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PGC-1 α and ERR α target gene downregulation is a signature of the failing human heart

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Abstract

Heart failure is a cause of significant morbidity and mortality in developed nations, and results from a complex interplay between genetic and environmental factors. To discover gene regulatory networks underlying heart failure, we analyzed DNA microarray data based on left ventricular free-wall myocardium from 59 failing (32 ischemic cardiomyopathy, 27 idiopathic dilated cardiomyopathy) and 33 non-failing explanted human hearts from the Cardiogenomics Consortium. In particular, we sought to investigate cardiac gene expression changes at the level of individual genes, as well as biological pathways which contain groups of functionally related genes. Utilizing a combination of computational techniques, including Comparative Marker Selection and Gene Set Enrichment Analysis, we identified a subset of downstream gene targets of the master mitochondrial transcriptional regulator, peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), whose expression is collectively decreased in failing human hearts. We also observed decreased expression of the key PGC-1 α regulatory partner, estrogen-related receptor α (ERR α), as well as ERR α target genes which may participate in the downregulation of mitochondrial metabolic capacity. Gene expression of the antiapoptotic Raf-1/extracellular signal-regulated kinase (ERK) pathway was decreased in failing hearts. Alterations in PGC-1 α and ERR α target gene sets were significantly correlated with an important clinical parameter of disease severity - left ventricular ejection fraction, and were predictive of failing vs. non-failing phenotypes. Overall, our results implicate PGC-1 α and ERR α in the pathophysiology of human heart failure, and define dynamic target gene sets sharing known interrelated regulatory mechanisms capable of contributing to the mitochondrial dysfunction characteristic of this disease process.

Keywords

Heart failure; Mitochondria; Fatty acid oxidation; PGC-1 α ; ERR α ; Raf-1; ERK; LCAD; Gene expression profiling

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1. Introduction

Congestive heart failure is a cause of significant morbidity and mortality in developed nations. For individuals free of heart failure at age 40, data from the Framingham Heart Study indicates that the lifetime risk of developing heart failure is 20% [1]. Among the most common etiologies of end-stage human heart failure are idiopathic dilated cardiomyopathy (DCM) and ischemic cardiomyopathy (ICM). To investigate pathophysiologic mechanisms of heart failure, microarray technology has been utilized extensively in the characterization of global gene expression changes in both ischemic and idiopathic dilated (or non-ischemic) cardiomyopathy [2,3]. A number of prior microarray studies of heart failure have noted no significant gene expression changes between DCM and ICM [4] or have grouped ischemic and non-ischemic samples into a single failing category when comparing against non-failing controls [5,6]. Although end-stage heart failure may represent a common clinical endpoint with a uniform gene expression signature irrespective of etiology, additional studies have identified both shared and distinct gene expression changes between ischemic and non-ischemic heart failure [7–9].

Gene regulatory networks, or pathways, previously associated with heart failure include immune and inflammatory response [10], cell signaling and metabolism [4,6,8], sarcomere and cytoskeletal organization [6,9,11], transcription/translation [12], and apoptosis [13,14]. Conventional methods used to discover dysregulated pathways in heart failure have relied fundamentally on single-gene approaches combined with Gene Ontology or self-defined functional annotations [7,10,11,15]. Our current study differs in that it also employs biological pathway-based analytic techniques to detect over-arching patterns of gene expression in relatively large populations of failing (DCM/ICM) vs. non-failing (NF) human hearts, as well as in individual comparisons of DCM and ICM groups.

Given that insufficient energy delivery and altered substrate utilization occur in the failing heart, gene regulatory networks modulating mitochondrial function have been implicated in the pathogenesis of heart failure [16–19]. In particular, studies investigating mechanisms of defective energy transduction in heart failure have defined a role for the transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α), a key regulator of post-natal cardiac mitochondrial function [16,20–22]. In the mouse heart, PGC-1 α is essential for maximal and efficient cardiac mitochondrial fatty acid oxidation, ATP synthesis, and lipid homeostasis, especially in the setting of increased work [23]. Forced expression of PGC-1 α in hearts of transgenic mice increases mitochondrial number and stimulates respiration via several downstream transcriptional regulatory circuits, one of which involves the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) [24]. Additionally, the ability of PGC-1 α to coactivate the peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptor α (ERR α) gives it a critical role in the regulation of genes central to the mitochondrial β -oxidation of fatty acids as well as overall mitochondrial oxidative phosphorylation [16,25–30].

Reduced PGC-1 α activity has been observed in the evolution of several important diseases associated with metabolic derangement, including diabetes mellitus, pathologic cardiac hypertrophy, and heart failure. Oxidative phosphorylation genes regulated by PGC-1 α are coordinately depressed in human skeletal muscle with diabetes mellitus type 2 [28,31]. In pressure-overload cardiac hypertrophy in mice, PGC-1 α gene expression is decreased [21], and its level of expression correlates with mitochondrial oxidative capacity in both healthy and failing rat hearts subjected to chronic pressure overload [32]. Consistent with involvement of PGC-1 α in the maintenance of cardiac function following the stress of pressure overload, mice with generalized loss of PGC-1 α develop accelerated heart failure two months following transverse aortic constriction [33]. The current study attempts to extend to human heart failure

a role for mitochondrial transcriptional regulatory circuits characterized predominantly in normal and failing mouse hearts. This work implicates the PGC-1 α /ERR α regulatory circuit in the pathophysiology of human heart failure, suggesting transcriptional regulatory mechanisms involving PGC-1 α and its key partners which may contribute to the mitochondrial dysfunction characteristic of the failing heart.

2. Methods

2.1 Source of Microarray Data

Publicly available microarray data was obtained from the Cardiogenomics Consortium at Brigham and Women's Hospital in Boston, Massachusetts (<http://cardiogenomics.med.harvard.edu>), an NHLBI sponsored Program in Genomic Applications. RNA was isolated from left ventricular free-wall myocardium extracted from 92 explanted human hearts and hybridized to the Affymetrix HG-U133 plus 2.0 oligonucleotide microarray (54,675 probe sets) according to the guidelines and protocols as outlined on the website. (The 'Quality Control' section of the Cardiogenomics Consortium website (above) offers further details.) These studies were approved by the Committee for the Protection of Human Subjects at Brigham and Women's Hospital and Harvard Medical School. The gene expression analysis component reported in this paper was further reviewed by the Washington University Human Research Protection Office. Fifty-nine of the 92 explanted hearts were from patients with end-stage heart failure at the time of cardiac transplantation, where the etiology of failure was ischemic cardiomyopathy (ICM) in 32 patients and idiopathic dilated cardiomyopathy (DCM) in 27 patients. The remaining 33 samples were from normal, non-failing (NF) cardiac transplant donors whose cause of death was non-cardiac in origin, but whose heart could not be used for transplantation. Left ventricular tissue was harvested intraoperatively at the time of transplantation and snap frozen immediately (within 1 min) in liquid nitrogen, then stored at -80°C until RNA was isolated. Demographics and limited clinical parameters were available for the majority of patients. For the non-failing group, data on body mass index, hypertension, diabetes, and left ventricular ejection fraction were available for less than 50% of patients, while pulmonary capillary wedge pressures were not available for any of the patients in the non-failing group.

2.2 Data Scaling and Processing

The data were subjected to global scaling to correct for intensity-related biases as described on the website. Only scans with a 3'/5' ratio < 1.33 were included in our study. A single ICM sample was eliminated by this criterion, leaving a total of 91 samples - 27 DCMs, 31 ICMs, and 33 NFs.

All probe sets which were found to have greater than 93% absent calls across 91 samples were eliminated. These probe sets were considered absent, or under-expressed, in our study and were designated as 'filtered'. Of 54,675 total probe sets on the HG-U133 plus 2.0 array, 30,608 probe sets subsequently remained after filtering. We further collapsed the data from 30,608 probe sets to 17,419 non-redundant genes with distinct HUGO gene symbols, since multiple probe sets often map to a single gene. In this instance, the probe set with the maximum absolute expression value was uniformly assigned to the gene.

In order to reduce the age variability in our population and given the imbalanced number of samples derived from female subjects, all females and marked age outliers were eliminated from our study. A simple algorithm was constructed to remove age outliers in an objective fashion, such that the oldest and youngest were systematically subtracted from each population until the mean age of the three populations was not significantly different. In effect, an age and gender 'matched' subset was created, consisting of 49 samples - 13 DCMs, 20 ICMs, and 16

NFs, while still preserving sample size and power. This subset is referred to as the ‘matched’ population. The remaining 42 samples were defined as the ‘unmatched’ population and used for independent validation of our class prediction model.

2.3 Single-gene Analysis

The Comparative Marker Selection algorithm from the GenePattern 2.0 software package [34,35] was employed to identify individual genes that were differentially expressed in all four pairwise comparisons (DCM/ICM vs. NF, DCM vs. NF, ICM vs. NF, DCM vs. ICM). A 2-sided *t*-test followed by 1000 permutations of phenotype labels was performed for this analysis. A false discovery rate *q* value (FDR)<0.05, accounting for multiple hypothesis testing [35], and a fold change (FC)>1.2 served as a cutoff for significant genes. A family-wise error rate (FWER)<0.05 was used as another, more stringent statistic to discover highly significant genes.

2.4 Pathway Analysis

Gene Set Enrichment Analysis (GSEA) was utilized to probe pathways, or groups of functionally related genes, dysregulated in heart failure [36]. In discovery mode, gene sets with an FDR<0.25 and a nominal *p* value<0.05 were considered significant [31,36]. The gene ranking metric was a signal-to-noise ratio and the number of permutations specified was 1000.

502 curated gene sets representing generic biological pathways were downloaded from the Broad Institute Molecular Signature Database (MSigDb) (<http://www.broad.mit.edu/gsea>). The original sources of these pathways include BioCarta, GenMapp, Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Broad Institute. The ‘PGC-1 α targets’ gene set was derived from the MSigDb pathway annotated as ‘PGC’, and is based on genes responsive to adenoviral-mediated PGC-1 α gain-of-function in cultured mouse myoblasts (C2-C12 cells) [31]. The ‘ERR α targets 1’ and ‘ERR α targets 2’ gene sets were mined from the literature, and are based on genes influenced by adenoviral-mediated ERR α gain-of-function in primary rat neonatal cardiac myocytes [25] and ChIP-on-chip gene promoter occupancy assays [37], respectively. A total of 504 pathways were initially compiled for investigation. Gene sets with less than 15 genes or more than 500 genes were excluded from the analysis, leaving 252 pathways after application of this threshold. Gene classification using Gene Ontology (GO) Biological Process and Cellular Component terms was performed for core enrichment set genes via FatiGo online web tool [38].

Given that multiple probe sets may map to a single gene, the GSEA software package offers two options with respect to collapsing multiple probe sets into a single expression vector to represent a gene: the maximum probe set expression value or the median probe set expression value. In this study, the maximal probe set expression value was set as the default in the algorithm, as in other previous studies that rely on GSEA. According to the authors of the GSEA algorithm, this approach allows for more widespread and accurate signal detection [36]. Moreover, we performed GSEA employing both options and found the difference between the two analyses minimal (e.g., the PGC-1 α and ERR α target gene pathways remain significantly downregulated with human heart failure in both cases).

2.5 Linear Regression Model

Linear regression models were generated using Microsoft Excel’s Regression Package. Expression levels of the PGC-1 α target, ERR α target 1, and ERR α target 2 core enrichment set genes were designated as independent, explanatory variables, and left ventricular ejection fraction (LVEF) as a dependent variable. Expression values of genes in each core enrichment set were normalized to $\mu=0$ and $\sigma=1$ across samples from the matched population. A core enrichment set mean expression level was then computed for each of the samples and regressed

versus LVEF to illustrate the explanatory power of this gene set. LVEF was known for 27 of the 49 samples in the matched population.

2.6 Class Prediction Model

Using the K-nearest neighbors (KNN) algorithm of the GenePattern 2.0 software package with a Euclidean distance metric [34], prediction models were built to distinguish between failing and non-failing classes based on genes in the PGC-1 α target, ERR α target 1, ERR α target 2, and ERK pathway core enrichment sets (CES). For comparison, a parallel prediction model was created employing the most significantly up and downregulated markers at FWER<0.05. All models were initially trained on samples from the matched population, and then tested on samples from the unmatched population. It is important to note that these are two mutually exclusive populations, the sum of which makes up the total population.

2.7 Source of Left Ventricular Tissue for Protein Level Quantification

For determination of left ventricular protein levels in failing versus nonfailing hearts, cardiac tissue samples were obtained from 8 hearts with no history of myocardial dysfunction and with normal left ventricular ejection fraction by echocardiography. These ‘nonfailing’ hearts (from 4 men and 4 women) were donated for orthotopic cardiac transplantation but declined for reasons related to size or ABO blood type mismatch. Consent for donation of heart tissue for research purposes was obtained from family members by the organ donor organization covering the Colorado-Wyoming-Montana region (Donor Alliance). Eight ‘failing’ heart samples were obtained from cardiac transplant candidates (4 men and 4 women) with idiopathic dilated cardiomyopathy whose age was nearly identical to the ‘nonfailing’ population. In particular, the mean ages of the ‘nonfailing’ and ‘failing’ populations were both 58 \pm 2 years, with age ranges of 52–69 years and 52–65 years, respectively. The left ventricular ejection fraction by echocardiography was 62 \pm 2% for the ‘nonfailing’ hearts and 17 \pm 5% for the ‘failing’ hearts.

2.8 Western Blotting

Generation of left ventricular whole cell protein extracts, protein immunoblotting, and immunoreactive signal quantification were performed as previously described [39]. Protein gels were transferred to a nitrocellulose membrane, prior to incubation with the following antibodies: calnexin (C4731, Sigma-Aldrich, St. Louis, MO), long-chain acyl-CoA dehydrogenase (LCAD, a gift of Arnold W. Strauss), citrate synthase (a gift of John O. Holloszy), and cytochrome c (556433, BD Pharmingen, San Jose, CA). Antibodies were diluted in 1 \times phosphate-buffered saline containing 3% bovine serum albumin (BSA) and 0.1% Tween, then incubated with the blot overnight at 4 C. Anti-rabbit and anti-mouse secondary antibodies were used at 1:10,000 dilution.

3. Results

We analyzed microarray data from 58 failing (31 ICMs, 27 DCMs) and 33 non-failing explanted human hearts. Since age and gender are known confounders in transcriptional profiling studies of heart failure [40,41], we created an age and gender ‘matched’ subset by eliminating all females and age outliers to minimize variability in our population (Table 1). The matched population consisted of 49 males (20 ICM, 13 DCM, 16 NF), where the difference in mean ages was not statistically significant. Consistent with the association between hypertension, diabetes, and coronary artery disease, the ICM group in the total population exhibited the highest prevalence of hypertension, along with a trend toward increased prevalence of diabetes (Table 1). In the total population, the DCM group displayed the most severe heart failure, with a mean left ventricular ejection fraction (LVEF) of 12.9 \pm 1.3% vs. ICM mean LVEF of 21.0 \pm 2.7%, although both groups demonstrated marked left ventricular

systolic dysfunction. A similar trend toward greater impairment of systolic function was observed in the DCM vs. ICM groups of the matched population; this apparent difference, however, was not statistically significant.

In our initial approach, the Comparative Marker Selection algorithm [34,35] was used to identify individual genes that were differentially expressed between non-failing (NF) and combined failing (DCM/ICM) classes. Out of a total of 17,419 distinct human genes in our dataset after filtering (as per Methods), 9,991 genes (57%) exhibited a fold change >0 , while 7,428 genes (43%) exhibited a fold change <0 . At a false discovery rate q value (FDR) <0.05 and a fold change (FC) >1.2 , 2,236 genes (13% of total) were significantly upregulated and 1,509 genes (9% of total) were significantly downregulated (Fig. 1A). Using an even more stringent statistic to define up and downregulated genes, a family-wise error rate (FWER) <0.05 and FC >1.2 , only 340 genes (2% of total) were upregulated and 144 genes (1% of total) were downregulated (Figs. 1A and 1B). For a complete list of significant genes in all categories, see Supplemental Table S1. This analysis was also performed for the following pair-wise comparisons: DCM vs. NF, ICM vs. NF, and DCM vs. ICM (Supplemental Tables S2, S3, and S4 respectively).

Since heart failure represents a complex disease caused by alterations in multiple genes or gene sets such that single-gene strategies may be less revealing in terms of overall physiology, we next chose to pursue a pathway-based methodology for analysis. In particular, we applied Gene Set Enrichment Analysis (GSEA) [31,36] to investigate 504 curated, generic biological pathways in an unbiased fashion for the failing (DCM/ICM) vs. non-failing (NF) comparison (see Supplemental Table S5 for list of pathways). At an FDR <0.25 and $p<0.05$ [36], no pathways were found to be significantly upregulated, despite the fact that 57% of all genes in our dataset exhibited a fold change >0 . However, 41 pathways were significantly downregulated in failing hearts by these criteria (Table 2). We also evaluated these pathways for DCM vs. NF and ICM vs. NF comparisons and found comparable patterns of downregulation (data not shown). Additionally, no pathways were significantly differentially regulated between the DCM and ICM groups (data not shown). Given that the left ventricular free-wall myocardium from which RNA was isolated for determination of gene expression contains a mixture of cardiac myocytes, fibroblasts, and vascular cells, the results of this study reflect gene expression changes integrated across several important cardiac cell types.

The vast majority of downregulated pathways clustered into two broad functional categories –metabolism and cell signaling/growth, with remaining pathways related to inflammatory response, as well as cytoskeletal and membrane organization (Table 2). Cardiac remodeling in the setting of cardiac hypertrophy and heart failure progression is mediated in part by mitogen-activated protein kinase (MAPK) signaling cascades [extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs/JNK), and p38] [42,43]. Our data indicate that gene expression profiles for these signaling and growth pathways are downregulated in heart failure. This potentially represents either a later, end-stage event or negative gene regulation in response to increased activation of pathways which are regulated more at the post-translational than transcriptional level. It is possible, though, that the observed downregulated gene expression of the ERK pathway, which favors physiologic growth and protection from apoptosis, may contribute to the progression of heart failure [44]. Indeed, the ERK gene pathway was the most significantly downregulated pathway in human heart failure by the gene set enrichment analysis (Table 2), indicating that this downregulation of gene expression is a potential contributor to decreased ERK pathway activity in severe heart failure. Gene set enrichment analysis of failing human hearts in this study revealed that 14 genes in the ERK pathway form the ERK core enrichment set (ERK CES, Supplemental Table S6), driving downregulation of the ERK pathway. Members of the ERK CES include the following genes which encode key proteins of the ERK pathway three-component kinase cascade: RAF1

(Raf-1), MAP2K2 (MKK2), MAP2K1 (MKK1), and MAPK3 (ERK1). Other related pro-growth and antiapoptotic pathways demonstrated by the GSEA analysis to be downregulated with human heart failure include the insulin-like growth factor 1 receptor (IGF1R) pathway, the IGF1/MTOR pathway, the insulin signaling, and insulin receptor pathways (Table 2). Consistent with the considerable number of downregulated metabolic pathways in the failing (DCM/ICM) state, genes regulated by PGC-1 α and ERR α which are central to mitochondrial energy metabolism were also significantly downregulated ($p < 0.035$) (Table 2). Downregulation of PGC-1 α and ERR α target genes may participate in the mitochondrial dysfunction previously characterized in heart failure [16]. Concomitant downregulation in heart failure of PGC-1 α , ERR α , and ERK pathways would result in increased susceptibility for apoptosis in the failing heart, exacerbated by impaired overall mitochondrial oxidative capacity.

Of the 327 genes in the 'PGC-1 α targets' gene set which were also present in our data (Supplemental Table S7), 158 genes comprised what is termed the 'core enrichment set' (PGC-1 α target CES) based on the GSEA results (Supplemental Table S8). A core enrichment set may also be referred to as a leading edge subset [36], as it is the genes in this set that drive the statistical significance of a pathway by determining the enrichment score (Fig. 2A). Thus, the PGC-1 α target CES represents PGC-1 α responsive genes that are significantly downregulated in heart failure (Fig. 2A). Similarly, the 'ERR α targets 1' and 'ERR α targets 2' gene sets contained 70 and 104 genes [25,37] (Supplemental Tables S9 and S11), 24 and 38 of which formed the core enrichment sets (ERR α target 1 CES and ERR α target 2 CES), respectively (Supplemental Tables S10 and S12). Classification of genes in the PGC-1 α target CES, ERR α target 1 CES, and ERR α target 2 CES using GO Biological Process terms revealed marked functional overlap (Fig. 2B). A high percentage of genes in all three core enrichment sets were involved in glucose metabolism, apoptosis, and the core mitochondrial functions of fatty acid metabolism, the citric acid cycle, electron transport, and oxidative phosphorylation – pathways known to be regulated by PGC-1 α and ERR α . Additional highly represented categories in the PGC-1 α target and both ERR α target core enrichment sets included transcription/translation, protein metabolism/localization, and protein phosphorylation/signaling. In contrast to the PGC-1 α target CES, the ERR α target 1 and 2 CES contained a significant number of genes encoding cardiac contractile proteins. Further classification, using GO Cellular Component terms, demonstrated that mitochondria-associated genes comprised 50% of the PGC-1 α target CES, 26% of the ERR α target 1 CES, and 40% of the ERR α target 2 CES.

Given that diminished mitochondrial energy transduction has been linked with progression of heart failure [45,46], we investigated the relationships between PGC-1 α and ERR α target CES gene expression and left ventricular ejection fraction (LVEF), a clinical parameter decreased in systolic heart failure. We utilized linear regression models to define these potential relationships (Fig. 3). As depicted (Fig. 3A), a significant positive correlation existed between LVEF and PGC-1 α target CES mean expression (adjusted R²=0.32 and $p = 0.001$, where p refers to the F statistic of the model). The coefficients of the regression were also significant at $p < 0.01$. Parallel analyses demonstrated positive correlation between LVEF and both ERR α target 1 and 2 CES mean expression (Figs. 3B and 3C). Thus, reduced expression of PGC-1 α and ERR α target CES genes are associated with impaired left ventricular systolic function and, as such, may serve as indicators of heart failure severity.

In light of the association of PGC-1 α target CES expression with heart failure severity, we explored whether the relatively small number of genes comprising the PGC-1 α target CES was capable of independently predicting the heart failure phenotype. Utilizing a K-nearest neighbor (KNN) class prediction model, we found that expression of PGC-1 α target CES genes correctly distinguished between failing and non-failing classes perfectly (100%) in the matched

population, on which the model was trained, and in 80.9% of the unmatched population, on which the model is tested (Table 3). The unmatched population consisted of the remaining members of the total population not included in the matched population (see “Methods”). The sensitivity of the PGC-1 α target CES gene set in detecting heart failure was 96.0% in the unmatched population, suggesting that PGC-1 α target gene downregulation serves as a signature for severe cardiac dysfunction. The related ERR α target 1 and 2 CES genes similarly predicted the heart failure phenotype in the unmatched population with a sensitivity of 96.0% and 84.0%, respectively (Table 3). The specificity of the PGC-1 α target CES gene set was 58.8% in the unmatched population (Table 3), while ERR α target 1 and 2 CES genes demonstrated specificities less than 50%. Decreased specificity consistently observed in the unmatched population may reflect known increased age and gender heterogeneity of this population among the non-failing subjects (Table 1). ERK pathway CES gene set sensitivity in detecting heart failure was 88.0% in the unmatched population, indicating that the ERK pathway CES, representing the most downregulated pathway in heart failure by the GSEA analysis, is also predictive of heart failure status (Table 3). For comparison, we built a parallel model using the most significantly differentially expressed gene markers at FWER<0.05. This set contains 484 genes spanning broad functional categories in contrast to the smaller PGC-1 α target CES of 158 genes which are strongly associated with mitochondrial function. Employing these significant markers at FWER<0.05 for the unmatched population, failing vs. non-failing samples were correctly assigned in 85.7% of instances, with a sensitivity and specificity of 92.0% and 84.7%, respectively (Table 3). Overall, these results demonstrated that PGC-1 α and ERR α target CES downregulation is predictive of end-stage human heart failure, illustrating the power of this small, coordinately regulated, and focused gene network as an independent signature of a complex disease.

To investigate potential mechanisms contributing to the downregulation of PGC-1 α target CES genes, we reviewed the Comparative Marker Selection results (described earlier) for altered expression of PGC-1 α and its regulatory partners in heart failure. Despite probable decreased activity of PGC-1 α as evidenced by downregulation of PGC-1 α target genes, expression of the PGC-1 α gene itself was modestly increased by 36%, perhaps in a compensatory effort (Table 4). Gene expression of the key PGC-1 α partner ERR α was significantly reduced by 35%, consistent with the above described downregulation of ERR α target genes and potentially contributing to decreased functional activity of PGC-1 α . Similarly, we observed decreased expression of peroxisome proliferator-activated receptor δ (PPAR δ), another important PGC-1 α partner which regulates expression of genes encoding key mitochondrial proteins involved in fatty acid and glucose oxidation [47,48]. A less well characterized family member of PGC-1 α , PGC-1-related coactivator (PRC) was also significantly decreased by 53% in failing (DCM/ICM) vs. non-failing hearts. These data suggest a role for ERR α , PPAR δ , and PRC in the dysregulation of PGC-1 α target genes in heart failure.

The PGC-1 β and nuclear respiratory factor-1 (NRF-1) genes were filtered from our analysis due to absent signal, while gene expression of mitochondrial transcription factor A (Tfam) was unchanged. Expression of nuclear respiratory factor-2 (NRF-2) was increased by 29% (Table 4), another possible attempt at compensation in response to decreased expression of mitochondrial genes. Although the known PGC-1 α partner peroxisome proliferator-activated receptor γ (PPAR γ) is typically the least represented of the PPARs in the heart, its expression was increased by 24% in heart failure, while expression of the more abundant peroxisome proliferator-activated receptor α (PPAR α) was unchanged. Interestingly, a murine model of ERR α null hearts also exhibited a potentially compensatory elevation in PGC-1 α and PPAR γ gene expression [29], paralleling the above described findings in human heart failure. Consistent with previous studies of altered gene expression in the failing heart, expression of additional PGC-1 α partners, myocyte enhancing factor 2A (MEF2A) and forkhead box C2 and O1A (FOXO1A), was increased [49]. Overall, these results both confirm prior

heart-failure associated alterations in key transcription factors and propose important novel transcriptional regulatory patterns in the failing human heart which require further validation.

To begin to understand how downregulation of PGC-1 α and ERR α heart target gene expression influences the failing heart at the level of the proteome, protein levels of three PGC-1 α target genes [long-chain acyl-CoA dehydrogenase (LCAD), citrate synthase, and cytochrome c] were assessed by western blot analysis. These three mitochondrial enzymes are central to the fatty acid β -oxidation spiral, the tricarboxylic acid (TCA) cycle, and the electron transport chain, respectively. For determination of left ventricular protein levels in failing versus nonfailing hearts, cardiac tissue samples were obtained from 8 hearts with no history of myocardial dysfunction and with normal left ventricular ejection fraction by echocardiography (see “Methods”). These nonfailing hearts (from 4 men and 4 women, age range 52–69 y) were compared with failing hearts from age and gender-matched patients with idiopathic dilated cardiomyopathy. (The left ventricular ejection fraction by echocardiography was $62\pm 2\%$ for the nonfailing hearts and $17\pm 5\%$ for the failing hearts.) The protein level of the PGC-1 α target gene LCAD, which catalyzes the first reaction in the mitochondrial β -oxidation of straight-chain fatty acids, was significantly reduced by 40% in failing versus non-failing hearts (Fig. 4A). LCAD protein levels were normalized to the level of calnexin, used as a loading control (Fig. 4B). (Calnexin, an integral protein of the endoplasmic reticulum, was not regulated with heart failure.) The protein level of citrate synthase, the first enzyme of the mitochondrial TCA cycle, trended toward a reduced level in the failing left ventricles, although this was not statistically significant in this limited population (Fig. 4B). The absence of reduction in cytochrome c protein levels in the failing heart (Fig. 4B) is likely consistent with complex post-transcriptional regulatory mechanisms influencing the balance of protein synthesis and degradation. Nevertheless, marked reduction in the failing heart of the PGC-1 α target gene LCAD, a mitochondrial enzyme central to the heart’s ability to transduce energy from its dominant metabolic substrate – fatty acid, suggests that core components of mitochondria may be altered selectively in the failing heart.

4. Discussion

The ERK gene pathway, which favors physiologic growth and protection from apoptosis [44], was the most significantly downregulated pathway in human heart failure by gene set enrichment analysis in this study, with regulation at the gene expression level a consistent finding and a potential contributor to decreased ERK pathway activity in severe heart failure. Ultrasensitivity in the MAPK cascade [50] may allow changes in gene expression (and subsequent protein levels) to alter signaling of an activated MAPK pathway. Concomitant downregulation in heart failure of PGC-1 α , ERR α , and ERK pathways would result in increased susceptibility for apoptosis in the failing heart, exacerbated by impaired overall mitochondrial oxidative capacity, increased reactive oxygen species, and diminished mitochondrial membrane potential. ERK pathway core enrichment set genes driving the downregulation of the ERK pathway included RAF1 (Raf-1), MAP2K2 (MKK2), MAP2K1 (MKK1), and MAPK3 (ERK1). Raf protein kinases phosphorylate and activate mitogen-activated protein kinase kinases (MKKs), which in turn phosphorylate and activate extracellular signal-regulated kinases (ERKs) [44]. ERKs regulate cardiac cellular physiology by phosphorylating numerous substrates, including transcription factors, apoptotic proteins, and translational factors. The MKK1-ERK signaling pathway stimulates physiologic cardiac hypertrophy associated with augmented cardiac function and partial resistance to apoptosis, without inducing p38 or c-Jun N-terminal kinases 1/2 (JNK 1/2) [51]. Studies in mice have demonstrated that, in response to pressure overload, Raf-1 kinase activity is essential for cardiac hypertrophy and cardiac myocyte survival through inhibition of apoptosis [52]. ERK phosphorylation of the proapoptotic caspase-9 and BIM proteins mediates its antiapoptotic function [53,54], while Raf-1 has additional antiapoptotic targets (independent of the ERK

cascade) which include apoptosis signal-regulating kinase 1 (ASK1) and the mammalian sterile 20-like kinase 2 (MST2) [44]. Given a role for apoptosis in the failing human heart [55,56], the physiologic growth promoting and potent antiapoptotic functions of the Raf-1/MKK/ERK cascade suggest that reductions in the protein levels or activity of this pathway may sensitize patients to the development of heart failure [44,51]. In contrast, antagonism of the JNK and p38 activation associated with cardiomyopathy may be therapeutic [51]. Other related pro-growth and antiapoptotic pathways demonstrated by the GSEA analysis to be downregulated with human heart failure include the insulin-like growth factor 1 receptor (IGF1R) pathway, the IGF1/MTOR pathway, the insulin signaling, and insulin receptor pathways. Clinical studies suggest that growth hormone therapy may have beneficial effects in congestive heart failure in humans [57], while growth hormone therapy in failing rat hearts reduces oxidative stress and enhances energy metabolism [58].

This study implicates the transcriptional energy metabolic regulator PGC-1 α , along with its target genes, in the pathogenesis of human heart failure. Furthermore, regulatory mechanisms involving PGC-1 α and its key transcriptional partners, including ERR α and PPAR δ , may contribute to the mitochondrial dysfunction characteristic of the failing heart. Impaired mitochondrial function leads to insufficient energy delivery in heart failure by reducing overall mitochondrial oxidative efficiency and by altering the heart's use of its preferred energy substrate – fatty acid [16,17,19,59]. Studies in humans with pathologic left ventricular hypertrophy demonstrate not only a decrease in myocardial fatty acid uptake and oxidation but also compromised myocardial efficiency (i.e., the ratio of myocardial work to myocardial oxygen consumption) [60–62]. Moreover, the phosphocreatine-to-ATP ratio (PCr/ATP), a gauge of cellular high energy stores, is reduced in heart failure. Magnetic resonance spectroscopy has demonstrated in animals and humans that the reduction in high-energy phosphate stores in compensated pressure overload-induced cardiac hypertrophy further declines with the transition to heart failure [18,63–65]. Indeed, PCr/ATP ratios are correlated with heart failure severity as defined by New York Heart Association (NYHA) classification [66] and are strongly predictive of total and cardiovascular mortality [45,46,67].

Potential mechanisms underlying the imbalance in energy metabolism associated with pathologic cardiac hypertrophy and heart failure include reduced activity both of PGC-1 α , a critical transcriptional regulator of mitochondrial function [21,22], and of ERR α , which recruits PGC-1 α to cardiac metabolic target genes. Interestingly, mice lacking PGC-1 α exhibit decreased levels of ATP [22], as do failing canine hearts [68]. In the mouse heart, PGC-1 α is essential for maximal and efficient cardiac mitochondrial fatty acid oxidation, ATP synthesis, and lipid homeostasis, especially in the setting of increased work [23]. Loss of PGC-1 α in the mouse heart reduces mitochondrial coupling as evidenced by reduction in the maximal ATP/O ratio (ratio of ATP produced to oxygen consumed with State 3 respiration) [23]. Furthermore, hearts of mice deficient in ERR α demonstrate abnormal phosphocreatine depletion and functional decline when subjected to hemodynamic stress [30]. Our current finding of a positive correlation between PGC-1 α and ERR α target CES gene expression and left ventricular systolic function parallels the previously described relationship between the phosphocreatine-to-ATP ratio and heart failure severity, and is consistent with a role for PGC-1 α and ERR α as important regulators of myocardial energy homeostasis.

In this study, the ability of PGC-1 α and ERR α target gene expression to predict the heart failure phenotype with high sensitivity suggests that this transcriptional profile represents a signature of human heart failure. Despite the multiple biologic pathways dysregulated in the failing heart, it is notable that the PGC-1 α regulatory network, with its relatively confined metabolic focus, is so uniformly downregulated with severe cardiac dysfunction. As with any changes observed in end-stage heart failure, these alterations may reflect primary mechanisms of pathology or compensatory changes, potentially adaptive initially but maladaptive chronically. Gene

expression patterns with heart failure may also be influenced by treatment of patients with extensive pharmacologic regimens, for which limited information was available in our study population. Although logistically difficult, obtaining human cardiac tissue at different stages in the progression of heart failure may clarify a causal link between these gene expression changes, impaired cardiac energy transduction, and the evolution of heart failure. Interestingly, recent work demonstrates that polymorphisms in the PGC-1 α gene are associated with hypertrophic cardiomyopathy [69].

Despite the downregulation of PGC-1 α target genes, expression of PGC-1 α itself was minimally induced, potentially consistent with multiple complex mechanisms influencing PGC-1 α target gene expression. Given that the PGC-1 α family members, PGC-1 β and PRC, may have overlapping gene expression profiles [16,70], one such mechanism may involve reduced transcriptional activity of one or both of these family members. The PGC-1 β gene was filtered in our study due to absent signal. PRC, which also coactivates transcription factors involved in mitochondrial biogenesis including NRF-1 [70–72], was found to be significantly decreased, suggestive of a novel role for PRC in heart failure in reducing expression of nuclear respiratory factor gene targets. Further detailed knowledge of PRC regulation and its target gene set in the cardiac myocyte is necessary. The observed minimal increased expression of the PGC-1 α gene in the setting of energetic insufficiency in heart failure may be compensatory and reflective of altered signaling or energy sensing pathways involving AMP-activated protein kinase [73].

Mechanisms by which PGC-1 α target gene expression may be decreased independent of the expression of the PGC-1 α gene itself include reduced activity of PGC-1 α as a transcriptional coactivator secondary to its post-translational modification or diminished activity of its key transcriptional partners. PGC-1 α integrates input from developmental cues, fasting, and exercise, as well as calcium and stress-activated pathways, such as the p38 MAPK pathway [16]. Alterations in p38 MAPK with associated post-translational modification of PGC-1 α have been demonstrated to impact the transcriptional capacity of PGC-1 α by increasing PGC-1 α protein stability [74], promoting dissociation of a repressor [75,76], and enhancing selective activation of PPAR α [77]. Deacetylation of PGC-1 α by the NAD⁺-dependent histone deacetylase SIRT1 has also been shown to modify PGC-1 α activity [78]. Decreased gene expression and possibly decreased activity of the key PGC-1 α transcriptional partners ERR α and PPAR δ likely represent an additional mechanism negatively influencing expression of PGC-1 α target genes, including those encoding mitochondrial proteins central to fat and glucose oxidation. Indeed, both gene expression and activity of ERR α are reduced, as indirectly evidenced by ERR α target gene downregulation. Given that ERR α target genes encoding key mitochondrial proteins are enriched in nuclear respiratory factor promoters [37], decreased ERR α gene expression and activity observed in heart failure may contribute to a decrease in overlapping NRF target gene induction, despite the modest 29% increase in NRF-2 gene expression observed in this study with heart failure.

Notably, the gene encoding pyruvate dehydrogenase kinase 4 (PDK4) contributes to the PGC-1 α target core enrichment set of genes which demonstrate decreased expression in heart failure (Supplemental Table S8). This finding is consistent with prior single-gene analysis of failing hearts [4]. A negative regulator of glucose oxidation, PDK4 has previously been described as a PGC-1 α /ERR α target in skeletal muscle and provides one mechanism whereby PGC-1 α exerts reciprocal inhibition of glucose catabolism while increasing fatty acid oxidation pathways [79]. A model by which altered PGC-1 α and ERR α target gene expression contributes to energy metabolic substrate switches in the developing and failing heart is depicted in Fig. 5. Further confirmation of this model in human heart failure will require tissue based validation in the form of RT-PCR, gene promoter occupancy assays, and proteomics studies which explore regulation at the level of protein abundance and activity rather than gene expression

[80]. A previous survey of the mitochondrial proteome in various tissue types, including cardiac tissue, however, does suggest overall concordance between mRNA abundance and protein expression [81]. Indeed, the marked 40% protein level reduction in the failing heart of the PGC-1 α target gene LCAD, a mitochondrial enzyme which catalyzes the first step of the mitochondrial fatty acid β -oxidation (FAO) spiral, suggests that core components of mitochondria are altered selectively in the failing heart leading to the observed metabolic remodeling. Downregulation of LCAD protein in heart failure is consistent with the previously described downregulation in human heart failure of the mitochondrial FAO enzyme medium-chain acyl-CoA dehydrogenase (MCAD) [59]. Gene expression of both LCAD and MCAD is under cooperative control by PGC-1 α and PPAR α [82,83], and LCAD gene expression is reduced in the hearts of mice deficient in PGC-1 α [23]. Through their ability to restore appropriate energy metabolic balance in the pathologic hypertrophied and failing human heart, PGC-1 α and its key transcriptional partner ERR α may represent novel therapeutic targets in heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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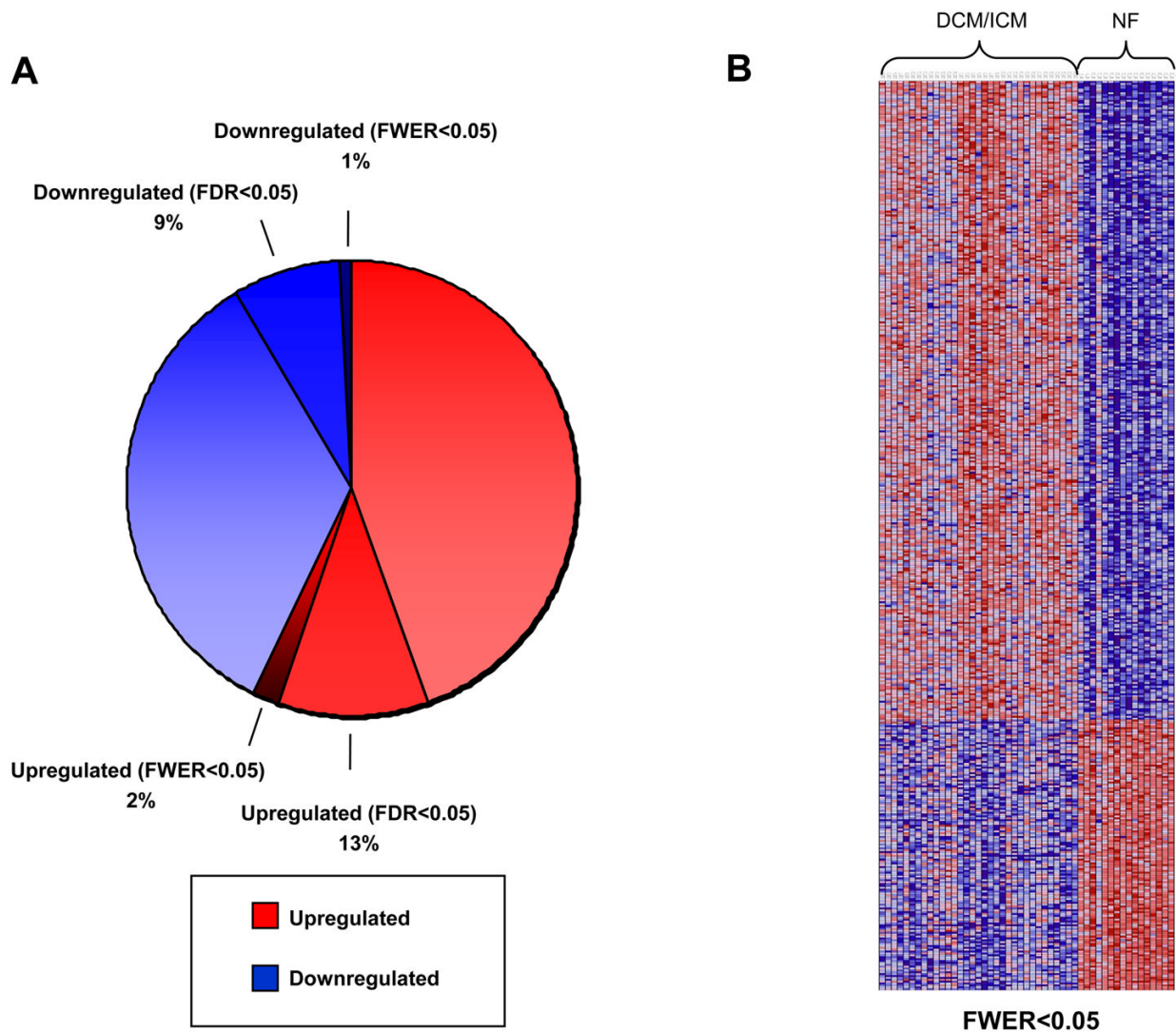
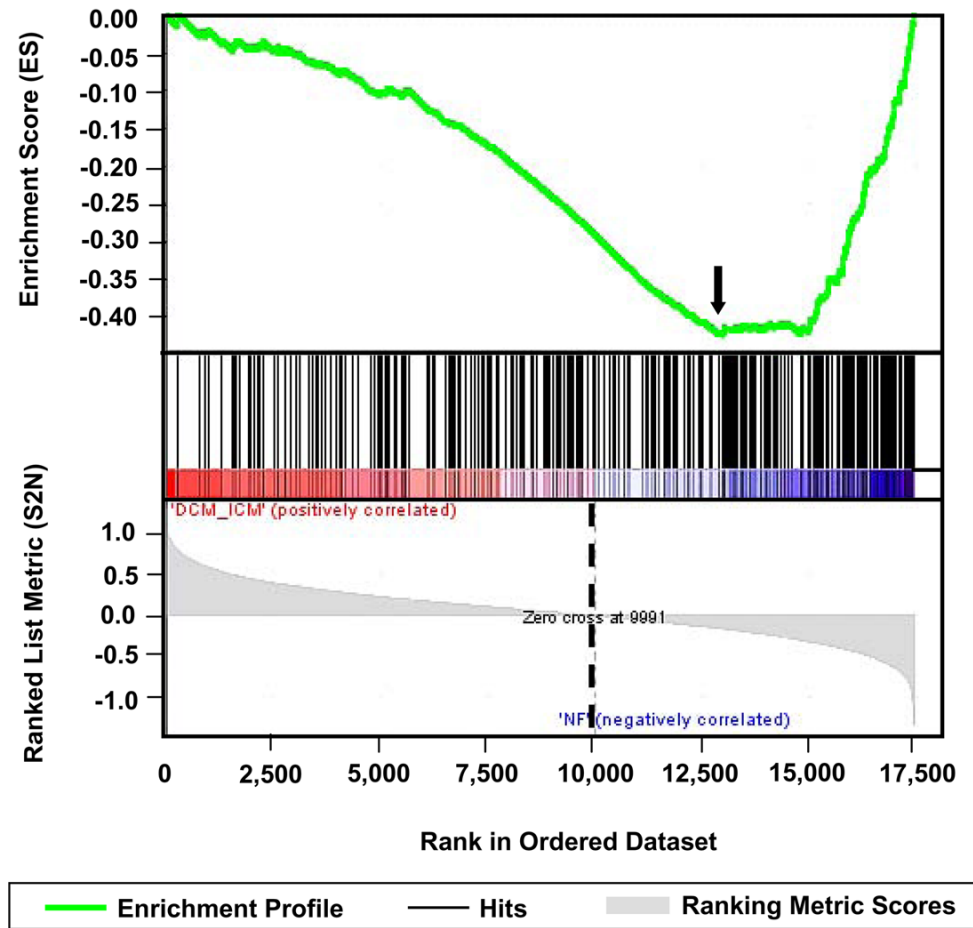
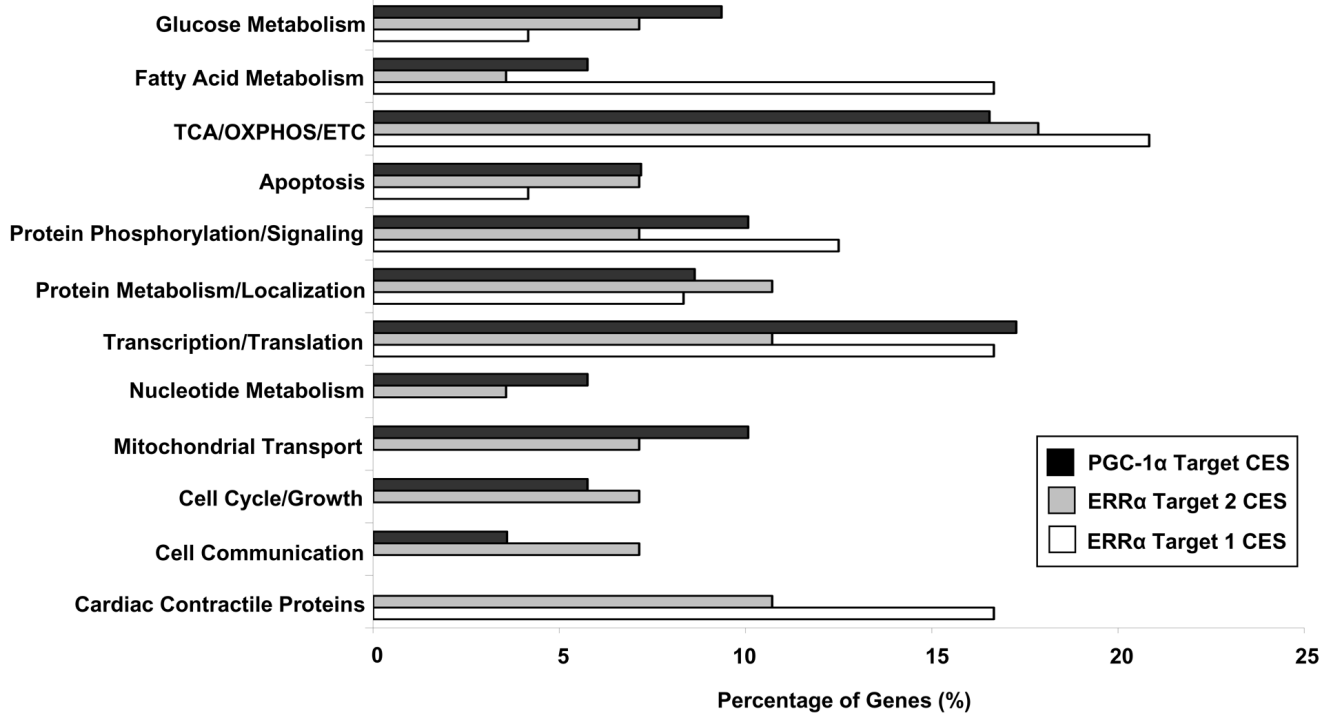


Fig. 1. Results of comparative marker selection analysis in DCM/ICM vs. NF groups. Red indicates increased expression, while blue indicates decreased expression relative to the non-failing population mean. (A) The pie chart conveys the percentage of genes up and downregulated of 17,419 total genes. Those genes that are upregulated at FWER<0.05 are a subset of those upregulated at FDR<0.05, which are in turn a subset of all upregulated genes; the same applies to downregulated genes. (B) Heatmap of genes significantly up and downregulated at FWER<0.05.

A

B**Fig. 2.**

Characterization of PGC-1 α , ERR α target1, and ERR α target 2 core enrichment sets defined by the DCM/ICM vs. NF comparison. (A) Upper plot depicts the enrichment profile in green of the 'PGC-1 α targets' pathway by Gene Set Enrichment Analysis (GSEA). The arrow denotes the point of maximal deviation of the Enrichment Score (ES) from the baseline, which is determined by genes of the 'Core Enrichment Set' (CES). Lower plot shows all 17,419 genes rank-ordered according to the signal-to-noise metric, with the dotted line demarcating genes that are positively (red) and negatively (blue) correlated with heart failure. (B) Comparative functional distributions using GO Biological Process terms of the 158 genes of the PGC-1 α target CES, the 24 genes of the ERR α target 1 CES, and the 38 genes of the ERR α target 2 CES.

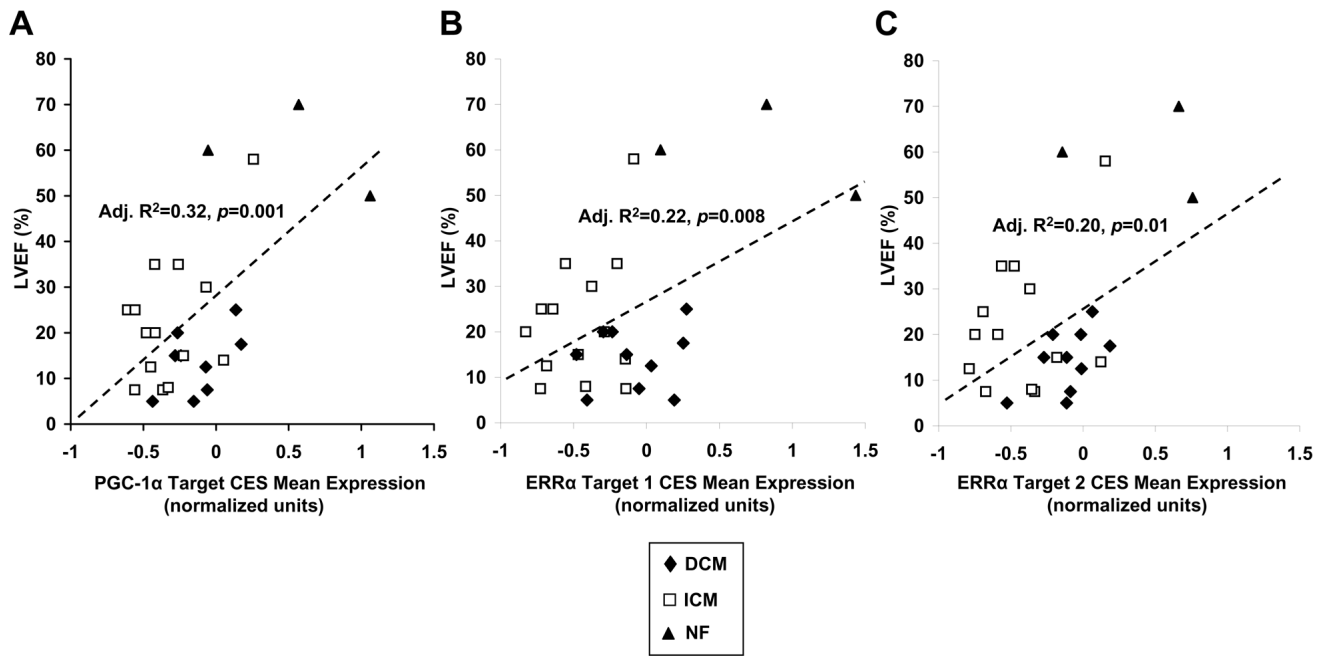


Fig. 3. PGC-1 α target, ERR α target 1, and ERR α target 2 core enrichment set (CES) mean expression correlate with severity of heart failure. Linear regression was used to model the relationship between left ventricular ejection fraction (LVEF), a marker of disease severity, with normalized, mean expression levels of genes in the PGC-1 α target CES (A), ERR α target 1 CES (B), and ERR α target 2 CES (C). The Adjusted R² and p -value of each model is shown.

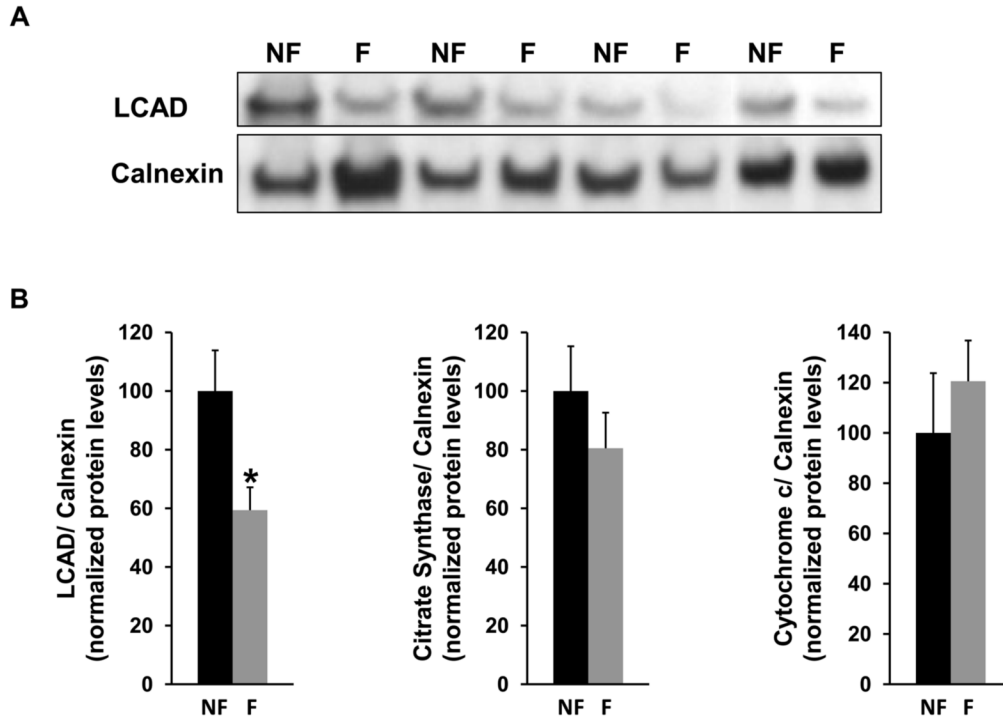
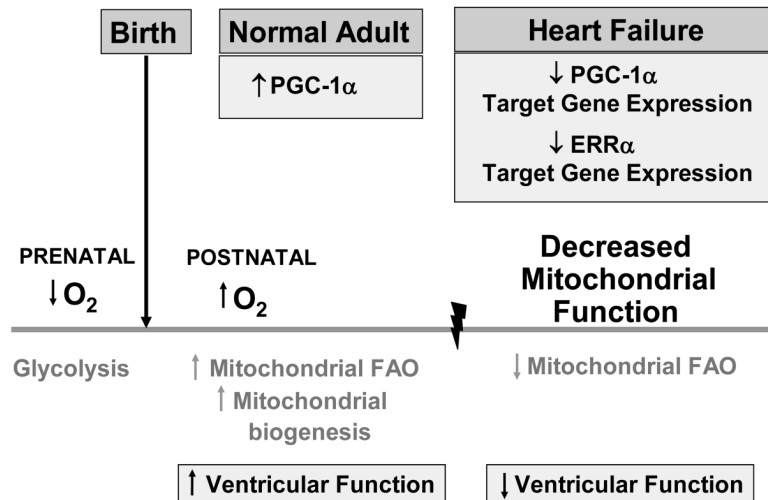


Fig. 4. Protein levels of PGC-1 α target genes in left ventricular tissue obtained from failing and nonfailing hearts. (A) Representative western blot panel depicts levels of the PGC-1 α target gene long-chain acyl-CoA dehydrogenase (LCAD) and calnexin (used as a loading control) in nonfailing (NF) versus failing (F) hearts. Failing heart tissue was obtained from the left ventricle of patients with idiopathic dilated cardiomyopathy. Patients with failing and nonfailing hearts were age and gender-matched. (B) Bar graphs depict western blot protein levels of three PGC-1 α target genes (LCAD, citrate synthase, and cytochrome c) normalized to the level of calnexin in nonfailing (NF) versus failing (F) left ventricles (n=8). * significantly different ($p < 0.05$) compared to nonfailing hearts.

**Fig. 5.**

A role for altered PGC-1 α and ERR α target gene expression in energy metabolic substrate switches in the developing and failing heart. In the setting of dietary and physiologic cues, developmentally programmed gene regulatory events facilitate the postnatal “switch” in myocardial fuel preference from glucose as the predominant source for ATP production to fatty acids metabolized via the mitochondrial fatty acid oxidation (FAO) pathway in the adult heart. Coordinate with this increased mitochondrial FAO is mitochondrial biogenesis promoted by PGC-1 α , and associated enhanced left ventricular function. With heart failure, cardiac mitochondrial function declines with changes reminiscent of fetal metabolic re-programming, concomitant with decline in PGC-1 α and ERR α target gene expression.

Table 1
 Characteristics of Total and Matched Patient Populations

	Total Population				Matched Population			
	NF	ICM	DCM		NF	ICM	DCM	
n	33	31	27		16	20	13	
Gender	19M, 14F	26M, 5F	17M, 10F		16M	20M	13M	
Age (y)	39.8 ± 3.2	52.1 ± 1.9*	46.9 ± 3.6		46.9 ± 4.1	49.5 ± 2.2	49.6 ± 4.0	
Age Range (y)	1–69	20–69	10–79		20–69	20–69	20–79	
BMI (kg/m²)	24.4 ± 1.5	25.7 ± 0.8	23.6 ± 1.1		24.6 ± 1.5	25.2 ± 1.0	23.1 ± 2.1	
HTN	18.2%	58.1%*	22.2%#		25.0%	50.0%	15.4%#	
DM	3.0%	35.5%*	22.2%*		6.3%	30.0%	15.4%	
LVEF (%)	61.3 ± 4.3	21.0 ± 2.7*	12.9 ± 1.3*##		60.0 ± 5.8	22.3 ± 3.7*	14.3 ± 2.2*	
PCWP (mmHg)	N/A	17.6 ± 1.5	23.5 ± 1.6#		N/A	17.8 ± 2.0	24.7 ± 2.6#	

M, male; F, female. HTN, hypertension; DM, diabetes mellitus; BMI, body mass index; LVEF, left ventricular ejection fraction. PCWP, pulmonary capillary wedge pressure. Age, BMI, LVEF, and PCWP are reported as mean ± S.E.M.

* Comparison with NF is significant by 2-sided Student's *t*-test or Chi-square test at $p < 0.05$.

Comparison with ICM is significant by 2-sided Student's *t*-test or Chi-square test at $p < 0.05$. N/A denotes data not available.

Table 2
Significantly Downregulated Pathways in Heart Failure by Gene Set Enrichment Analysis

Pathway	Description	FDR	p -value
ERK PATHWAY	Cell growth is promoted by Ras activation of the anti-apoptotic p44/42 MAP kinase pathway	0.103	<0.001
INTEGRIN PATHWAY	Integrins are cell surface receptors that interact with the extracellular matrix and transduce signaling	0.105	<0.001
INSULIN SIGNALING	Genes related to insulin signaling	0.136	<0.001
B CELL ANTIGEN RECEPTOR	B cell receptors bind antigens and promote B cell activation	0.166	<0.001
FMLP PATHWAY	The fMLP receptor in neutrophils recognizes bacterial peptides and activates NADPH oxidase	0.159	0.004
CCR3 PATHWAY	CCR3 is a G-protein coupled receptor that recruits eosinophils to inflammation sites via chemokines	0.131	0.006
MAPK PATHWAY	The MAP kinase pathway has four main sub-pathways: Erk, JNK/SAPK, p53, and ERK5	0.169	0.006
UCALPAIN PATHWAY	Calpains promote formation of integrin adhesion clusters which recruit Rac	0.182	0.008
PYK2 PATHWAY	Pyk2 and Rac1 stimulate the JNK cascade and activate MKK3, which activates p38	0.161	0.008
HEART FAILURE DOWNREGULATED (VENTRICLE)	Downregulated genes in the ventricles of failing hearts compared to healthy controls	0.147	0.012
GALACTOSE METABOLISM	Genes involved in galactose metabolism	0.181	0.012
ERRα TARGETS 1	Genes regulated by estrogen-related receptor α (Huss et al., Mol Cell Biol 2004)	0.213	0.012
PURINE METABOLISM	Genes involved in purine metabolism	0.165	0.013
NDK/DYNAMIN PATHWAY	NDK, Phosphins and Dynamin have a role in endocytosis	0.186	0.013
PGC-1α TARGETS	Genes regulated by peroxisome proliferator-activated receptor gamma coactivator-1α	0.197	0.014
RAS PATHWAY	Ras activation stimulates many signaling cascades, including PI3K/AKT, to inhibit apoptosis	0.166	0.015
TRANSLATION FACTORS	Genes involved in translation	0.143	0.015
ERK1/ERK2/MAPK PATHWAY	The Erk1 and Erk2 MAP kinase pathways are regulated by Raf, Mos, and Tpl-2	0.181	0.015
BCR SIGNALING PATHWAY	Members of the BCR signaling pathway	0.205	0.016
MET PATHWAY	The hepatocyte growth factor receptor c-Met stimulates proliferation and alters cell adhesion	0.187	0.017
IL2RB PATHWAY	The IL-2 receptor is required for IL-2 and IL-15 signal recognition and activates JAK kinase	0.191	0.018
NFKB PATHWAY	Active NF- κ B is localized in the nucleus and regulates transcription of a variety of genes	0.172	0.019
INSULIN RECEPTOR PATHWAY IN CARDIAC MYOCYTES	Genes related to the insulin receptor pathway in cardiac myocytes	0.164	0.021
IGF1/MTOR PATHWAY	Growth factor IGF-1 activates AKT, Gsk3-beta, and mTOR to promote muscle hypertrophy	0.197	0.024
PROSTAGLANDIN AND LEUKOTRIENE METABOLISM	Genes involved in prostaglandin and leukotriene metabolism	0.179	0.024
SPRY PATHWAY	The Sprouty protein family bind Grb-2, preventing Ras and MAP kinase activation	0.174	0.024

Pathway	Description	FDR	<i>p</i> -value
INTEGRIN MEDIATED CELL ADHESION	Genes involved in integrin-mediated cell adhesion	0.220	0.026
ACTIN CYTOSKELETON REGULATION	Genes related to regulation of the actin cytoskeleton, in particular Rho GTPases	0.220	0.027
PIP3 SIGNALING IN CARDIAC MYOCYTES	Genes related to PIP3 signaling in cardiac myocytes	0.228	0.027
FRUCTOSE AND MANNOSE METABOLISM	Genes involved in fructose and mannose metabolism	0.216	0.032
ERRα TARGETS 2	Genes regulated by estrogen-related receptor α (DuFour et al., Cell Met 2007)	0.217	0.033
cAMP CHEMOTAXIS PATHWAY	Genes involved in cytoskeletal organization during chemotaxis	0.210	0.034
TRKA RECEPTOR	TrkA receptor binds nerve growth factor to activate MAP kinase pathways and promote cell growth	0.170	0.035
ECM PATHWAY	Extracellular matrix induces integrin-mediated FAK phosphorylation, activating PI3 and MAP kinase	0.170	0.035
G13 SIGNALING PATHWAY	Genes involved in G13 signaling pathway	0.170	0.037
GLYCOLYSIS AND GLUCONEOGENESIS	Genes involved in glycolysis and gluconeogenesis	0.220	0.040
CERAMIDE PATHWAY	Ceramide is a lipid signaling molecule that can activate proliferative or apoptotic pathways	0.217	0.041
FAS SIGNALING PATHWAY	Fas receptor induces apoptosis and NF- κ B activation when bound to Fas ligand	0.227	0.042
IGF1R PATHWAY	IGF-1R promotes cell growth and inhibits apoptosis via Ras activation and the AKT pathway	0.224	0.042
MTOR PATHWAY	Target of rapamycin, mTOR senses mitogenic factors and nutrients and induces cell proliferation	0.213	0.043
EGF PATHWAY	Epidermal growth factor promotes cell proliferation via the MAP kinase and Ras pathways	0.241	0.048

Significance is defined by FDR<0.25 and *p*<0.05. Pathways are ordered by *p*-value. Descriptions are based on those provided in MSigDb.

Table 3
PGC-1 α , ERR α , and ERK CES genes predict Failing vs. Non-failing Phenotypes

		Unmatched (Test) Population	Matched (Training) Population
PGC-1α Target CES	Correct/Total	34/42 (80.9%)	49/49 (100%)
	Sensitivity	96.0%	
	Specificity	58.8%	
ERRα Target 1 CES	Correct/Total	32/42 (76.2%)	49/49 (100%)
	Sensitivity	96.0%	
	Specificity	47.1%	
ERRα Target 2 CES	Correct/Total	26/42 (63.4%)	49/49 (100%)
	Sensitivity	84.0%	
	Specificity	29.4%	
ERK Pathway CES	Correct/Total	31/42 (73.8%)	49/49 (100%)
	Sensitivity	88.0%	
	Specificity	52.9%	
Significant Markers (FWER<0.05)	Correct/Total	36/42 (85.7%)	49/49 (100%)
	Sensitivity	92.0%	
	Specificity	76.5%	

Results of KNN prediction models using the PGC-1 α target, ERR α target 1, ERR α target 2, and ERK pathway CES genes vs. the most significantly differentially expressed markers at FWER < 0.05 are shown, including the ratio of samples correctly assigned by the model, as well as sensitivity and specificity. CES, 'core enrichment set' by Gene Set Enrichment Analysis.

Table 4
Degree of Differential Expression of PGC-1 α and Key Transcriptional Partners

Gene	Symbol	Fold Change	FDR
Peroxisome proliferator-activated receptor gamma coactivator 1α	PPARGC1A	1.36	0.013
Peroxisome proliferator-activated receptor gamma coactivator 1 β	PPARGC1B	Filtered	
Peroxisome proliferator-activated receptor gamma coactivator-related 1	PPRC1	-1.53	0.013
Nuclear respiratory factor 1	NRF1	Filtered	
Nuclear respiratory factor 2	NRF2/GABPA	1.29	0.013
Mitochondrial transcription factor A	TFAM	1.05	0.558
Estrogen-related receptor α	ESRRA	-1.35	0.013
Estrogen-related receptor β	ESRRB	1.09	0.743
Estrogen-related receptor γ	ESRRG	-1.09	0.480
Peroxisome proliferator-activated receptor α	PPARA	1.11	0.275
Peroxisome proliferator-activated receptor β/δ	PPARB/D	-1.35	0.034
Peroxisome proliferator-activated receptor γ	PPARG	1.24	0.013
Myocyte enhancing factor 2A	MEF2A	1.16	0.028
Myocyte enhancing factor 2B	MEF2B	1.06	0.369
Myocyte enhancing factor 2C	MEF2C	-1.01	0.957
Myocyte enhancing factor 2D	MEF2D	1.24	0.166
Forkhead box C1	FOXC1	1.32	0.263
Forkhead box C2	FOXC2	1.22	0.040
Forkhead box P1	FOXP1	-1.07	0.336
Forkhead box P4	FOXP4	1.03	0.789
Forkhead box O1A	FOXO1A	1.22	0.021
Menage-a-trois 1	MNAT1	Filtered	

Comparison for differential expression is DCM/ICM vs. NF. Fold change is positive if upregulated, negative if downregulated. Those genes with an FDR<0.05 by Comparative Marker Selection are highlighted in bold.