) according to the manufacturer's instruction.

SUPPLEMENTAL FIGURES AND LEGENDS

Figure S1 Huang et al.

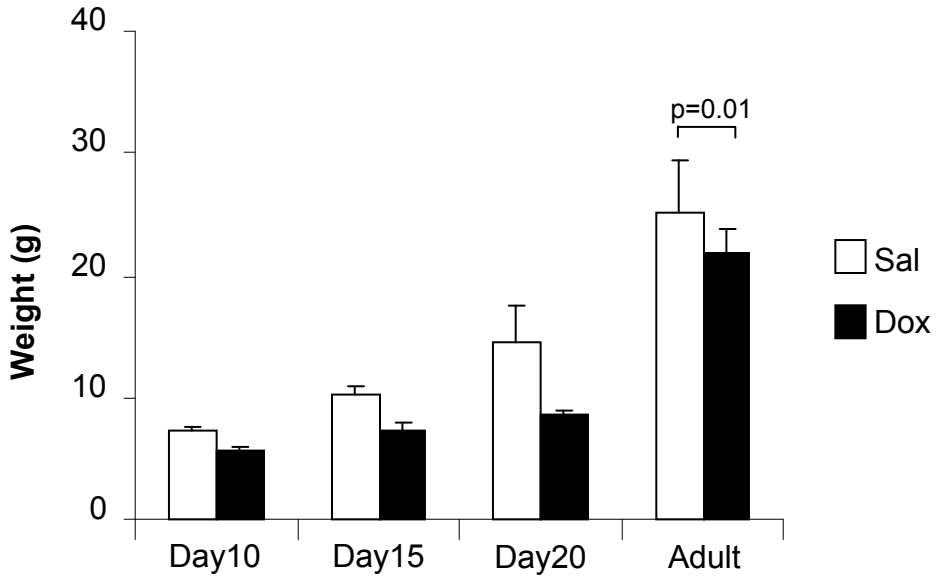

Figure S1. Weight of mice after injections with saline or doxorubicin (n=10).

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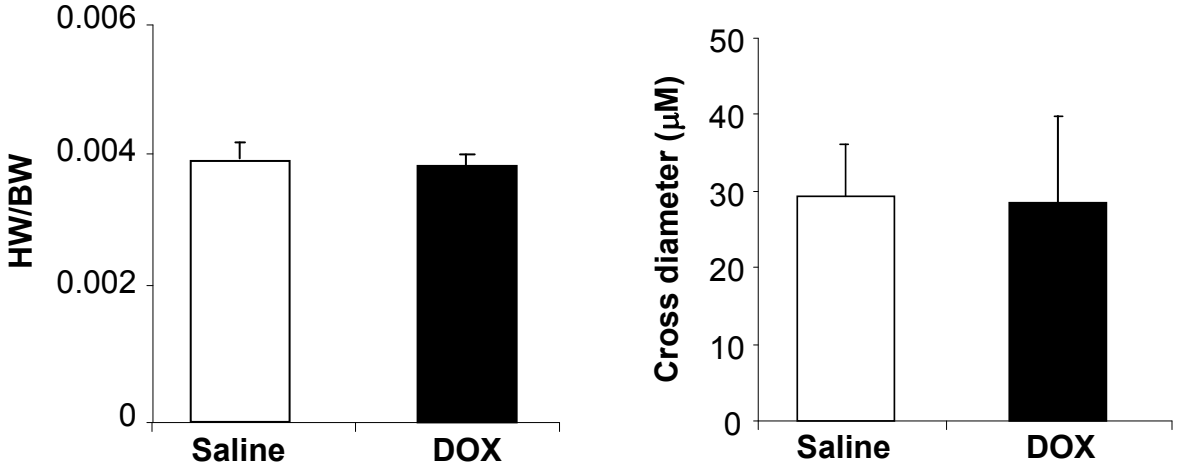
A.



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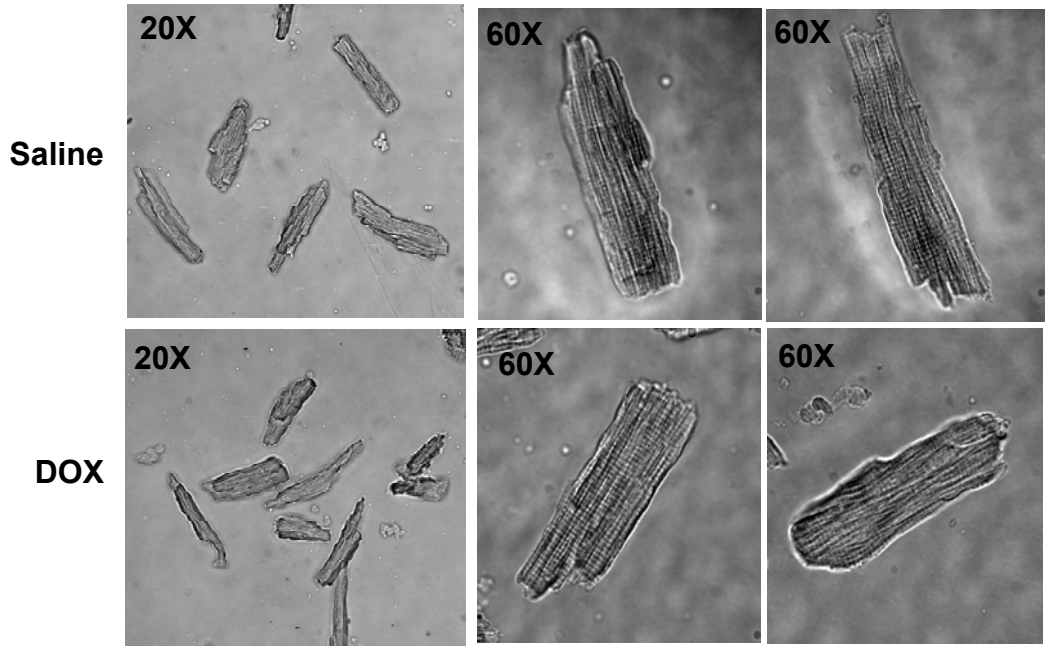
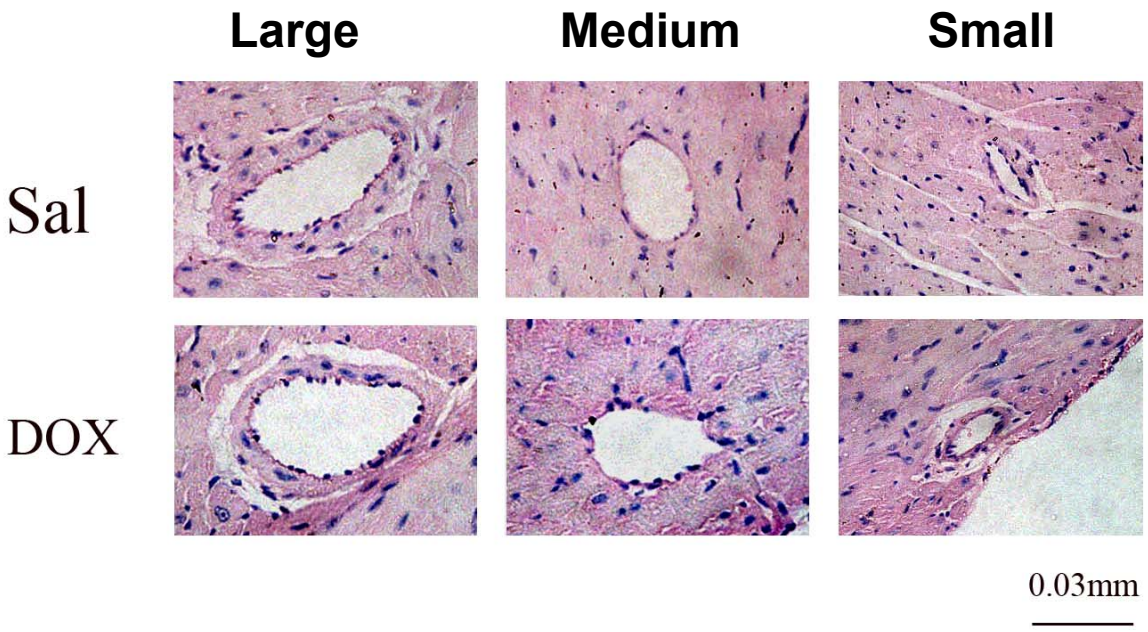


Figure S2. A. HW/BW ratio of non-stressed mice (n=10). **B.** Cross sectional measurement of myocytes. **C**. Bright field images of myocytes isolated from adult Saline and DOX hearts.

Figure S3 Huang et al.

A.



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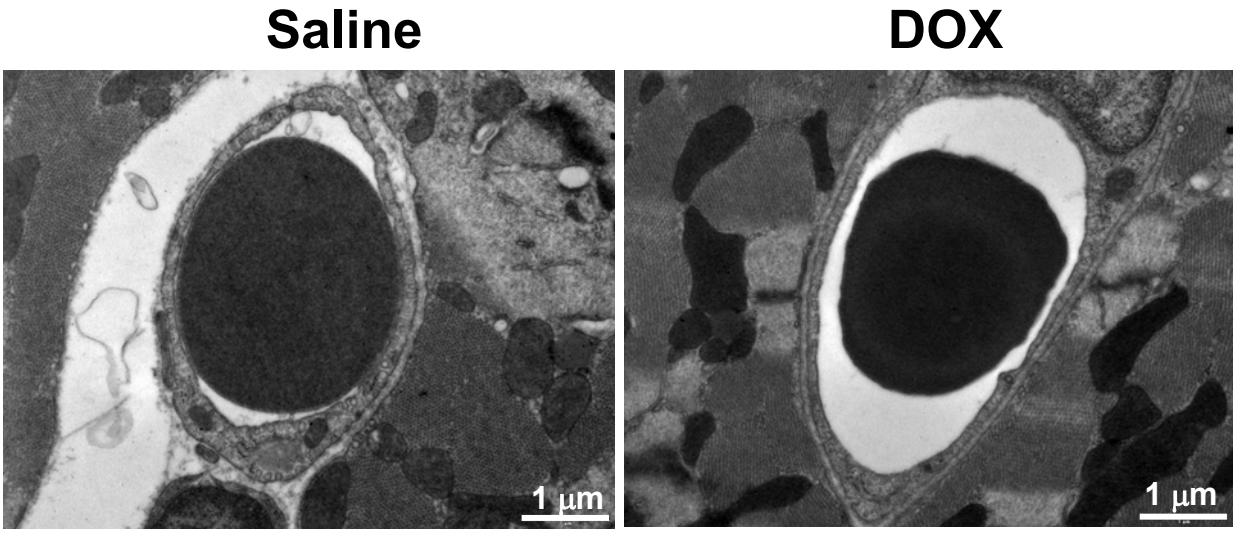


Figure S3. A. Representative H & E staining of vessels in heart sections prepared from adult mice that were treated with saline or DOX as juveniles. There were no obvious differences in the structure of vessels. **B.** Transmission electron micrograph of vessel in adult saline and DOX hearts.

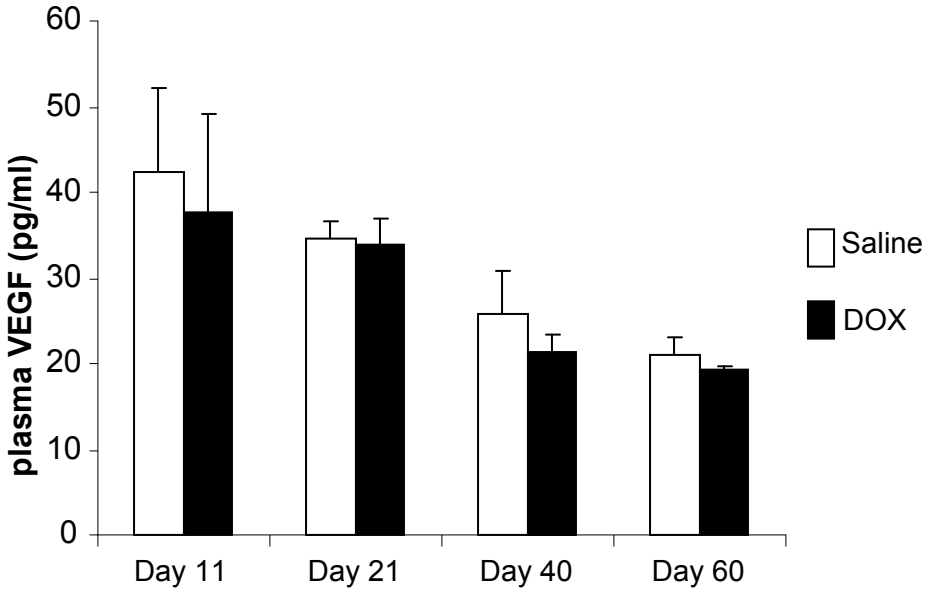


Figure S4. Plasma levels of VEGF in saline and DOX treated mice were determined by ELISA (n=4).

Figure S5 Huang et al.

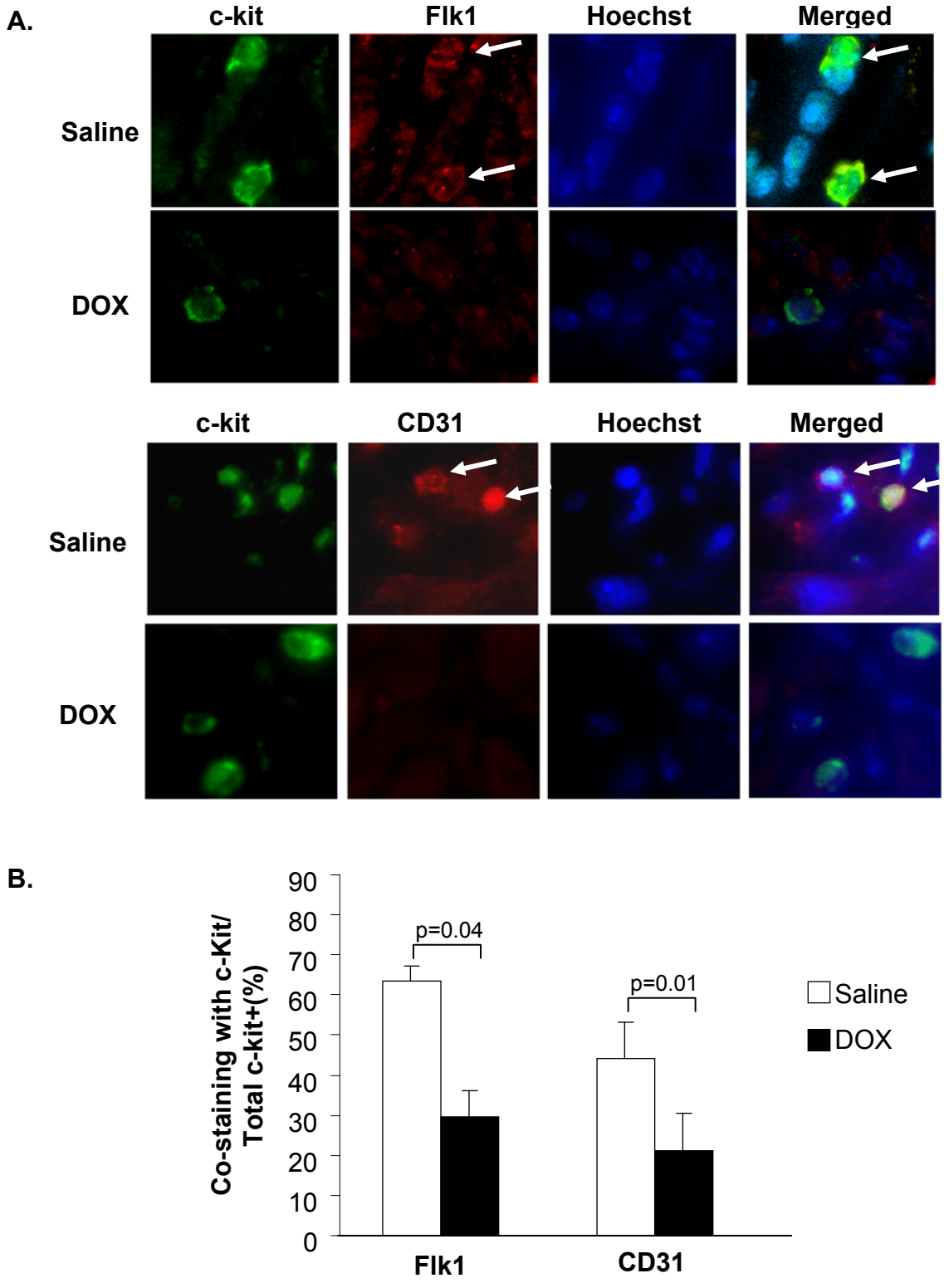


Figure S5. Staining of CPCs for markers of endothelial cell lineage. **A.** Co-localization of c-kit+ and Flk1 or CD31 in heart sections from saline and DOX mice 7 days after the myocardial infaction. **B.** Quantitation of c-kit+ cells positive for Flk1 or CD31 (n=3).

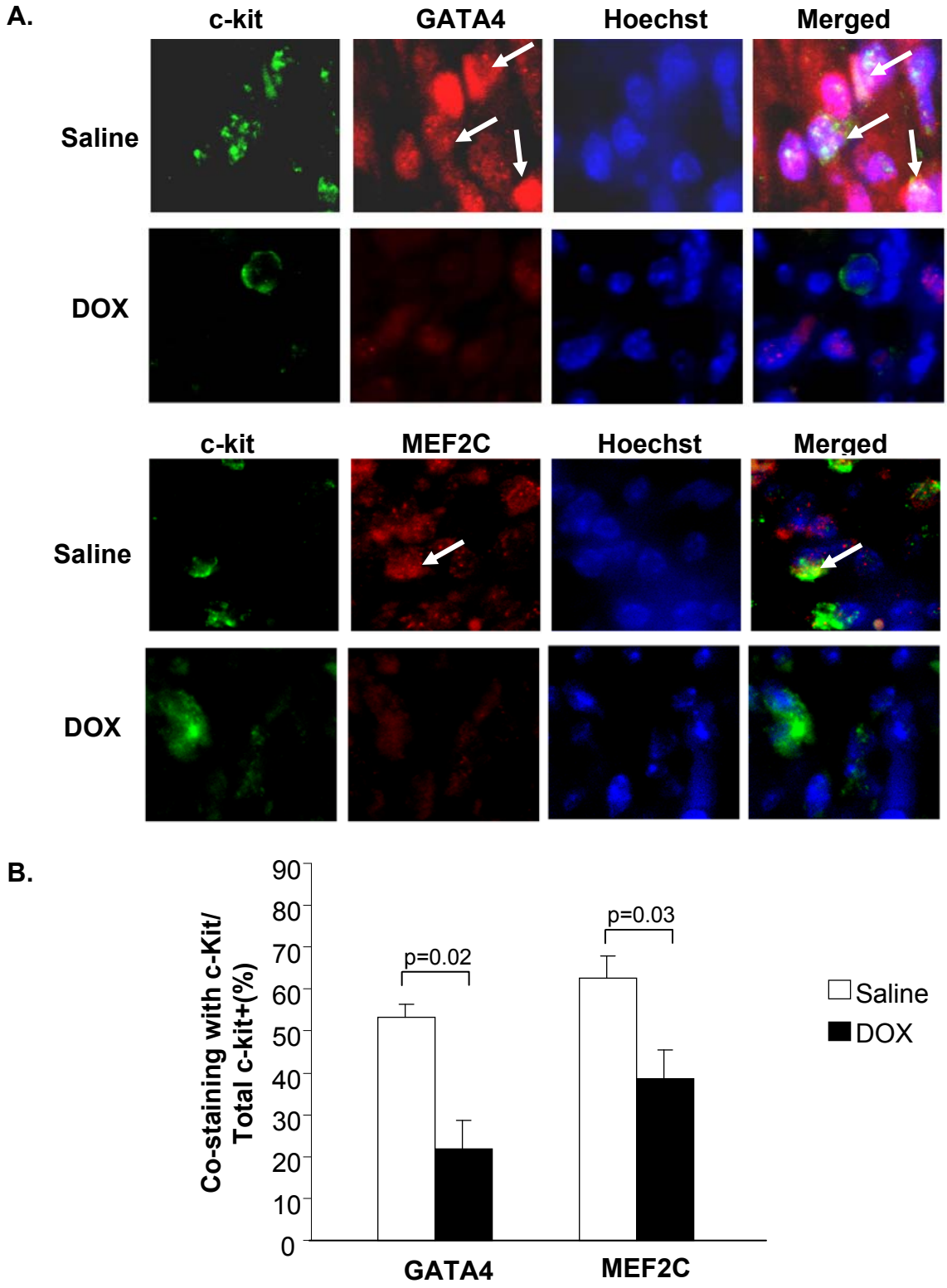
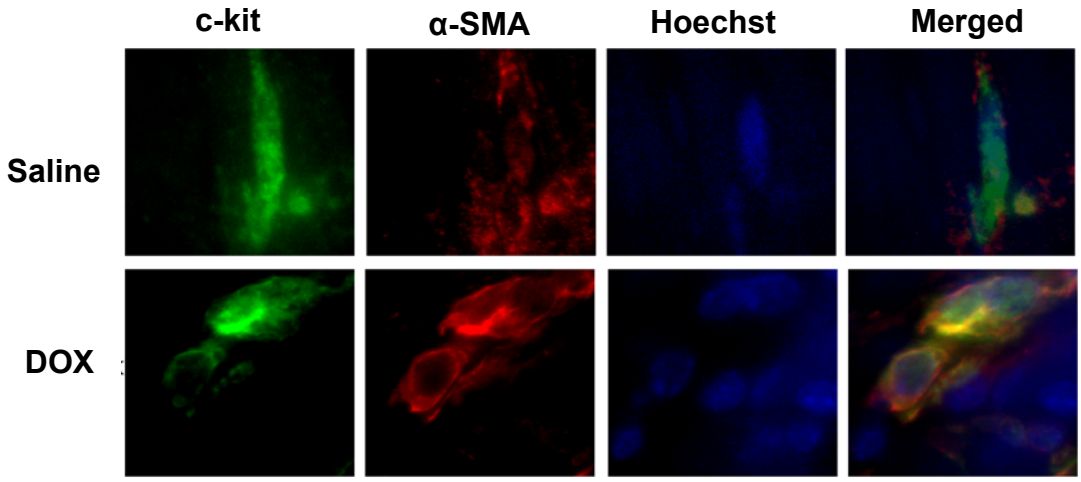


Figure S6. Staining of CPCs for markers of myocyte cell lineage. **A.** Co-localization of c-kit+ and GATA4 or MEF2C in heart sections from saline and DOX mice 7 days after the myocardial infaction. **B.** Quantitation of c-kit+ cells positive for GATA4 or MEF2C (n=3).

A.



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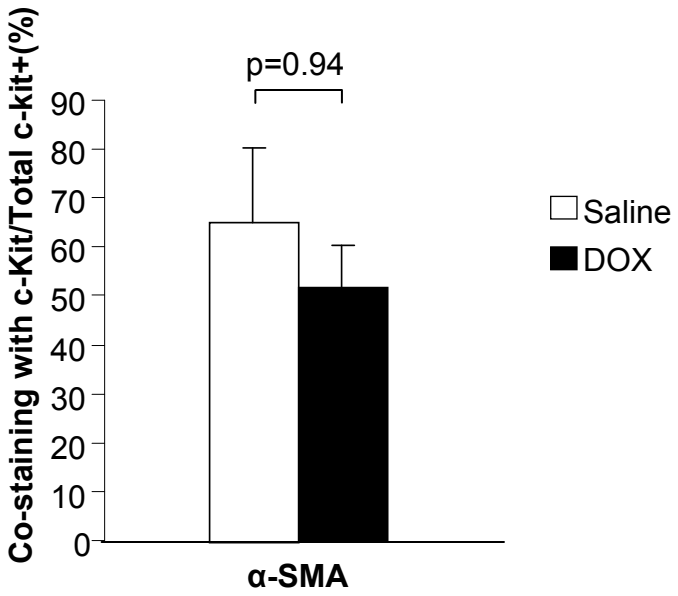

Figure S7. Staining of CPCs for markers of smooth muscle cell lineage. **A.** Co-localization of c-kit+ and α-smooth muscle actin (α-SMA) in heart sections from saline and DOX mice 7 days after the myocardial infarction. **B.** Quantitation of c-kit+ cells positive for α-SMA (n=3).

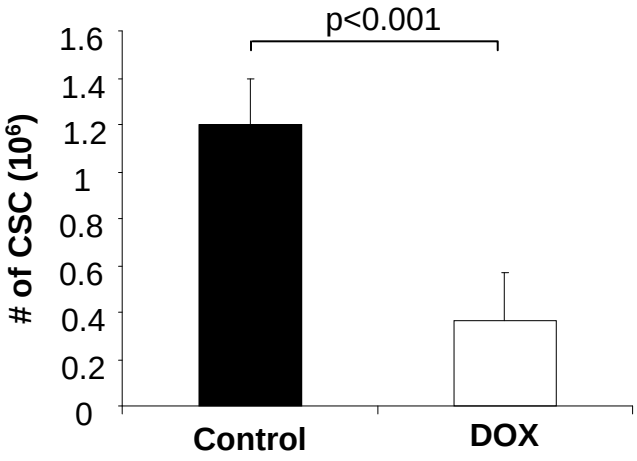

Figure S8. An equal number of cells were plated on 35 mm plates, treated with 100 nM doxorubicin for 72 h, and then counted on a hematocytometer (n=4).

SUPPLEMENTAL REFERENCES

1. Hilal-Dandan R, Kanter JR, Brunton LL. Characterization of G-protein signaling in ventricular myocytes from the adult mouse heart: differences from the rat. *J Mol Cell Cardiol.* 2000;32(7):1211-1221.

2. Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA. Apoptosis repressor with caspase recruitment domain protects against cell death by interfering with Bax activation. *J Biol Chem.* 2004;279(20):21233-21238.

3. Kaneko K, Li X, Zhang X, Lamberti JJ, Jamieson SW, Thistlethwaite PA. Endothelial expression of bone morphogenetic protein receptor type 1a is required for atrioventricular valve formation. *Ann Thorac Surg.* 2008;85(6):2090-2098.