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Original article

Phenotypic screen quantifying differential regulation of cardiac myocyte hypertrophy identifies CITED4 regulation of myocyte elongation

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ABSTRACT

Cardiac hypertrophy is controlled by a highly connected signaling network with many effectors of cardiac myocyte size. Quantification of the contribution of individual pathways to specific changes in shape and transcript abundance is needed to better understand hypertrophy signaling and to improve heart failure therapies. We stimulated cardiac myocytes with 15 hypertrophic agonists and quantitatively characterized differential regulation of 5 shape features using high-throughput microscopy and transcript levels of 12 genes using qPCR. Transcripts measured were associated with phenotypes including fibrosis, cell death, contractility, proliferation, angiogenesis, inflammation, and the fetal cardiac gene program. While hypertrophy pathways are highly connected, the agonist screen revealed distinct hypertrophy phenotypic signatures for the 15 receptor agonists. We then used k-means clustering of inputs and outputs to identify a network map linking input modules to output modules. Five modules were identified within inputs and outputs with many maladaptive outputs grouping together in one module: Bax, C/EBPβ, Serca2a, TNFα, and CTGF. Subsequently, we identified mechanisms underlying two correlations revealed in the agonist screen: correlation between regulators of fibrosis and cell death signaling (CTGF and Bax mRNA) caused by AngII; and myocyte proliferation (CITED4 mRNA) and elongation caused by Nrg1. Follow-up experiments revealed positive regulation of Bax mRNA level by CTGF and an incoherent feedforward loop linking Nrg1, CITED4 and elongation. With this agonist screen, we identified the most influential inputs in the cardiac hypertrophy signaling network for a variety of features related to pathological and protective hypertrophy signaling and shared regulation among cardiac myocyte phenotypes.

1. Introduction

Cardiac hypertrophy develops with increased biochemical and mechanical stresses on the heart [1] and is a major predictor of heart failure and sudden cardiac death [2–4]. Specific features of cardiac hypertrophic remodeling depend on the type of cardiac stress [5,6]. Physiological stresses such as exercise lead to hypertrophy without cardiac dysfunction, but pathological stresses such as high blood pressure and myocardial infarction lead to hypertrophy with increased fibrosis, cell death, and cardiac dysfunction [7]. Moreover, myocytes grow distinctly in response to different mechanical stimuli. Pressure overload induces concentric hypertrophy, characterized by thickening of the heart wall, and volume overload of the heart induces eccentric hypertrophy, characterized by myocyte elongation and dilation of the heart wall [8]. Since eccentric hypertrophy is a greater risk to patients than concentric hypertrophy [9], increased knowledge of the unique signaling pathways controlling myocyte shape will be important in improving therapies for heart failure. Previous work suggests that myocyte size is regulated by common signaling pathways and myocyte shape is regulated by distinct signaling pathways [10,11]. But little is known about the specific signaling pathways that induce distinct characteristics of hypertrophy [12,13].

Many signaling pathways and genes manage the hypertrophic response [14]. While many of the components of this network have been identified, the distinct contributions to different features of hypertrophy such as shape, fibrosis, and cell death between pathways are not well understood [15]. Furthermore, the pathways governing hypertrophy are highly connected with much cross-talk between pathways [16]. It is unclear how all of the parts of such an interconnected network function together as a coordinated system that can induce distinct, context-dependent hypertrophy features. Commonly measured markers of hypertrophy such as cell size and fetal gene expression are not markedly differentially regulated between receptor pathways in the signaling network [16].

Here, we test the hypothesis that features such as myocyte shape, fibrosis, cell death, and inflammation may better differentiate hypertrophic signaling pathways. We quantified differential regulation of 5 shape features using high-throughput myocyte imaging and transcript

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levels of 12 genes induced by 15 predominant hypertrophic agonists. These genes have previously been associated with phenotypes such as fibrosis, cell death, contractility, proliferation, angiogenesis, inflammation, and the fetal cardiac gene program, providing a phenotypic signature for each agonist. We clustered pathway inputs and outputs to identify a network map linking input modules to output modules. Among these, we found strong correlations between Bax and CTGF mRNA abundance in response to AngII and between myocyte elongation and CITED4 mRNA abundance in response to Nrg1. Follow-up experiments validated these correlations, revealing regulation of pro-apoptotic Bax by fibrosis marker CTGF and negative regulation of myocyte elongation by CITED4 gene expression.

2. Methods

2.1. Cell culture and microscopy

Cardiac myocytes were harvested from 1 to 2 day old Sprague Dawley rats using the Neomyts isolation kit (Cellutron, Baltimore, MD). Myocytes were cultured in plating media (Dulbecco's modified Eagle media, 17% M199, 10% horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 50 mg/mL: streptomycin) at a density of 100,000 cells per well of a 96-well plate coated with SureCoat (a combination of collagen and laminin, Cellutron). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the University of Virginia Institutional Animal Care and Use Committee. Two days after isolation, myocytes were transfected with GFP under a cardiac myocyte specific troponin T promoter [17] using Lipofectamine 2000 (Invitrogen, Carlsbad, California; transfection efficiency: 10–15%). Two days after transfection, myocytes were imaged using automated image acquisition scripts, which collect a 5 × 5 grid of images in each well of interest in the 96-well plate (Fig. 1) [18]. Images were collected using an Olympus IX81 inverted microscope with 10× UPlanSApo 0.40 NA objective, Orca-AG CCD camera (Hamamatsu, Bridgewater, NJ), automated stage (Prior Scientific, Rockland, MA), and IPLab software (Scanalytics, Fairfax, VA).

2.2. Quantifying changes in shape

After initial images were collected, myocytes were rinsed and cultured in serum-free media containing one of three concentrations of a hypertrophic agonist (dilution factor = 10, intermediate concentration listed): 1 μM atrial natriuretic factor (ANF), 1 μM angiotensin II (AngII), 1 nM cardiotrophin-1 (CT1), 10 nM epidermal growth factor (EGF), 100 nM endothelin-1 (ET1 while maintaining cell health in the serum free control condition), 20 ng/mL fibroblast growth factor-2 (FGF2), 10 nM insulin growth factor-1 (IGF1), 10 ng/mL interleukin-6 (IL6), isoproterenol (ISO), 1 nM leukemia inhibitory factor (LIF), 1 μM norepinephrine (NE), 10 ng/mL neuregulin-1 (Nrg1), 1 μM phenylephrine (PE), 1 ng/mL transforming growth factor-beta (TGFβ), or 10 ng/mL tumor necrosis factor-alpha (TNFα). A comprehensive list of agonist concentrations is shown in Supplementary Table S1. After 48 h, myocytes were imaged again. 48 h stimulation allowed for robust changes in cell size and shape to be measured while maintaining cell health in serum free conditions.

Changes in myocyte area, perimeter, form factor, elongation, and integrated fluorescence intensity were calculated using automated custom Matlab image analysis algorithms. Form factor is a measure of circularity and is calculated as $4\pi^*\text{area/perimeter}^2$. A circle therefore has a form factor value of 1 and all other shapes have form factors less than 1. Elongation is a ratio of the major axis length to the minor axis length of the myocyte. All shape measurements were recorded from two wells from three independent myocyte isolations.

2.3. Quantifying changes in transcript abundance

48 h after stimulation with the hypertrophic agonists, total RNA was purified from myocytes given the intermediate concentration of the agonist using the RNeasy Mini kit (Qiagen, Valencia, CA). Complementary DNA was synthesized from 85.5 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad). mRNA levels of twelve genes (Bcl-2, Bax, C/EBPβ, CITED4, VEGF, Serca2a, BNP, Skeletal α-actin, IκB, TNFα, CTGF, and GAPDH) were measured using qPCR (BioRad CFX Connect) using iTaq Universal SYBR Green Supermix (Bio-Rad), 2 ng of cDNA, and 400 nM of each primer set. GAPDH served as internal control. Gene-specific primers were designed on PrimerQuest (Integrated DNA Technologies, Inc.) A list of primers used is shown in Supplementary Table S2. Data were analyzed using the comparative C_T method with efficiency correction [19]. Measurements were collected from three independent myocyte isolations.

2.4. siRNA knockdown

Two days after isolation, 10 nM silencer select siRNA (Ambion) was transfected into cells using Lipofectamine RNAiMAX, as described by the manufacturer's protocol. Two siRNA sequences were tested for each target. Cells were transfected again the next day with 10 nM siRNA in fresh

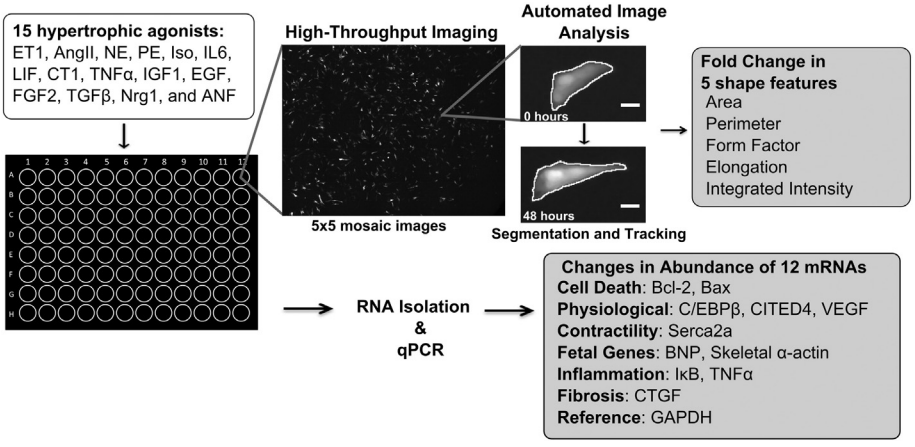

Fig. 1. 15 ligand hypertrophic agonist screen in neonatal rat cardiac myocytes, measuring changes in 5 shape features and abundance of 12 mRNAs. Myocytes were treated for 48 h with 1 of 15 hypertrophic agonists. Fold change in 5 shape features was calculated using our automated image acquisition and analysis platform. We measured fold change in abundance of 12 mRNAs related to cell death, physiological hypertrophy, contractility, fetal genes, inflammation, and fibrosis using qPCR.