SUPPLEMENT MATERIAL

β-Myosin Heavy Chain Is Induced by Pressure Overload in a Minor Sub-Population of Smaller Mouse Cardiac Myocytes

DETAILED METHODS

Cell staining and flow cytometry.

Fixed cells in Calcium- and Magnesium-Free PBS (CMF-PBS, mmol/L: NaCl 137, KCl 2.68, KH_2PO_4 1.452, and Na_2HPO_4-$7H_2O$ 8.058) were aliquoted into tubes (~200,000 adult myocytes/ml; ~600,000-800,000 neonatal total cells/ml), pelleted at 800x*g* for 3min, incubated 30min at 37°C in CMF-PBS 100µl with donkey serum 5%, mouse IgG 5µg/ml (Invitrogen), Triton X-100 0.1%, DNAse-free RNAse A 2µg/ml (Sigma, St. Louis, MO), and finally incubated overnight with primary antibodies at 4°C.

Cells were labeled with the following monoclonal IgG antibodies: (1) rat anti-CD45 conjugated to allophycocyanin (APC)-Cy7 (0.25µg/ml, Clone 30-F11, BD Pharmingen); (2) mouse anti-β-MyHC (1:500, ascites, Clone NOQ7.5.4D, Sigma, MO); (3) mouse anti-sarcomeric MyHC that does not distinguish between α- and β-MyHC isoforms (1µg/ml, Clone MF-20, Developmental Studies Hybridoma Bank, Iowa); (4) mouse anti-troponin T (0.25µg/ml, Clone 13-11, Lab Vision); (5) mouse anti-α-MyHC (0.5µg/ml, Clone BA-G5, Abcam); and (6) mouse anti-Ki67 Alexa 647 (1:50 pre-diluted, Clone B56, BD Pharmigen). Rabbit anti-phospho-histone H3 serine 10 polyclonal IgG (1:600) was from Millipore (06-750). Mouse monoclonal IgGs (MF20, NOQ7.5.4D, BA-G5, and 13-11) were labeled with Zenon kit (Invitrogen) conjugated to Alexa 488 (green), Alexa 647 (red), or biotin followed by streptavidin-APC-Alexa 780 (far red), following manufacture's recommendations.

After the Abs above, cells were stained for the incorporation of the thymidine analogue ethynyl deoxyuridine (EdU) using the Click-iT EdU Flow Cytometry Assay Kit with Alexa Fluor 647 azide, following the manufactures recommendations.

Optimal titration of these reagents used the coexisting non-muscle cells in these cardiac preparations as the in-tube negative control, and preliminary studies defined the Ab dilution that detected the maximum number of cells.

Prior to flow cytometry, cells were washed with CMF-PBS containing calf serum 2% and re-suspended in CMF-PBS 500µl with calf serum 0.2% and propidium iodide (PI) 10µg/ml or 7-Amino-actinomycin D 4µg/ml (7-AAD; BD Pharmingen) to detect DNA content.

Data were collected using 3 different flow cytometers: (1) a standard FACScan (BD Biosciences, San Jose, CA) upgraded to a dual laser system with the addition of a blue laser (15mW at 488nm) and a red laser (25mW at 637nm) (Cytek Development, Inc, Fremont, CA); (2) a standard dual laser BD FACSCalibur with a blue laser (15mW at 488nm) and a red laser (12mW at 635nm); or (3) a BD LSR II with 3 solid state lasers, violet (50mW at 405nm), blue (20mW at 488nm), and red (18mW at 633nm). All instruments had standard manufacturer-supplied filter sets, and flow cells measured 430µm x 180µm. To enhance the ability of these standard bench top flow cytometers to analyze adult cardiac cells, we made modifications in routine collection procedures to avoid instrument clogging, the main complication of sampling adult cardiac myocytes. We did the following: (1) fixed myocytes in a large volume (12ml) to

avoid cell clumping; (2) adjusted the concentration of large myocytes at the time of cytometry to ~200,000/ml (standard 1-2 million/ml); (3) removed the outer cylinder of the collection port to minimize cell aggregation at the port site and to facilitate backflow rinsing between samples; (4) re-suspended cells by gentle vortexing every 2min during collection; and (5) treated routinely with 10% bleach for 10min before and after each experiment to minimize debris build up in the system. Once all these procedures were in place, there was no need for service on clogged cytometers to collect the data presented in this manuscript.

The flow rate was set as "Hi" in FACScan and "MED" for FACSCalibur and LSR II. Event rates ranged from 50-300 particles/sec. Thresholds were set at 200 arbitrary units on FL3-log channel, and voltage was adjusted in every preparation to locate the NMC G1 peak at ~1000 arbitrary units in FL3, to gate the nucleated cells and minimize debris from preparation to preparation (Figure 1A in main text).

Alexa 488, Alexa 647, PI or 7-AAD, APC-Alexa 780, and APC-Cy7 signals were collected for ≥10,000 nucleated myosin heavy chain expressing cells (myocytes) in each tube. In all experiments, the positive and negative gates were determined by collecting fluorescence of the same cells stained with nonspecific mouse IgG as a staining negative control. Post collection, data were analyzed using FlowJo software (Tree Star, Inc., OR) on a Mac OSX platform.

Mice, rats, and rabbits.

Adult C57Bl/6J mice were from Jackson Laboratory. The same strain was bred in our Veterinary Medical Units to produce fetal mice at 17-19 day post-coital (dpc) and newborn litters. Female Sprague-Dawley rats with newborn litters were from Charles River. Adult New Zealand White male rabbits were from Charles River. The SF VAMC or UC Davis IACUC approved all protocols.

Transverse aortic constriction (TAC) and echocardiography.

Male mice age 11-12w had TAC by the method of Rockman.[1] Anesthesia without endotracheal intubation was with isoflurane, 3% in 100% oxygen for induction in a chamber, then 1.5% for maintenance by nose cone at 0.5-1 L/min. The transverse aorta was dissected above the left rib cage, and tied under direct visualization with a 7-0 nylon suture against a 27-gauge needle. The wound was closed, and the incision was injected with bupivacaine HCl 8mg/kg as a 2.5mg/ml solution (Hospira, Inc., Lake Forest, IL).

This procedure produces a pressure gradient at 3w of ~100mm Hg measured by Doppler echocardiography.[2,3] The pressure gradient at 1w or 3w was confirmed in a subset of conscious, gently restrained mice, using an Acuson Sequoia C256 (Siemens) with a 15-MHz linear array transducer. Control animals were age-matched littermate male mice that were sham operated or had no surgery, since no difference in β-MyHC protein expression was noted between these two groups in preliminary experiments.

EdU labeling.

To label DNA, EdU 10 μg/g body weight was given intraperitoneal (ip) in 500 μl PBS just prior to TAC or sham surgery, then daily ip until the time of sacrifice. Stock EdU was 12.5 μg/μl in DMSO, which was diluted 25-fold in PBS before injection.

Adult mouse myocardial cell isolation and fixation.

We used the detailed protocol of O'Connell et al. to obtain single cell suspensions of adult RV and LV.[4] The heart was removed by anterior thoracotomy under deep anesthesia with

isoflurane 3% in oxygen 0.5-1L/min, the proximal aorta was cannulated, and the coronaries were perfused anterograde with nominal calcium-free perfusion buffer (PB) at 37°C containing (mmol/L): NaCl 120, KCl 14.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$-$7H_2O$ 1.2, Na-Hepes 10, $NaHCO_3$ 4.6, taurine 30, 2,3-butanedione monoxime 10, glucose 5.5 (Sigma, MO). After perfusion for 4min at 3ml/min to flush blood from the vasculature, the heart was digested for 2min at 3ml/min with PB supplemented with collagenase II 2.4 mg/ml (Worthington Biochemical, Lakewood, N.J.) without additional calcium, then for 8min at 3ml/min with PB with collagenase with additional $CaCl_2$ 40μmol/L.

The flow-through PB was collected to recover digested vascular cells, made 10% calf serum, and chilled on ice. After digestion, the heart was placed in 12ml Stop Buffer (SB; PB with $CaCl_2$ 12.5 μmol/L and 10% calf serum [HyClone Defined Bovine Calf Serum SH30073]) to inactivate collagenase, the atria were discarded, and the RV free wall and LV with septum were separated. Each ventricle was dispersed into single cells by mincing into ~10-15 small pieces using fine tip forceps, then triturating sequentially (~10-15 times each) through plastic pipettes with 3mm, 2mm and 1mm openings. Any undigested fibrotic tissue was removed with fine forceps. Based on morphology, adult myocytes were counted with a hemocytometer and an ECLIPSE E600 microscope (Nikon, Melville, NY).

Cells in the flow-through PB were centrifuged at 800x*g* for 5min, and the pellet was combined with the live myocytes in 12ml SB. Cells were fixed for 10min at room temperature by gently mixing with neutral buffered formalin (final 1%, #23-245-684, Fisher, Pittsburg, PA), washed by centrifugation at 800x*g* 5min at room temperature, and re-suspended by gentle pipetting and vortexing in CMF-PBS 4ml. Fixed cell suspensions were filtered through a 200μm metal mesh, centrifuged at 800x*g* 3min at room temperature, re-suspended by gentle pipetting in CMF-PBS 800μl, and made 50% ethanol by adding with gentle vortexing 2ml of 70% ethanol in glycerol at -20°C. After 1h on ice, cells were pelleted at 800x*g*, and gently re-suspended in CMF-PBS 4ml. Cells could be stored up to 4w at 4°C without change in β-MyHC expression.

Myocyte volume by Coulter Multisizer.

To quantify myocyte volume, myocardial cells fixed in neutral buffered formalin without ethanol were analyzed with a Coulter Multisizer (Beckman Coulter, Brea, CA). At least 10,000 adult myocytes were quantified, and the mean of the myocyte volume distribution was calculated from the distribution curves generated by Coulter AccuComp 1.15 software.

Neonatal rat ventricular myocyte (NRVM) culture, treatment, and fixation.

The current protocol is modified slightly from the original.[5,6] Ventricles from 1 day-old Sprague-Dawley rats (Charles River, CA) were minced with fine scissors and digested at room temperature in a 50ml Falcon tube with trypsin 1.5 mg/ml (BD Difco, Franklin Lakes, NJ) and DNAse 0.02 mg/ml (Sigma, MO), in 10ml Calcium- and Bicarbonate-Free Hank's salts with Hepes (CBFHH) containing (mmol/L): NaCl 137, KCL 5.36, $MgSO_4$*$7H_2O$ 0.81, dextrose 5.55, KH_2PO_4 0.44, Na_2HPO_4*$7H_2O$ 0.34, and Na-Hepes 20 at pH 7.4. Tissue pieces were disrupted using a stir bar at the slowest speed (~30rpm), and a 10ml wide-tip serological pipette. The first 2 supernatants were removed at 10min intervals and discarded. The next 4 dissociations at 7min intervals were combined in a 50ml tube with 7ml calf serum to inhibit enzymes (HyClone Defined Bovine Calf Serum SH30073). This process was repeated 3-4 times for a total 12-16 dissociation steps. Cells in 50ml tubes were centrifuged gently, re-suspended in minimal essential media (MEM) with Hank's salts (UCSF Media Service, San Francisco, CA), supplemented with calf serum 5%, penicillin G 50U/ml (Sigma, MO), and

vitamin B12 1.5μmol/L (Sigma, MO), combined in a single tube, and centrifuged and re-suspended again in the same culture medium.

To minimize contaminating non-muscle cells, cells were pre-plated for 45-60min, and the myocyte-enriched cell suspension from the pre-plates was removed was counted. The yield of viable myocytes (excluding trypan blue) was approximately 3.5-4 million per neonatal heart. Myocytes were plated onto Falcon culture dishes at a density of 500 viable cells/mm^2 in the same culture medium plus BrdU100μmol/L (#B-5002 Sigma, MO), to inhibit any residual non-muscle cells.

The next day, cultures were washed twice with serum-free MEM, and re-fed with serum-free MEM supplemented with vitamin B12, penicillin, BrdU, human transferrin 10μg/ml (#T-2252), bovine insulin 10μg/ml (#I-1882), and bovine serum albumin 1mg/ml (Intergen, now Millipore, Life Sciences Grade bovine serum albumin) (all supplements from Sigma, except albumin). Plating efficiency was approximately 25-30% of viable myocytes plated. After 24h, myocytes were treated with triiodothyronine 100nmol/L (Sigma #T-2752) or vehicle (NaOH) for 3d, harvested into a single cell suspension with Difco trypsin 1 mg/ml in CBFHH, pelleted at 800x*g* for 5min at room temperature, re-suspended in CBFHH 1ml, and fixed by adding neutral buffered formalin 1% final. After 10min at room temperature, cells were washed and re-suspended in CMF-PBS 2ml for storage up to 4w at 4°C without change in β-MyHC.

Fetal and neonatal mouse myocardial cell isolation and fixation.

Ventricles from 17-19 dpc fetal C57Bl/6J mice or 1- to 2-day old neonatal mice were minced, dispersed at 37°C with collagenase type II 0.75mg/ml (Worthington Biochemicals, Lakewood, N.J), and mechanically disaggregated with a 10ml wide-tip serological pipette. The single cell suspension from each neonatal heart was placed in one tube, and treated as a separate sample. The fetal hearts were pooled prior to fixation. Cells were pelleted at 800x*g* for 5min at room temperature, re-suspended in CBFHH 1ml, and fixed by adding neutral buffered formalin 1% final. After 20min at room temperature, cells were washed and re-suspended in CMF-PBS 2ml for storage up to 4w at 4°C without change in β-MyHC.

Rabbit myocardial cell isolation and fixation.[7]

Hearts from adult (3mos) male New Zealand White rabbits anaesthetized with pentobarbital sodium (80-100 mg/kg iv) were rinsed in ice-cold Ca^{2+}-free DMEM, then mounted on a Langendorff apparatus and perfused by gravity using constant pressure (80mmHg) at 37°C. DMEM was gassed with 100% O_2 and pH was maintained at 7.2 with $NaHCO_3$ and HEPES. After 5min, collagenase (0.8-1.0 mg/ml, Worthington) and Ca^{2+} (20μmol/L) were added. After 15-25min, perfusion was stopped, and ventricles were minced into ~2mm^3 pieces, and incubated for 5-20min more in fresh enzyme, if needed. Finally, enzyme activity was stopped with DMEM containing 1% BSA, and tissue was agitated or triturated to liberate single cells, which were filtered through a 240μm nylon mesh, washed, and stored until use in DMEM with Ca^{2+} 150 μmol/L.

Cells were fixed for 10min at room temperature by gently mixing with neutral buffered formalin (final 1%, #23-245-684, Fisher, Pittsburg, PA), washed by centrifugation at 800x*g* 5min at room temperature, and re-suspended by gentle pipetting and vortexing in CMF-PBS 4ml. Fixed cell suspensions were filtered through a 200μm metal mesh, centrifuged at 800x*g* 3min at room temperature, and gently re-suspended in CMF-PBS 4ml. Cells could be stored up to 4w at 4°C without change in β-MyHC expression.

Western blot.

Live myocytes (~400,000) from single cell preparations were aliquoted on ice into RIPA buffer with protease inhibitors (Tris 50mmol/L, NaCl 150mmol/L, SDS 0.1%, Na deoxycholate 0.5%, Triton X-100 1%, and 1 Complete Mini Protease Inhibitor Cocktail Tablet per 10ml [Roche, #11836153001]), homogenized with a Polytron at speed 7 out of 10, snap frozen in liquid nitrogen, and stored at -80°C. Lysates were diluted to 500 myocytes per μl in RIPA buffer. Then 100μl of myocyte lysate were mixed with 25μl of 4X Loading Buffer (Tris-HCl pH 6.8, SDS, glycerol, bromophenol blue, and protease inhibitors, AfCS Protocol PS00000052 plus protease inhibitor tablet, at http://www.signaling-gateway.org/data/ProtocolLinks.html, made in a 10ml batch), resulting in 400 myocytes per μl in loading buffer. This myocyte lysate (100μl) was mixed with 900μl 1.5X Loading Buffer (see AfCS protocols PS00000051, PS00000052, and PS00000050), giving the equivalent of 40 myocytes per μl.

Proteins from 400 myocytes per lane in 10μl were separated on precast 7.5% SDS-PAGE gels (Criterion Gel, BioRad, CA) in AfCS Tris, glycine, SDS Running Buffer (AfCS Protocols PS00000054 and PS00000055), at a constant 75V, until the dye front reached the resolving gel, then 120V constant until the dye front reached the bottom of the gel. Proteins were transferred to nitrocellulose in Running Buffer without SDS plus 20% (v/v) anhydrous methanol (Mallinckrodt #3016, 300ml in 1.5L final volume) at a constant 250mA for 45min at 4°C. Blots were blocked for 1h at room temperature in TBS-Tween 20 (TBS-T: Tris-buffered saline with 0.1% Tween 20, AfCS protocol PS00000064) plus 5% non-fat dry milk (5g in 100ml); rinsed briefly in TBS-T; incubated overnight with anti-β-MyHC mAb NOQ 7.5.4D at 1:20,000 dilution in TBS-T plus 5% BSA (5g in 100ml); rinsed 10min 3 times in 50ml TBS-T; incubated for 1h at room temperature with anti-mouse IgG HRP secondary antibody diluted at 1:10,000 in TBS-T plus 5% non-fat dry milk; and rinsed 10min 4 times with 50ml TBS-T. Bands were developed using ECL reagent SuperSignal West Dura (Thermo Scientific #34076) according to product instructions; visualized using BioRad ChemiDoc XRS system; and quantified using BioRad Quantity One Software version 4.6.5.

Immunohistochemistry.

Mice under deep anesthesia with isoflurane were perfused through the cardiac apex with cold KCl in CMF-PBS (60 mmol/L, pH 7.3) to arrest the heart in diastole, and for an additional 10min to clear blood from coronary vessels and ventricular cavities. The heart was then perfusion-fixed in situ 10min at room temperature with neutral buffered formalin 10% final, dissected out, and incubated with rocking at 4°C successively with neutral buffered formalin 10% final for 2h, 15% sucrose in PBS for 2h, and 30% sucrose in CMF-PBS overnight, and finally embedded in freezing medium (Tissue-Tek O.C.T., Sakura Finetek), frozen in a dry ice and ethanol bath, and cut in 7μm sections.

Frozen tissue sections were thawed for 5min at room temperature, washed in CMF-PBS 5min 3 times, permeabilized with Triton X-100 1% in CMF-PBS 30min at room temperature, and blocked 1h with donkey serum 5%, mouse IgG 5μg/ml (Invitrogen), and Triton X-100 0.1% in PBS, using 2 sections per slide, 25μl per section. Anti-β-MyHC mAb NOQ7.4.5D was conjugated to Zenon-488 (red) or Zenon-546 (orange) reagent following manufacture's recommendations. Sections were incubated with conjugated mAb overnight at 4°C in a moisture chamber (1:100 Ab dilution in CMF-PBS with Triton X-100 0.2% and donkey serum 1%); washed 3 times in Tris-buffered saline with Tween 20 0.1%; stained with fluorescein-conjugated wheat germ agglutinin (2μg/ml in 25μl, Molecular Probes #W834) for 10min at room temperature; washed in CMF-PBS 3 times; and mounted in Fluoromount-G (Southern Biotech #OB100-01). Slides were examined with an ECLIPSE E600 (Nikon), and photographed with a

Spot digital camera (BT1900-Spot Boost EMCCD; Diagnostic Instruments Inc, Sterling Heights, Michigan).

Data analysis.

Results are presented as mean ± SD. Significant differences ($p<0.05$) were tested using one-way ANOVA and Bonferroni's multiple comparison test for more than two groups, or Student's unpaired t-test for two groups. A normal distribution was assumed for all continuous variables. Linear regression tested for association between mean volumes ratios of Control/TAC and mean side scatter ratios of Control/TAC (GraphPad Prism v5.0).

SUPPLEMENT REFERENCES

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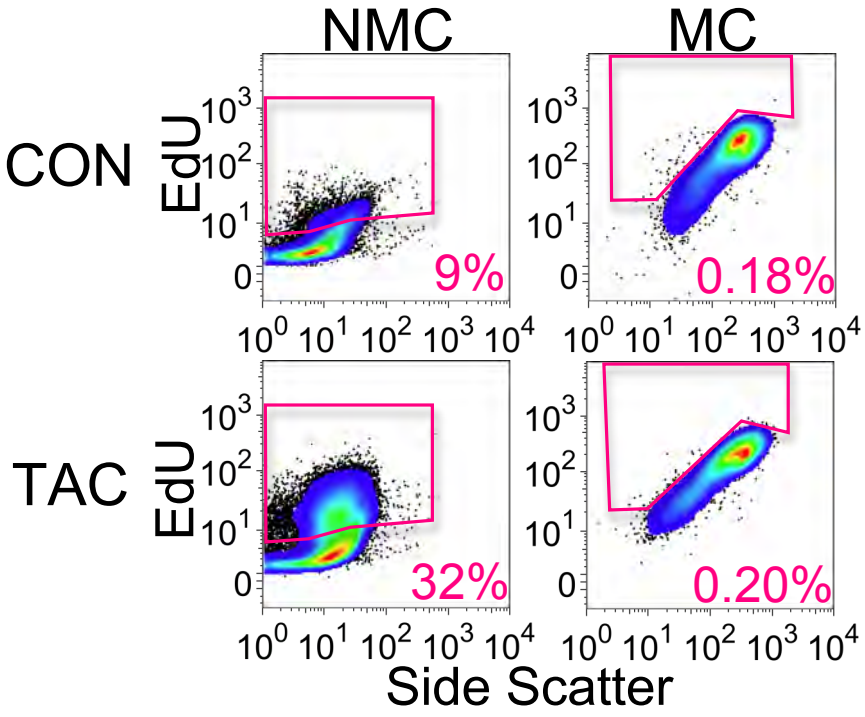
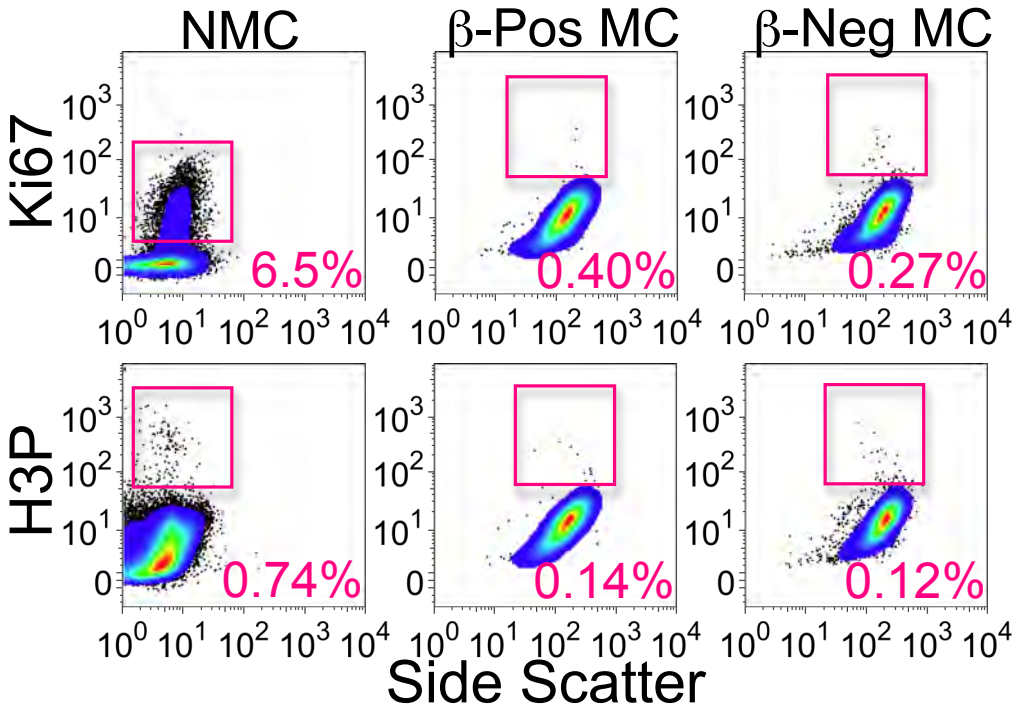
SUPPLEMENT FIGURES I-III

(A) Flow cytometry for EdU incorporation in CON and TAC cells



(B) Flow cytometry for Ki67 and H3P in TAC cells

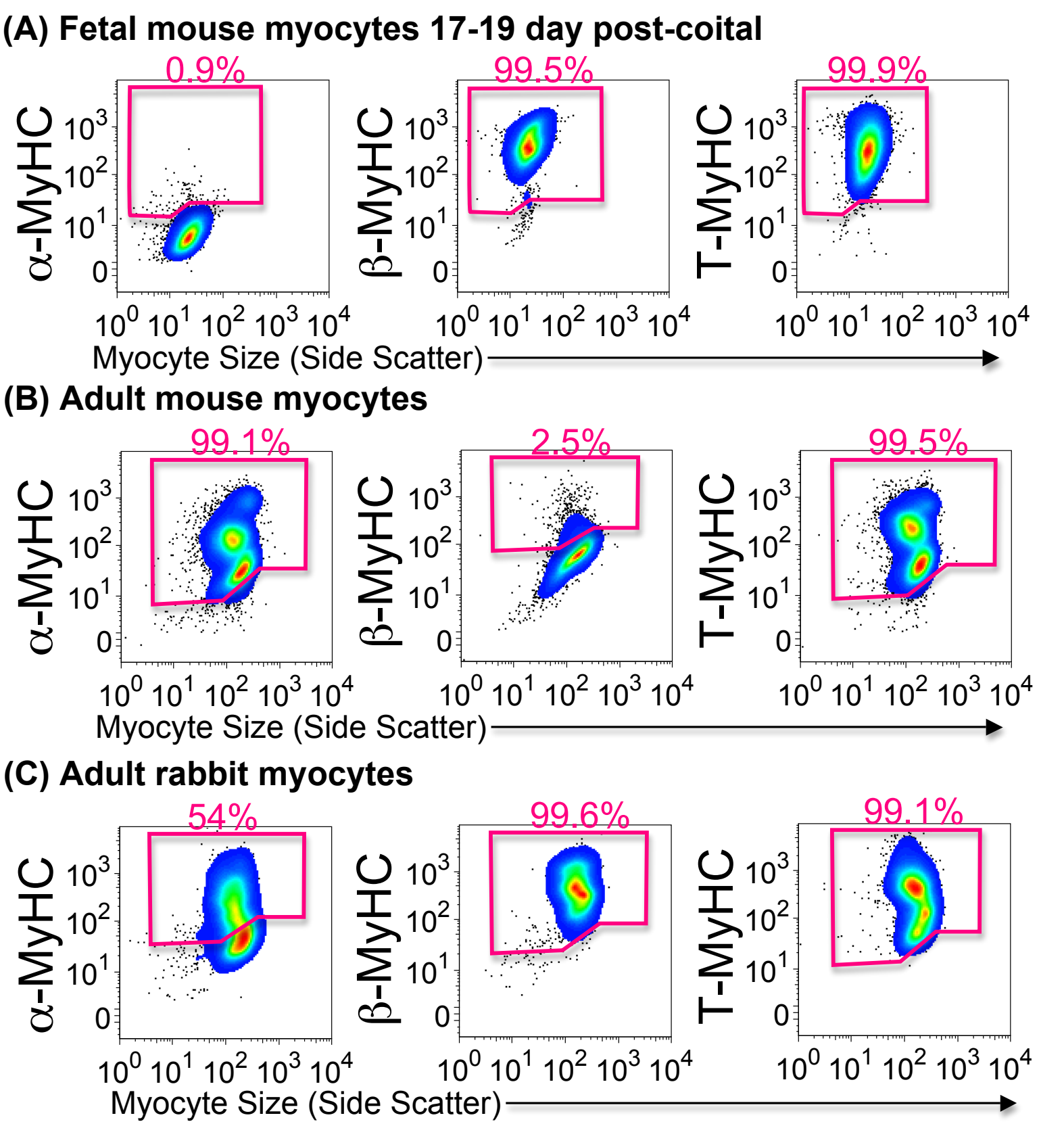


Online Figure I. DNA synthesis and cell cycle proteins were increased after TAC in nonmyocytes only.

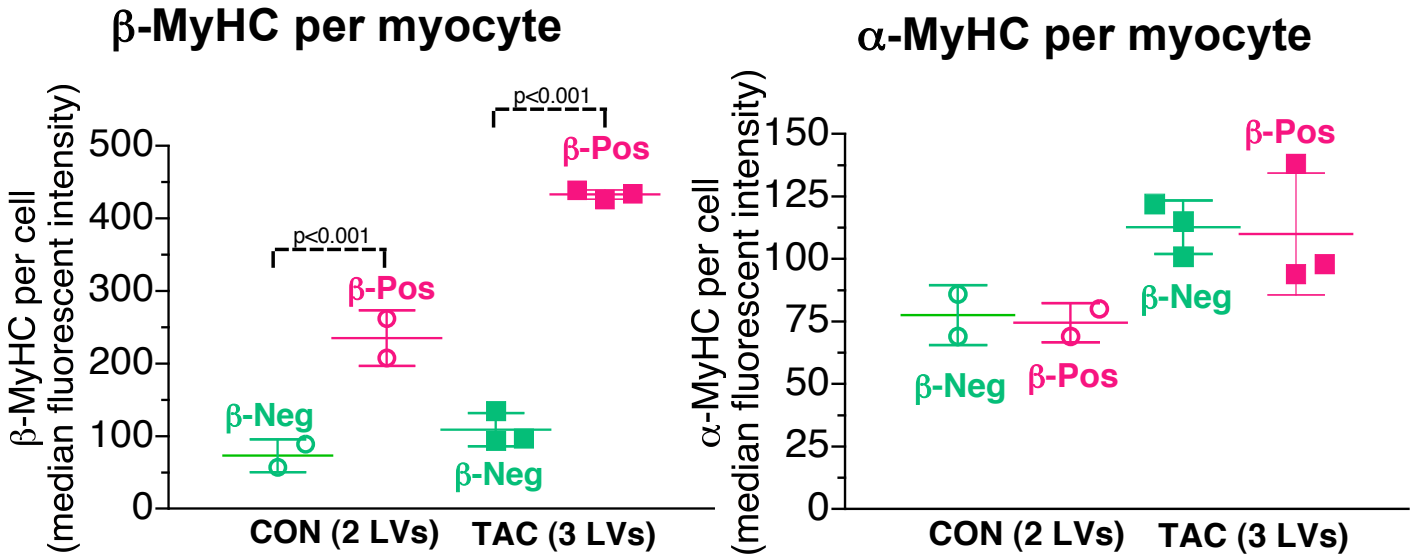
EdU (10μg/g) to label DNA synthesis was injected intraperitoneal on the day of TAC or sham surgery (CON) and then daily, and MCs and NMCs from the same LV were isolated on d7 for flow cytometry. MCs (10,000-20,000 per LV) were identified as positive for troponin T (mAb 13-11, Lab Vision), and further characterized with the β-MyHC mAb NOQ7.5.4D as negative (β-Neg) or positive (β-Pos) for β-MyHC. NMCs (70,000-80,000 per LV) were defined as negative for both troponin T and CD45, and were positive controls for proliferation markers. Shown are pseudo-color contours, with dots for outlier cells. Positive cell gates (pink, with percent of total cells indicated) were set to include <0.5% positive cells when cells from the same LV were stained with isotype Ab, as illustrated in Figure 1A, right panel.

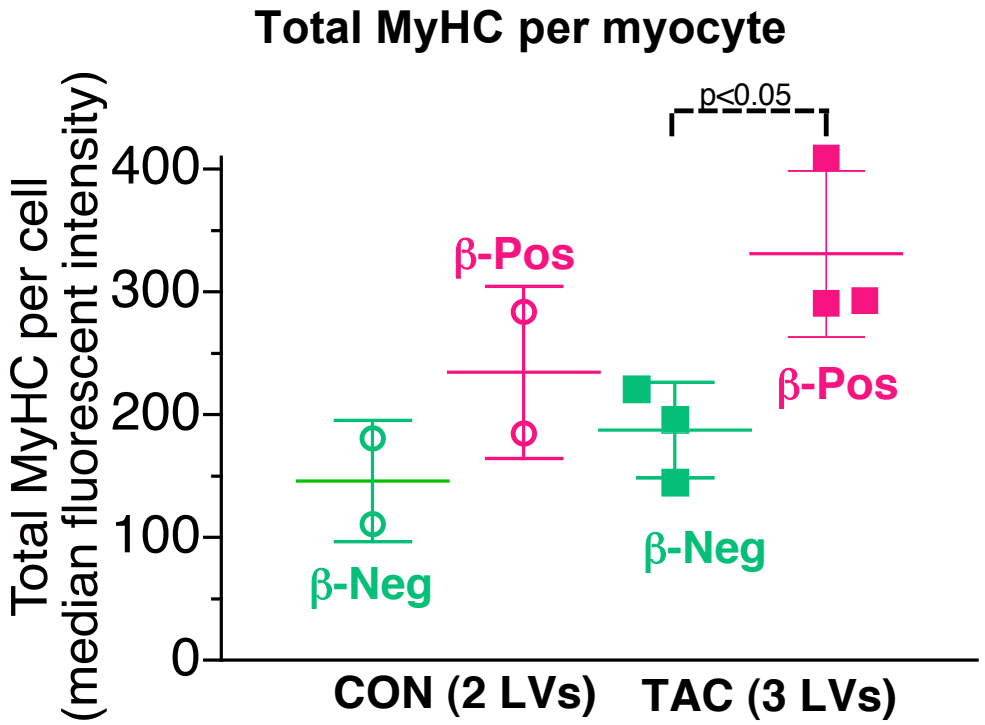
(A) EdU incorporation into DNA of TAC and CON NMCs and MCs. EdU staining artifactually quenched β-MyHC staining (not shown), so only total MCs are shown (positive for troponin T).

(B) Staining for Ki67 and histone H3 phosphorylation (H3P) in cells after TAC.



Online Figure II. Validation of a mAb for α-MyHC. Myocytes were isolated from the fetal mouse ventricle, and from the adult rat and rabbit LV, and 10,000-20,000 myocytes per LV were used in flow cytometry. Myocytes were defined as positive for troponin T, and further characterized with Abs for α-MyHC (BA-G5, Abcam), β-MyHC (NOQ7.5.4D, Sigma), and total (T)-MyHC (MF-20, DSHB). Shown are pseudo-color contours, with dots for outlier myocytes. Gates (pink) were set by isotype staining of myocytes from the same heart, and include positive myocytes above the 99% of myocytes stained with isotype Ab, as shown in Figure 1A, right panel. Percents are number of positive cells as a fraction of total troponin T-positive myocytes. The α-MyHC Ab detects no α-MyHC in fetal mouse myocytes **(A)**; and >99% α-MyHC-positive cells in adult mouse LV **(B)**, both as expected. The α-MyHC Ab also finds a 54% sub-population of myocytes in adult rabbit LV that have both α- and β-MyHC **(C)**.





Online Figure III. β-MyHC-positive myocytes also contain α-MyHC, and have greater total MyHC. LV myocytes (>10,000) from 2 CON LVs and 3 TAC LVs at 1w after banding were identified in flow cytometry by staining with the troponin T Ab 13-11, and MyHC proteins were quantified by staining with the β-MyHC mAb NOQ7.5.4D, the α-MyHC mAb BA-G5, and the total MyHC Ab MF20. The median fluorescent intensity per cell for each protein was determined for β-MyHC-positive and β-MyHC-negative myocytes. Values are mean±SD, each point is one LV. p by 1-way ANOVA and Newman Keuls post-test. By echocardiography in awake mice, the mean pressure gradient in TAC mice at 1w was 95±9 mmHg, and fractional shortening had dropped from 59±1% in pre-TAC mice and in sham CON mice to 41±1% in TAC mice (a 31% decrease, p<0.001).