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Novel large-particle FACS purification of adult ventricular myocytes reveals accumulation of myosin and actin disproportionate to cell size and proteome in normal post-weaning development

Javier E. López [a,*], Janhavi Sharma [a], Jorge Avila [a], Taylor S. Wood [a], Jonathan E. VanDyke [a], Bridget McLaughlin [a], Craig K. Abbey [b], Andrew Wong [a], Bat-Erdene Myagmar [c], Philip M. Swigart [c], Paul C. Simpson [c], Nipavan Chiamvimonvat [a,d]

[a] University of California, Davis, United States
[b] University of California, Santa Barbara, United States
[c] VA Medical Center, University of California, San Francisco, CA
[d] VA Medical Center, Mather, CA, United States

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ABSTRACT

Rationale: Quantifying cellular proteins in ventricular myocytes (MCs) is challenging due to tissue heterogeneity and the variety of cell sizes in the heart. In post-weaning cardiac ontogeny, rod-shaped MCs make up the majority of the cardiac mass while remaining a minority of cardiac cells in number. Current biochemical analyses of cardiac proteins do not correlate well the content of MC-specific proteins to cell type or size in normally developing tissue.
Objective: To develop a new large-particle fluorescent-activated cell sorting (LP-FACS) strategy for the purification of adult rod-shaped MCs. This approach is developed to enable growth-scaled measurements per-cell of the MC proteome and sarcomeric proteins (i.e. myosin heavy chain (MyHC) and alpha-actin (α-actin)) content.
Methods and results: Individual cardiac cells were isolated from 21 to 94 days old mice. An LP-FACS jet-in-air system with a 200-μm nozzle was defined for the first time to purify adult MCs. Cell-type specific immunophenotyping and sorting yielded ≥95% purity of adult MCs independently of cell morphology and size. This approach excluded other cell types and tissue contaminants from further analysis. MC proteome, MyHC and α-actin proteins were measured in linear biochemical assays normalized to cell numbers. Using the allometric coefficient α, we scaled the MC-specific rate of protein accumulation to growth post-weaning. MC-specific volumes ($\alpha = 1.02$) and global protein accumulation ($\alpha = 0.94$) were proportional (i.e. isometric) to body mass. In contrast, MyHC and α-actin accumulated at a much greater rate (i.e. hyperallometric) than body mass ($\alpha = 1.79$ and 2.19 respectively) and MC volumes ($\alpha = 1.76$ and 1.45 respectively).
Conclusion: Changes in MC proteome and cell volumes measured in LP-FACS purified MCs are proportional to body mass post-weaning. Oppositely, MyHC and α-actin are concentrated more rapidly than what would be expected from MC proteome accumulation, cell enlargement, or animal growth alone. LP-FACS provides a new standard for adult MC purification and an approach to scale the biochemical content of specific proteins or group of proteins per cell in enlarging MCs.

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1. Introduction

Cardiac tissue heterogeneity and extracellular matrix (ECM) composition complicate linking protein changes during normal myocyte (MC) growth (i.e. ontogeny) or hypertrophy to specific cell types. Adult ventricular tissues are normally composed of non-muscle cells (NMCs) and MCs in a 2:1 ratio [1]. Hence, accounting for specific changes in cellular proteins during cardiac ontogeny or disease is challenging if tissue sampling does not reflect the cell-specific contribution to the measured proteins. Standard biochemical analyses of enlarging tissues are usually normalized to equal amounts of global proteins loaded onto an assay

Abbreviations: Ab, antibody; α-actin, alpha-actin; APC, allophycocyanin; BDM, 2, 3-butanedione monoxime; cTnT, cardiac troponin T; DAPI, 4′, 6-diamidino-2-phenylindole; DSHB, Developmental Studies Hybridoma Bank; ECM, extra cellular matrix; FCM, Flow cytometric analysis; H/BM, heart to body mass ratio; HRP, horseradish peroxidase; HT, hypertrophy; LP-FACS, large-particle fluorescent-activated cell sorting; mAb, monoclonal antibody; MC, myocyte; MF20, anti-myosin heavy chain monoclonal antibody; MyHC, myosin heavy chain; NMC, non-muscle cell; PI, propidium iodide; PFA, paraformaldehyde.

* Corresponding author at: Cellular and Molecular Cardiology, 451 E. Health Science Dr., GBSF Suite #6303, Davis, CA 95616, United States.
E-mail address: drjlopez@ucdavis.edu (J.E. López).

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(e.g. Bradford assay, immunoblotting, etc.). This standard practice, however, makes normalizing one specific protein signal to another difficult because the source of proteins in tissues may be unclear. Whole tissue preparations offer at best a "bulk sum" of all proteins from MCs, NMCs, and ECM that may mask cell-type specific changes. Single-cell analysis is preferred to limit proteome redundancy where global and/or specific proteins arise from multiple cell types or ECM [2,3].

Maturing cardiac MCs increase in size after cessation of proliferation shortly after birth and with adult specialization post-weaning [1]. While adult MCs make up a minority of cells in number, they enlarge >10-fold into adulthood to account for most of the adult ventricular mass [4,5]. Consideration of cell size changes is also critical when quantitating proteins. If we hypothetically load equal amounts of protein from cell lysates into a biochemical assay to compare enlarging MCs that have doubled in size and protein content, we would effectively survey half as many cells of the larger MCs as the smaller ones. An equal protein signal between samples, in fact, would not mean equal protein content per-cell, but rather that the larger MCs actually have twice as much protein as the smaller MCs. Similarly, a 50% reduction in signal on the larger MCs would mean equal protein content per-cell. This is especially problematic when changes in specific protein content or cell size occur at different rates from global proteins. Comparing developmental or diseased conditions in this scenario can potentially obscure real differences in the rate of protein accumulation or result in "false-positive" variations [6–8]. Although sarcomeres and other specialized proteins increase with cardiac mass [9], an inability to biochemically sample MC proteins separate from NMCs and ECM contaminant can limit the validity of normalizing MC proteins to global proteins from tissues [10,11].

To overcome these challenges, we developed a highly reproducible fluorescence-activated cell-sorting (FACS) strategy that purifies known numbers of adult MCs from NMCs and ECM in single cell preparations. We have designed and optimized this new large-particle protocol (LP-FACS) using a conventional jet-in-air sorter especially fitted with a 200-μm nozzle. In this new configuration, the sorter counts and purifies adult ventricular MCs regardless of morphology. Purified MCs are then sized and used to generate protein lysates. Analyzing protein lysates from known numbers of purified and well-sized MCs permits an independent scaling of proteins of interest to global proteins (i.e. proteome) and/or myocyte growth.

To gain a new insight into the cell size-sarcomere content relationship of normally growing MCs, we purified ventricular MCs post-weaning with LP-FACS and biochemically measured MyHC and α-actin content per-cell. These two proteins are integral constituents of the sarcomere and responsible for muscle contraction. Both proteins have two isoforms differentially expressed in the heart throughout development: α & β − MyHCs [12] and α − cardiac & skeletal actins [13]. To scale the biochemical content of MC proteins to animal growth and MC sizes, we applied the simple model of ontogenic allometry [14]. This model relates the rate of change of physiological and/or molecular features to growth during development [15,16]. Using this new analytical system, we identified that sarcomeric proteins accumulate at a disproportionately higher rate than the MC-specific proteome during normal growth. Scaling specific proteins to cell size in LP-FACS purified MCs provides a new framework to understand variability in normal tissue growth at the level of individual cell types in health and disease.

2. Brief methods

2.1. Single-cell preparations

The Institutional Animal Care and Use Committee at the University of California, Davis, approved all animal care and procedures. C57BL/6 mice were fed standard mouse diet ad libitum. Mouse hearts were obtained during the postnatal period from the 1st to the 94th day of life. On postnatal day 20–21, litters were weaned and single-cell suspensions were obtained at least 1 day post-weaning. Post-weaning mice from postnatal days 21–25 were referred to as weanlings, and mice from postnatal days 75 to 94 were referred to as adults. Cardiac single-cell preparations from post-weaning mice were obtained by the methods of López, JE et al. [10] with modifications (see Supplemental methods) aimed to increase the proportion of healthy and mostly rod-shaped MCs in ventricular preparations. Briefly, coronary arteries were perfused with a nominal calcium-free perfusion buffer containing 2,3-butanedione monoxime (BDM) at 37 °C followed by collagenase enzymatic digestion and a high-K^+ HEPES containing solution at pH 7.4. This buffer sequence was optimized to yield ~85% rod-shaped MCs. To preserve the morphology of isolated adult MCs post-sorting, cells were gently fixed with 0.4% paraformaldehyde (PFA) prior to LP-FACS sorting. Neonatal and adult cell isolation followed by β-MyHC, α-MyHC, α-actin, and NPPA flow cytometric analysis (FCM) was done on a FACScan cytometer (BD Biosciences, San Jose, CA) as previously described [10].

2.2. Adult myocyte cell sorting and analysis

LP-FACS was performed with a jet-in-air Influx Cell Sorter (BD Biosciences, San Jose, CA) modified with a 200-μm nozzle and a small particle detector upgrade on the FSC channel (threshold channel). New sort settings, single-cell preparation, cell sizing, and protein analysis methods are detailed in Supplemental materials. Briefly, low-pressure settings and reduced oscillator frequency for droplet stability were developed to accommodate the larger size of adult MCs (up to ~110 × 40 μm) with variable fractions of cell shapes (i.e. rods vs. rounds). Cell shape is a critical factor in MC flow cytometry because it affects the propensity of large particles to align in a flow stream. Single-cell preparation, handling and cell concentration were optimized to maximize rod-shaped MC alignment in the flow stream without excluding round MCs. Immunophenotyping with four monoclonal antibodies (mAbs) and a DNA marker provided a sort logic that can identify MCs (labeled with anti-cardiac Troponin T mAb) and NMCs (labeled with anti-CD31, CD45, and Sca-1 mAbs) independently. All primary mAbs were fluorochrome conjugated to avoid cross-contamination. Influx settings and cell labeling were designed to reach a pre-specified ≥95% MC purity (independent of morphology) with ≥90% accuracy on MC counts. The median volumes of 5000–7000 MCs post-sort were measured with a Coulter Multisizer 4 (Beckman Coulter, Brea, CA) [17,18].

2.3. Protein analysis

Global protein analysis using the Bradford method was performed on LP-FACS-purified MC lysates to measure MC proteome. MyHC content was determined by immunoblotting densitometry of MF20 mAb conjugated to HRP to avoid non-specific binding and/or cross-reactivity of secondary Abs binding to primary Abs used for cell sorting. α-actin was measured similarly using 5c5 IgM mAb. Secondary anti-IgM Ab conjugated to HRP was selected to avoid cross reactivity with primary Abs used for cell sorting.

MyHC, α-actin and global protein signals were first standardized to cell numbers that provided a signal in linear range. The number of MCs needed for each assay was selected based on the linear range of the signal. These cell numbers were different for weanlings and adults due to ontogenic differences in cell size and protein content. Global protein content was calculated in nanograms per MC (ng/MC) for each animal. The MyHC and α-actin content per-MC in arbitrary units was normalized to the weanling samples in the same slot blot.

2.4. Ontogenic allometry and statistical analysis

Results are shown as mean ± SD unless otherwise noted. Significant differences ($p < 0.05$) among groups were tested in GraphPad Prism version 6.0 with 1-way ANOVA and Bonferroni multiple comparison test for >2 groups, or unpaired Student's *t*-test for 2 groups. Protein content

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