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

Javier E. López, Bat-Erdene Myagmar, Philip M. Swigart, Megan D. Montgomery, Stephen Haynam, Marty Bigos, Manoj C. Rodrigo, Paul C. Simpson

Rationale: Induction of the fetal hypertrophic marker gene β-myosin heavy chain (β-MyHC) is a signature feature of pressure overload hypertrophy in rodents. β-MyHC is assumed present in all or most enlarged myocytes.

Objective: To quantify the number and size of myocytes expressing endogenous β-MyHC by a flow cytometry approach.

Methods and Results: Myocytes were isolated from the left ventricle of male C57BL/6J mice after transverse aortic constriction (TAC), and the fraction of cells expressing endogenous β-MyHC was quantified by flow cytometry on 10 000 to 20 000 myocytes with use of a validated β-MyHC antibody. Side scatter by flow cytometry in the same cells was validated as an index of myocyte size. β-MyHC-positive myocytes constituted 3±1% of myocytes in control hearts (n=12), increasing to 25±10% at 3 days to 6 weeks after TAC (n=24, P<0.01). β-MyHC-positive myocytes did not enlarge with TAC and were smaller at all times than myocytes without β-MyHC (70% as large, P<0.001). β-MyHC-positive myocytes arose by addition of β-MyHC to α-MyHC and had more total MyHC after TAC than did the hypertrophied myocytes that had α-MyHC only. Myocytes positive for β-MyHC were found in discrete regions of the left ventricle in 3 patterns: perivascular, in areas with fibrosis, and in apparently normal myocardium.

Conclusions: β-MyHC protein is induced by pressure overload in a minor subpopulation of smaller cardiac myocytes. The hypertrophied myocytes after TAC have α-MyHC only. These data challenge the current paradigm of the fetal hypertrophic gene program and identify a new subpopulation of smaller working ventricular myocytes with more myosin. (Circ Res. 2011;109:629-638.)

Key Words: β-myosin heavy chain ■ cardiac hypertrophy ■ fetal genes ■ flow cytometry ■ pressure overload ■ cardiac monocytes

In 1979, Lompre et al1 described the appearance in rat cardiac overload of a V3 myosin isoenzyme with reduced ATPase activity and slower mobility on nondenaturing gels. V3 myosin contains β-myosin heavy chain (β-MyHC), whereas the normal adult V1 myosin with higher ATPase activity contains α-MyHC, and protein changes are caused by changes in the mRNAs.2-4 In rats and mice, β-MyHC mRNA and protein are induced reliably by disease-related hypertrophic stimuli in vivo and by hypertrophic agonists in culture but not by physiological stimuli such as exercise training.6 Thus, β-MyHC has become the classic marker for pathological hypertrophy. Indeed, β-MyHC is emblematic of an entire “fetal gene program” that is normally low or absent in adult heart and myocytes and is induced in rodent models of pathological hypertrophy in vivo and in vitro, including skeletal α-actin, β-tropomyosin, atrial natriuretic factor, and proto-oncogenes such as myc, fos and jun.7-9

In the human ventricle, β-MyHC and skeletal α-actin are predominant normally,10,11 but the concept of fetal gene induction in human myocardial disease appears to be valid, as represented by upregulation of atrial natriuretic factor, MB creatine kinase, and atrial β-MyHC.12-14

If β-MyHC is indeed a valid marker for pathological cardiac myocyte hypertrophy, then 2 criteria would appear to follow. First, β-MyHC should be induced in most or all myocytes in a hypertrophied heart, and second, the myocytes expressing β-MyHC should be hypertrophied, or larger in size. However, a few reports indicate that endogenous β-MyHC is expressed heterogeneously in the overloaded myocardium,15,16 a mean 30% of myocytes in 1 report,15 and the same can be true for other fetal genes.16-18 The most extensive study used a knock-in reporter mouse with yellow fluorescent protein (YFP) fused to the N-terminus of β-MyHC (YFP-β-MyHC). This approach estimated that myocytes positive for YFP-β-MyHC constituted 38% of total myocytes after prolonged transverse aortic constriction (TAC); YFP-β-MyHC–expressing myocytes were found in areas of fibrosis in several hypertrophy models and were hypertrophied to the same extent as myocytes that did not express β-MyHC.19-22

Here, we sought to simultaneously quantify and size myocytes that expressed endogenous β-MyHC protein after TAC

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From the VA Medical Center (J.E.L., B.-E.M., P.M.S., M.D.M., M.C.R., P.C.S.), San Francisco, CA; University of California, Davis (S.H., J.E.L.); University of California, San Francisco (J.E.L., B.-E.M., M.D.M., P.C.S.); and Stanford Shared FACS Facility (M.B.), Stanford University School of Medicine, Stanford, CA.
The current affiliation for J.E.L. is the University of California, Davis. The current affiliation for M.C.R. is Entelos, Inc, Foster City, CA.
Correspondence to Paul C. Simpson, MD, VA Medical Center (111-C-8), 4150 Clement St, San Francisco, CA 94121. E-mail paul.simpson@ucsf.edu
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Flow cytometry used 3 different standard benchtop flow cytometers (FACScan, FACSCalibur, and LSRII; BD Biosciences, San Jose, CA) with a 430×180-μm flow cell. Fixed cells were permeabilized, blocked, and stained for DNA and with conjugated primary antibodies. Optimal titration of all reagents used the coexpressing nonmyocytes in each preparation as the in-tube negative control for myocyte-specific proteins and the positive control for proliferation markers. Preliminary studies defined the antibody dilution that detected the maximum number of cells. At least 10,000 myocytes were assayed in each run, setting positive and negative gates by fluorescence of negative control cells stained with nonspecific mouse IgG. Data were analyzed with FlowJo software on a Mac OSX.

The IgG mAbs included MF-20 mouse anti-sarcomeric MyHC (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); NOQ7.5.4D mouse anti-β-MyHC (Sigma-Aldrich, St Louis, MO); 13-11 mouse anti-troponin T (Lab Vision, Fremont, CA); mouse anti-α-MyHC (clone BA-G5; Abcam, Cambridge, MA); mouse anti-Ki67 (clone B56; BD Pharmingen, San Diego, CA); and rat anti-CD45 conjugated to allopheocyanin-Cy7 (clone 30-F11; BD Pharmingen). Anti-phospho-histone H3 serine 10 was a rabbit polyclonal IgG (Millipore, Billerica, MA). The mouse mAbs were labeled with Zenon yellow fluorescent protein YFP (Invitrogen, Carlsbad, CA) conjugated to Alexa 488 (green), Alexa 546 (orange), Alexa 647 (red), or biotin streptavidin-allophycocyanin-Alexa 780 (far red). For DNA synthesis, a flow cytometry kit quantified incorporation of the thymidine analog ethynyl deoxyuridine.

For immunohistochemistry, the heart was arrested and fixed in diastole so that 7-μm frozen sections could be cut. Results are mean±SD. Significant differences (P<0.05) were tested in GraphPad Prism version 5.0 with 1-way ANOVA and Bonferroni post test.

Results

Flow Cytometry of Isolated Adult Mouse Cardiac Cells Reliable analysis of myocytes expressing endogenous β-MyHC protein required a flow cytometry approach to

A Flow cytometry analysis of adult mouse LV cells

- Nucleated cells
  - Background Fluorescence (Isotype Abs)
  - Cell-specific Fluorescence (CD45 & MF-20 Abs)
  - WBCs
  - NMCs
  - Myocytes

- Side Scatter

B LV cell type percents

<table>
<thead>
<tr>
<th>Cell Type (%)</th>
<th>CON (n=10)</th>
<th>TAC (n=17)</th>
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<tr>
<td>WBC</td>
<td>p=0.01</td>
<td>p=0.01</td>
</tr>
<tr>
<td>MC</td>
<td>p=0.01</td>
<td>p=0.01</td>
</tr>
<tr>
<td>NMC</td>
<td>p=0.01</td>
<td>p=0.01</td>
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</table>

C LV myocyte numbers

<table>
<thead>
<tr>
<th>Myocytes/LV</th>
<th>CON (n=63)</th>
<th>TAC (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>p=0.67</td>
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</table>

Figure 1. Flow cytometry of single cells from adult mouse LV. A, Cells isolated from an adult mouse LV were fixed, stained, and analyzed by flow cytometry. Left, Cells were first gated for DNA content to eliminate debris and identify nucleated cells (boxed area). Middle (Left), Fluorescence of isotype antibodies in the same cell population defined background. Middle (Right), Cells were identified as white blood cells by CD45, as myocytes by MF-20, or as non-myocytes negative for both proteins. Note that CD45+ cells do not shift on the x-axis, which indicates absence of nonspecific binding of anti-myosin antibody. Right, Myocytes positive for myosin by MF-20 were also positive for troponin T. The 10% contour plots included at least 10,000 to 20,000 nucleated myocytes, and outliers (small dots) represented <2% of the cell population. Side scatter (right) is an index of cell size. Cell gates (pink) were set to include <1% positive cells when cells from the same LV were stained with isotype antibody. B, The fraction of different cell types in the LV was measured 1 to 3 weeks after TAC or sham control (CON). MC indicates myocyte. Values are mean±SD, P by 1-way ANOVA and Bonferroni post test. C, Myocytes isolated from TAC and control hearts were counted in a hemocytometer. Values are mean±SD, P by Student t test.
A Flow cytometry of cells from neonatal mouse heart

![Flow cytometry figure](image)

<table>
<thead>
<tr>
<th>Isotype Abs</th>
<th>MF-20 &amp; Isotype</th>
<th>MF-20 &amp; NOQ7.5.4D</th>
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<tbody>
<tr>
<td>β-MHC (NOQ7.5.4D)</td>
<td>NMC 34%</td>
<td>MC 66%</td>
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<td>β-MyHC-positive cells</td>
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Total sarcomeric myosin (MF-20)

B Flow cytometry of cultured NRVMs treated with T3

![Flow cytometry figure](image)

Total Myosin (MF-20) vs. Cell fluorescence

β-MyHC (NOQ7.5.4D) vs. Cell fluorescence

- **Vehicle**
- **T3**

**Validation of β-MyHC Antibody**

We screened 4 commercial mouse mAbs against the β or slow MyHC isoform in strained muscle (NOQ7.5.4D from Sigma, N2.261 and A4.840 from the Developmental Studies Hybridoma Bank, and NCL-MHCs from Novocastra), testing their specificity. NOQ7.5.4D was the single mAb that satisfied 3 criteria. First, NOQ7.5.4D recognized known developmental and species differences. NOQ7.5.4D recognized that almost all newborn mouse heart myocytes, as defined by MF-20, expressed β-MyHC (Figure 2A), whereas most adult myocytes did not (Figure 3 below). NOQ7.5.4D also

phenotype large numbers of isolated adult mouse cardiac myocytes and to measure cell size simultaneously. We avoided the usual problem of instrument clogging by attention to protocol details (supplemental Methods) and were able to analyze 10 000 to 20 000 myocytes per run on any 1 of 3 different standard flow cytometers.

Figure 1A illustrates the approach. All small and large cells isolated from mouse left ventricle (LV) were fixed and stained with propidium iodide or 7-amino-actinomycin D for DNA; with anti-CD45 to label white blood cells; and with MF-20 anti-sarcomeric myosin to label all myocytes. An aliquot of cells stained with isotype IgGs was the negative control to set the gates for fluorescent signals. As shown in Figure 1A left, we first eliminated debris by gating on nucleated cells with ≥2N DNA content, then gated nucleated cells for white blood cells, myocytes, and nonmyocytes, the last defined as cells that were negative for both CD45 and MF-20, the cells in Figure 1 were also stained with anti-sarcomeric myosin and all cells positive for MF-20 were also positive for β-MyHC with NOQ7.5.4D. At least 10 000 to 20 000 nucleated cells were analyzed for each heart, and 6 hearts from the same litter had similar results.

As an additional control for myocyte identification with MF-20, the cells in Figure 1 were also stained with anti-troponin T, and all cells positive for MF-20 were also positive for troponin T. In Figure 1A right, troponin reactivity is plotted against side scatter, a flow cytometry index of cell size, which shows the broad size range of these cells.

We tested the flow cytometry approach using cells isolated from the LV at 1 to 3 weeks after TAC or controls. TAC in these experiments was sufficient to cause dilated cardiomyopathy in C57BL/6J mice, with a pressure gradient at 3 weeks of 110±18 mm Hg (n=4; also see Online Figure III). As shown in Figure 1B, TAC caused a significant decrease in the fraction of LV cells that were myocytes (44±5% to 26±2%, P<0.01) and an increase in the fraction of white blood cells (5±1% to 13±1%, P<0.01) and nonmyocytes (52±5% to 61±2%, P<0.05), as expected from the inflammation and fibrosis in the model.24,25 The 40% relative decrease in myocytes as a fraction of total LV cells was a dilution effect caused by more white blood cells and nonmyocytes and was not caused by myocyte loss.

As shown in Figure 1C, TAC did not change the absolute number of myocytes isolated per LV, by hemocytometer counts (in millions: controls 2.1±0.5, TAC 2.0±0.6; n=63 to 25; P=0.7). Approximately 40% of myocytes in both groups were rod-shaped (controls 43±9% and TAC 40±13%, P=0.4). Notably, the fraction of myocytes in the mouse LV was lower than estimated by microscopic counts26 but similar to another approach could reproducibly phenotype large and equal numbers of myocytes from the mouse LV, before and after TAC.

Figure 2. Validation of β-MyHC antibody. **A**, Cells isolated from the neonatal mouse heart were stained with MF-20, NOQ7.5.4D, or isotype antibody controls and analyzed by bivariate flow cytometry. Left, Background defined by isotype antibodies. Middle, MF-20 defines myocytes (MCs; 66% of cells) and nonmyocytes (34% of cells). Right, Almost all myocytes stained positive for β-MyHC with NOQ7.5.4D. At least 10 000 to 20 000 nucleated cells were analyzed for each heart, and 6 hearts from the same litter had similar results. **B**, Cultured NRVMs in serum-free medium were treated 3 days with vehicle (dashed lines) or triiodothyronine (T3; 100 nmol/L; solid lines), removed from the dish, fixed, and stained for flow cytometry with (left) MF-20 for total sarcomeric myosin or (right) NOQ7.5.4D for β-MyHC. Triiodothyronine eliminated myocytes and to measure cell size simultaneously. We avoided the usual problem of instrument clogging by attention to protocol details (supplemental Methods) and were able to analyze 10 000 to 20 000 myocytes per run on any 1 of 3 different standard flow cytometers.

Figure 1A illustrates the approach. All small and large cells isolated from mouse left ventricle (LV) were fixed and stained with propidium iodide or 7-amino-actinomycin D for DNA; with anti-CD45 to label white blood cells; and with MF-20 anti-sarcomeric myosin to label all myocytes. An aliquot of cells stained with isotype IgGs was the negative control to set the gates for fluorescent signals. As shown in Figure 1A left, we first eliminated debris by gating on nucleated cells with ≥2N DNA content, then gated nucleated cells for white blood cells, myocytes, and nonmyocytes, the last defined as cells that were negative for both CD45 and MF-20 (Figure 1A middle).

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As shown in Figure 1C, TAC did not change the absolute number of myocytes isolated per LV, by hemocytometer counts (in millions: controls 2.1±0.5, TAC 2.0±0.6; n=63 to 25; P=0.7). Approximately 40% of myocytes in both groups were rod-shaped (controls 43±9% and TAC 40±13%, P=0.4). Notably, the fraction of myocytes in the mouse LV was lower than estimated by microscopic counts26 but similar to another flow cytometry analysis.27 As shown in Figure 2B, the neonatal heart had a higher fraction of myocytes (66%), as has also been observed by others.27 We concluded that our flow cytometry approach could reproducibly phenotype large and equal numbers of myocytes from the mouse LV, before and after TAC.

**Validation of a β-MyHC Antibody**

We screened 4 commercial mouse mAbs against the β or slow MyHC isoform in strained muscle (NOQ7.5.4D from Sigma, N2.261 and A4.840 from the Developmental Studies Hybridoma Bank, and NCL-MHCs from Novocastra), testing their specificity in identifying cardiac β-MyHC. NOQ7.5.4D was the single mAb that satisfied 3 criteria. First, NOQ7.5.4D recognized known developmental and species differences. NOQ7.5.4D recognized that almost all newborn mouse heart myocytes, as defined by MF-20, expressed β-MyHC (Figure 2A), whereas most adult myocytes did not (Figure 3 below). NOQ7.5.4D also
found >99% β-MyHC-positive myocytes in fetal mouse heart and adult rabbit heart (Online Figure II). Second, NOQ7.5.4D identified downregulation of β-MyHC by triiodothyronine in cultured NRVMs, as expected (Figure 2B). Third, NOQ7.5.4D identified a band of appropriate size (~200 kDa) in adult TAC myocytes, by Western blot of 400 myocytes. This band was absent in adult control myocytes (Figure 3A). In summary, NOQ7.5.4D satisfied 3 criteria for specific recognition of cardiac β-MyHC and was used in all subsequent experiments.

β-MyHC Is Induced in a Minor Subpopulation of LV Myocytes

As shown in Figure 3A, TAC caused a very robust induction of β-MyHC when assayed by Western blot of 400 LV myocytes. This finding would typically be interpreted as β-MyHC induction in all 400 myocytes. However, Figure 3B shows by immunocytochemistry with the mAb NOQ7.5.4D that β-MyHC was present in a minority of myocytes. We used flow cytometry to quantify the fraction of myocytes that expressed β-MyHC.

LV myocytes were isolated from control and TAC hearts at 3 weeks; stained with propidium iodide for DNA, CD45 for white blood cells, MF-20 for myocytes, and NOQ7.5.4D for β-MyHC; and analyzed by flow cytometry. Figure 3C shows a flow cytometry analysis of the total MF-20-positive myocyte population, similar to the right side of Figure 1A, with β-MyHC intensity on the y-axis, and side scatter, a size index, on the x-axis. In this control heart, 3% of total MF-20-positive myocytes were also positive for β-MyHC, whereas the TAC heart had 25% of myocytes positive (Figure 3C).

To test whether a higher fraction of myocytes expressed β-MyHC at times earlier or later than 3 weeks, we isolated LV myocytes from TAC hearts and concurrent sham controls at 3 days to 6 weeks after surgery. Figure 3D shows that the fraction of β-MyHC-positive myocytes was increased at 3 days (20±8%, n=5), reached a maximum at 1 week, and was stable for 6 weeks, with overall 25±10% positive (n=24, P<0.0001 versus sham; P=0.24 for TAC %β-MyHC-positive over time). Sham controls had 3±1% of myocytes positive for β-MyHC (n=12). The percentage of β-MyHC-positive myocytes was the same whether NOQ7.5.4D was labeled with Alexa 488 (green) or Alexa 647 (red; data not shown).

We concluded that endogenous β-MyHC was present in 3% of control myocytes and was induced in only 25% of myocytes after TAC.

Validation of Flow Cytometry to Quantify Myocyte Size

We wanted to use flow cytometry to quantify the size of the myocytes that did and did not express β-MyHC. The advantage of this strategy compared with microscopic methods would be a much larger sample size. To test whether myocyte side scatter by flow cytometry could be used to quantify size, we compared side scatter with myocyte volume from the Coulter Multisizer.

Figure 4A plots volume by Coulter, and side scatter by flow, versus cell number for LV and right ventricular (RV) myocytes from a control heart. Flow correctly identified that LV myocytes were larger than RV myocytes (left panels), and the correlation was excellent between the relative size of LV and RV myocytes detected by flow and by Coulter (Figure 4A, right). Similarly, as shown in Figure 4B, side scatter correctly identified the increased size of LV myocytes after TAC (left panels), and the
correlation was excellent between relative size determined by the 2 assays (right panel). The relative size increase was somewhat compressed by the side scatter assay compared with Coulter, which indicates that flow somewhat underestimated the cell size differences (right panels in Figures 4A and 4B).

We concluded that we could use side scatter by flow cytometry as a surrogate for relative myocyte size.

**β-MyHC-Positive Myocytes Are Smaller**

We used side scatter to quantify the relative size of LV myocytes in the same heart that did and did not express β-MyHC. Figure 5A presents side scatter versus myocyte number profiles for 3 control and 3 TAC hearts. In each case, β-MyHC protein and myocyte size were measured in the same cells from the same LV. Myocytes that express β-MyHC were barely detected in control LV but became readily apparent after TAC, and their size was clearly smaller relative to β-MyHC-negative myocytes from the same LV (Figure 5A).

Figure 5B summarizes the relative size of β-MyHC-positive and β-MyHC-negative myocytes from 11 control and 14 TAC LVs at 1 to 6 weeks after banding. Mean side scatter for each LV myocyte subpopulation was normalized to the side scatter of the control β-MyHC-negative myocytes run in an identical manner on the same cytometer on the same day.

In control LVs, the few β-MyHC-positive myocytes (3% of total myocytes) were only 65±16% as large as the majority of β-MyHC-negative myocytes (P<0.001; Figure 5B). In TAC LVs, there were now 25% β-MyHC-positive myocytes, and they were only 78±7% as large as the β-MyHC-negative myocytes (P<0.001).

Furthermore, the 75% of β-MyHC-negative myocytes were the only cells that enlarged after TAC. TAC myocytes negative for β-MyHC were 1.28±0.13-fold larger than the control β-MyHC-negative cells (P<0.001), equivalent to a 1.59-fold increase in size by cell volume (by extrapolation from the regression equation in the validation experiment; Figure 4B right). β-MyHC-negative myocytes enlarged to a plateau over the first week after TAC (Figure 5C).

In marked contrast, the 25% of β-MyHC-positive myocytes in TAC LVs were the same size as the 97% of β-MyHC-negative cells in control hearts (side scatter 1.02±0.12-fold, P=0.27; volume 1.10-fold by extrapolation from the regression in Figure 4B). β-MyHC-positive myocytes did not enlarge over the entire 6 weeks after TAC (Figure 5C). Therefore, β-MyHC was induced in smaller myocytes that did not enlarge with TAC.

In summary, LV myocytes that expressed endogenous β-MyHC were smaller than myocytes that did not express β-MyHC, both before and after TAC. Myocytes without β-MyHC, and therefore with α-MyHC only, were the myocytes that enlarged with TAC.

**β-MyHC-Positive Myocytes Are in Discrete Regions and Areas of the LV After TAC**

We used immunohistochemistry with the mAb NOQ7.5.4D to map the distribution of β-MyHC–expressing myocytes after TAC. The 3% of β-MyHC-positive cells in control hearts were too few to localize precisely. β-MyHC expression after TAC was restricted to the LV, where it was most abundant in the base of the heart, and was less toward the apex, except for a small area of intense expression at the apex (not shown). As shown in Figures 6A and 6B, β-MyHC-positive cells in the base of the heart were notable around larger coronary arteries and occurred infrequently in smaller vessels. Isolated clusters of β-MyHC-positive cells were also present in the LV septum close to the junction with the RV, as well as near the insertions of the mitral valve leaflets (Figure 6A). The tips of the papillary muscles had many β-MyHC-positive cells (not shown). Cells stained with the mAb NOQ7.5.4D had clear cross-striations, which confirmed them as myocytes (Figure 6C).
Prior studies using a reporter gene localized β-MyHC induction to areas of fibrosis. To test this localization for endogenous β-MyHC, we performed double staining for β-MyHC with wheat germ agglutinin to label collagen in fibrotic areas. As shown in Figure 7, a few myocytes positive for β-MyHC were found in sham control hearts (Figure 7A). After TAC, cells that expressed β-MyHC were observed perivascularly (Figure 7B; also seen in Figures 6A and 6B), in isolated areas away from vessels or fibrosis (Figures 7B and 7C), and in areas of fibrosis (Figure 7D). These data showed that cells that expressed endogenous β-MyHC after TAC were in discrete regions of the LV and in fibrotic, perivascular, and isolated areas.

β-MyHC-Positive Myocytes After TAC Arise From Myocytes With α-MyHC
To test whether the β-MyHC-positive myocytes after TAC arose by proliferation of the preexisting β-MyHC cells or some progenitor, we measured by flow cytometry the incorporation of ethynyl deoxyuridine into DNA and cell cycle markers Ki67 and phospho-histone H3. As shown in Online Figure I, these markers were positive in nonmyocytes after TAC but were not positive in myocytes, with or without β-MyHC.

Next, we validated an antibody for α-MyHC (Online Figure II) and quantified α-MyHC in control and TAC myocytes. As shown in Online Figure III, the relative level of α-MyHC per cell, quantified as median fluorescent intensity, was the same in cells with and without β-MyHC, before and after TAC. These data indicated that the β-MyHC-positive cells arose by new synthesis of β-MyHC in cells that already had α-MyHC. Importantly, the level of total MyHC, measured with the mAb MF-20, was significantly higher in the β-MyHC-positive cells after TAC than in β-MyHC-negative cells, which suggests that β-MyHC was added to α-MyHC, rather than that it replaced α-MyHC. Total MyHC in β-MyHC-negative cells increased with TAC by 28% versus a 59% increase in cell volume, whereas total MyHC increased by 41% in β-MyHC-positive cells that did not enlarge with TAC.

In summary, β-MyHC-positive myocytes arose without proliferation or hypertrophy after TAC by new synthesis of β-MyHC in α-MyHC–containing cells. β-MyHC-negative cells after TAC had hypertrophy but a smaller increase in total myosin.

Discussion
The major findings of the present study were as follows: (1) Endogenous β-MyHC protein is induced by pressure overload in only 25% of mouse myocytes; (2) β-MyHC-positive myocytes are smaller and do not enlarge with TAC; (3) β-MyHC-negative myocytes with α-MyHC only are the cells that enlarge with TAC; and (4) β-MyHC-positive myocytes arise without proliferation or hypertrophy by addition of β-MyHC to α-MyHC, and they have more total MyHC.

Figure 5. β-MyHC-positive myocytes are smaller. A, LV myocytes (10,000 to 20,000) from 3 control (CON) LVs (#1 to #3) and 3 TAC LVs at 3 to 6 weeks (#1 to #3) were stained with MF-20 and the β-MyHC mAb NOQ7.5.4D, and side-scatter profiles were determined by flow cytometry. β-MyHC-positive myocytes are indicated by solid black fill, β-MyHC-negative myocytes by gray dotted lines. Note the leftward shift to smaller sizes of β-MyHC-positive cells. B, Mean side scatter of β-MyHC-positive and negative myocytes from the same LV quantified in Figure 5A for 11 control mice and 14 mice at 1 to 6 weeks after TAC. Pairs of TAC and control LVs were assayed on the same cytometer on the same day, and side scatter of each subpopulation was normalized to the mean side scatter of control β-MyHC-negative myocytes, arbitrarily set at 1. In 1 assay, 3 TAC LVs were normalized to 1 control LV. Each point is 1 LV. Lines indicate mean ± SD. P by 1-way ANOVA and Bonferroni post test. C, Myocyte size was plotted vs time after TAC. Values are mean ± SE. Number of LVs was 5, 2, 1, 3, and 3 at weeks 1, 2, 3, 4, and 6, respectively. Pos indicates positive; Neg, negative.
These data challenge the current paradigm of the fetal hypertrophic gene program, showing a fetal gene in nonhypertrophied myocytes and no β-MyHC in hypertrophied cells. The data also identify an interesting subpopulation of smaller myocytes that contain β-MyHC, α-MyHC, and more total MyHC. Figure 8 is a model to summarize the main findings.

Flow cytometry allowed us to quantify β-MyHC expression and myocyte size simultaneously in large numbers of myocytes (Figure 1). Our approach is applicable to any standard cytometer, with careful attention to protocol details to avoid instrument clogging. Myocytes were identified by the well-vetted mAb MF-20, and β-MyHC–expressing myocytes were defined by a commercial mAb that was selective for β-MyHC by several criteria and was used at a dilution maximum for specific detection, in the green or red fluorescent spectra (Figure 2). We used a model of severe TAC (≈100-mm Hg gradient for 6 weeks) to induce remodeling and recruit the maximum number of β-MyHC-positive cells. Equal numbers of LV myocytes were isolated from control and TAC hearts (≈2 million), and 10 000 to 20 000 myocytes were analyzed in each run. Side scatter quantified myocyte size, which was as accurate as a Coulter Multisizer in detecting relative myocyte size differences in validation studies (Figure 4).

Our estimate of 25% of myocytes expressing β-MyHC after TAC for 3 days to 6 weeks (Figure 3) agrees well with 2 earlier studies, although we studied far more hearts (41 versus 6 to 8) and more myocytes per heart (>10 000 versus 200 to 3000). A mean 30% of rat myocytes were β-MyHC-positive at 2 to 14 days after ascending aortic banding, according to counts of 200 isolated cells stained with anti-V3 myosin antibody (mean calculated from Figure 3 in Rappaport et al19). Similarly, 38% of mouse myocytes had YFP-β-MyHC at 4 to 8 weeks after TAC, according to imaging of 3000 myocytes in LV sections.20 Therefore, the present data confirm in a large number of hearts and myocytes that endogenous β-MyHC is induced by pressure overload in only a minor subpopulation of LV myocytes.

However, our finding that β-MyHC-positive myocytes are smaller disagrees with previous reports that YFP-β-MyHC-positive and negative myocytes are equally hypertrophied.19,21,22 In contrast, we found that β-MyHC-negative cells were the only cells that enlarged with TAC. The difference might be explained by sampling. We quantified size in at least 10 000 myocytes in each of 25 hearts (Figure 5), whereas the laboratory of Smithies and colleagues measured cross-sectional area of 100 myocytes in each of 6 hearts.19,21,22 Alternatively, the YFP-β-MyHC fusion protein might itself have induced myocyte hypertrophy,32 whereas we studied endogenous β-MyHC.

An intriguing observation here was that the few β-MyHC–expressing myocytes were reproducibly present in discrete LV regions, including the LV-RV junction at the base, the insertion points of the mitral valve, the papillary muscles, and the apex (Figure 6). This regional distribution is extremely similar to that seen in 5- to 9-month-old FVB/N mice.33 We also found β-MyHC-positive cells around larger coronaries, similar to atrial natriuretic factor.34 Besides perivascular locations, β-MyHC myocytes were in areas of fibrosis and in isolated areas away from vessels or fibrosis (Figure 7). In this respect, induction of endogenous β-MyHC protein was not limited to areas of fibrosis, unlike the YFP-β-MyHC fusion protein.21,22 It is unknown what might determine the localization of β-MyHC cells, but there is an intriguing overlap with a stem cell niche in the apex,35 and atrial natriuretic factor and skeletal α-actin can also be found in fibrotic areas.17,18

We have shown that the β-MyHC-positive myocytes arise without proliferation or hypertrophy by new synthesis of β-MyHC in cells with α-MyHC. There was no isoform “switch.” Coexpression of α-MyHC in β-MyHC cells was noted in previous studies.15,36,37 However, because all neonatal myocytes can express β-MyHC (Figure 2), it is unknown why only a subpopulation of smaller adult myocytes in discrete LV regions retain the ability to transcribe β-MyHC with the stress of TAC. Also unknown is what else might distinguish the β-MyHC cells. For example, we have been unable to validate suitable antibodies for other fetal genes, such as atrial natriuretic factor. The small β-MyHC cells might be related to the small mononuclear, high-functioning myocytes identified in the cat and mouse heart,38,39 although we could not measure nuclear number reliably in the β-MyHC cells. In any case, the β-MyHC myocytes must uniquely have signaling molecules that can activate β-MyHC transcription with stress. One candidate is α1-adrenergic receptors, which induce β-MyHC24,40 and are also expressed in a subpopulation of myocytes.41

The present findings have important implications for the study of hypertrophic signaling. First and most obviously, β-MyHC induction does not indicate myocyte hypertrophy. Myocytes
with α-MyHC but not β-MyHC are the predominant population with hypertrophy after TAC. Therefore, signaling schemes focused on β-MyHC transcription might not be relevant to most myocytes (Figure 8). β-MyHC transcription is a poor marker for “hypertrophic signaling.” Second, the data caution against genetic approaches that test the functional role of β-MyHC by placing β-MyHC in most or all myocytes, such as adenovirus or transgenesis with the α-MyHC promoter.42,43

It is controversial whether β-MyHC induction and the fetal program are maladaptive41,44 or adaptive,45–47 and reliable induction as a function of disease severity does not answer this question. Transgenic models show that even high-level replacement of α-MyHC with β-MyHC causes no or mild pathology42,43,48,49 or can be beneficial.50,51 In the present experiments, the β-MyHC cells with more total MyHC did not have hypertrophy, whereas the cells with only α-MyHC did. β-MyHC

Figure 7. β-MyHC-positive LV myocytes after TAC in relation to vessels and fibrosis. Fixed frozen sections 3 weeks after TAC or sham surgery were stained with the mAb NOQ7.5.4D conjugated to Zenon-546 to label β-MyHC-positive myocytes (orange), plus wheat germ agglutinin to label membranes, vessels, and fibrosis (green). A. In sham, perivascular and isolated areas have a few β-MyHC-positive myocytes. After TAC, β-MyHC-positive myocytes are found (B) perivascularly and in an isolated area away from a vessel or fibrosis; (C) in an isolated area away from vessels or fibrosis; and (D) clustered in an area of fibrosis.

Figure 8. β-MyHC is found in a small subpopulation of small myocytes. The rectangles represent myocytes, which are shown in the relative number and volume measured in the present study. In control C57BL/6J male mouse hearts, 97% of myocytes had α-MyHC protein only (green), and 3% of myocytes coexpressed both β-MyHC (red) and α-MyHC proteins. After TAC, 25% of myocytes were positive for both β-MyHC and α-MyHC, which suggests that the β-MyHC cells arose by new synthesis of β-MyHC in α-MyHC-containing myocytes. Myocytes with β-MyHC were significantly smaller than myocytes with α-MyHC only, both before and after TAC. The hypertrophied myocytes after TAC expressed only α-MyHC, and the amount of α-MyHC per myocyte did not appear to increase as much as did cell size. β-MyHC cells had significantly more total MyHC.
might be antihypertrophic; for example, replacing α-MyHC with β-MyHC causes less hypertrophy in a troponin T mutant mouse.54 Increasing myocyte total MyHC and myofibrils causes improved function,52,53 whereas loss of myosin and myofibrils causes worse function in human cardiomyopathy and in an α1-adrenergic receptor knockout model in which β-MyHC and other fetal genes are not induced.45,54 Possibly, the β-MyHC-positive myocytes have a larger volume fraction of myofibrils, better function, and no deleterious secondary hypertrophy.

In summary, we used flow cytometry to show that β-MyHC is induced by pressure overload in only a minor subpopulation of smaller cardiac myocytes. These data challenge the current paradigm of the fetal hypertrophic gene program and identify a new subpopulation of smaller working ventricular myocytes with more myosin.

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Disclosures

None.

References

What Is Known?

- Reexression of the cardiac “fetal gene,” β-myosin heavy chain (β-MyHC), is a signature feature of pressure overload hypertrophy in rodents and is thought to be pathological.
- Endogenous β-MyHC mRNA and a fusion protein of yellow fluorescent protein-βMyHC have a heterogeneous myocardial expression pattern after stress.

What New Information Does This Article Contribute?

- Endogenous β-MyHC protein is induced by pressure overload in only a small subpopulation of mouse cardiac myocytes.
- Pressure-overloaded myocytes with β-MyHC are not hypertrophied and are smaller than myocytes that have only α-MyHC, the normal adult MyHC isoform.
- Myocytes with β-MyHC arise without proliferation or hypertrophy by adding β-MyHC to α-MyHC, and they have more total MyHC per cell than the hypertrophied myocytes with only α-MyHC.

- β-MyHC in rodent cardiac myocytes is expressed normally during fetal development and is replaced by α-MyHC after birth.

β-MyHC in rodent cardiac myocytes is expressed normally during fetal development and is replaced by α-MyHC after birth.
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Javier E. López, Bat-Erdene Myagmar, Philip M. Swigart, Megan D. Montgomery, Stephen Haynam, Marty Bigos, Manoj C. Rodrigo and Paul C. Simpson

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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, KH2PO4 1.452, and Na2HPO4-7H2O 8.058) were aliquoted into tubes (~200,000 adult myocytes/ml; ~600,000-800,000 neonatal total cells/ml), pelleted at 800xg 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, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄·7H₂O 1.2, Na·Hepes 10, NaHCO₃ 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 CaCl₂ 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 CaCl₂ 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 800xg 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 800xg 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 800xg 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 800xg, 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. 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, MgSO₄·7H₂O 0.81, dextrose 5.55, KH₂PO₄ 0.44, Na₂HPO₄·7H₂O 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 1.5\(\mu\)mol/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 BrdU100\(\mu\)mol/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 10\(\mu\)g/ml (#T-2252), bovine insulin 10\(\mu\)g/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 1mg/ml in CBFHH, pelleted at 800\(\times\)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\(^o\)C without change in \(\beta\)-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\(^o\)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 800\(x\)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\(^o\)C without change in \(\beta\)-MyHC.

**Rabbit myocardial cell isolation and fixation.**

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\(^o\)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\(^2+\) (20\(\mu\)mol/L) were added. After 15-25min, perfusion was stopped, and ventricles were minced into \(~2\)mm\(^3\) 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 240\(\mu\)m nylon mesh, washed, and stored until use in DMEM with Ca\(^2+\) 150 \(\mu\)mol/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 800\(x\)g 5min at room temperature, and re-suspended by gentle pipetting and vortexing in CMF-PBS 4ml. Fixed cell suspensions were filtered through a 200\(\mu\)m metal mesh, centrifuged at 800\(x\)g 3min at room temperature, and gently re-suspended in CMF-PBS 4ml. Cells could be stored up to 4w at 4\(^o\)C without change in \(\beta\)-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


SUPPLEMENT FIGURES I-III
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) Flow cytometry for EdU incorporation in CON and TAC cells. EdU staining artifactually quenched β-MyHC staining (not shown), so only total MCs are shown (positive for troponin T).

(B) Flow cytometry for Ki67 and H3P in TAC cells. Shown are pseudo-color contours, with dots for outlier cells.
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).
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).