Physiological genomics of cardiac disease: quantitative relationships between gene expression and left ventricular hypertrophy

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Mirotsou, Maria, Victor J. Dzau, Richard E. Pratt, and Ellen O. Weinberg. Physiological genomics of cardiac disease: quantitative relationships between gene expression and left ventricular hypertrophy. Physiol Genomics 27: 86–94, 2006. First published July 11, 2006; doi:10.1152/physiolgenomics.00028.2006.—The pathogenesis of cardiac left ventricular hypertrophy and failure is poorly defined due to the complexity of the disease phenotype. To gain a better understanding of the relationship between gene expression and left ventricular hypertrophy, we employed a quantitative approach to identify genes with expression patterns that correlate in a numerically continuous manner with parameters of cardiac structure and function in a mouse model of left ventricular hypertrophy due to transverse aortic constriction. Several genes showed expression patterns that were significantly correlated (Pearson’s correlation coefficient) with measurements of left ventricular weight, left ventricular wall thickness, and diastolic dimension. We validated our findings in two independent data sets and in a small subset of genes by real-time RT-PCR. Of genes with significant correlations to numerically continuous measurements of hypertrophy, we found enrichment for genes encoding extracellular matrix, growth-related and secreted proteins in the directly correlated subset, and for genes encoding mitochondria and metabolic/fatty acid oxidation proteins in the inversely correlated subset. The results of this filtering strategy suggest that this subset of transcripts with quantitative relationships between gene expression and left ventricular hypertrophy represents potentially important pathways that contribute to the progression to heart failure and are thus candidates for follow-up and functional analysis.

cardiac remodeling; hemodynamic overload; physiological parameters; Pearson correlation analysis; mouse model

PRESSURE OVERLOAD ON THE HEART resulting from chronic hypertension or aortic stenosis is a stimulus for left ventricular hypertrophy (LVH), an adaptive growth response to the increase in hemodynamic burden. In adaptive cardiac hypertrophy, the heart exhibits a pattern of remodeling characterized by increased muscle mass and left ventricular wall thickness accomplished by increased width (and not length) of individual cardiac myocytes. Left ventricular chamber diameter and systolic performance are usually preserved. If the stimulus for hypertrophy is persistent and severe, adaptive hypertrophy eventually progresses to decompensation and heart failure, a syndrome in which the left ventricle dilates and systolic contractile performance is diminished.

The process of adaptation to increased hemodynamic load and the progression to decompensation and heart failure are accomplished by activation of multiple pathways involving growth factors, regulators of cell cycle and cell death, cytokines, kinases and phosphatases, extracellular matrix proteins, contractile proteins, transcription factors, as well as several pathways still to be identified (5). Identification of novel pathways that participate in the adaptation to pressure overload is an important step toward the development of new therapeutic strategies aimed at preventing or delaying the progression to overt heart failure.

The application of DNA microarrays to animal models of LVH allows parallel monitoring of hundreds of genes to dissect and characterize the complex cardiac hypertrophy phenotype (1, 12, 19, 26). Most genomic studies have compared differential gene expression between two conditions (i.e., normal vs. diseased) to detect altered gene expression in a binary manner. Although highly informative, this approach can identify hundreds of genes with no clear definition of their association to the hypertrophic phenotype. A potentially more informative approach is to correlate the levels of gene expression with quantitative physiological parameters in a numerically continuous, rather than binary, manner. The advantage of such a process is revealed when applying additional levels of filtering to identify gene changes that are associated with defined functional endpoints.

In this study, we employed this quantitative approach to identify genes with expression patterns that correlate [Pearson’s correlation coefficient (CC)] with parameters of cardiac structure and function in LVH. Pressure overload LVH was achieved in mice by surgical transverse aortic constriction at the level of the aortic arch between the carotid arteries and hearts were studied during the adaptive, compensatory phase of LVH. Low stringency filtering first identified genes that were up- or downregulated in response to pressure overload. Expression profiles of this subset of load-responsive genes were then tested for significant correlations with measurements of left ventricular weight/body weight (LVW/BW, an index of cardiac hypertrophy), posterior left ventricular wall thickness (PWT), anterior left ventricular wall thickness (AWT), fractional shortening (FS, an index of cardiac systolic contractile function) and end-diastolic dimension (EDD, an index of left ventricular chamber size and dilation). We identified several genes that showed significant correlations to geometric and functional parameters of cardiac hypertrophy. To provide validation of our findings against an independent data set we queried these genes in the CardioGenomics Programs for Genomic Applications (PGA) database (http://cardiogenomics.med.harvard.edu/home), which is a National Institutes of Health (NIH)-funded PGA genomic profile database of left
ventricular samples at several time points during the development of LVH due to ascending aortic banding in mice.

Notably, a large number of the transcripts that showed strong direct correlations to the level of LVH were extracellular matrix and cell growth-related genes, whereas those that showed high significant inverse correlations to the level of LVH were mitochondrial and metabolic genes. This is one of the few studies to define quantitative relationships between gene expression and LVH at the genomic level and has led to the identification of novel candidate transcripts and molecular pathways relevant to the LVH phenotype.

**Materials and Methods**

**Animal cohorts.** Gene expression profiles from three separate mouse cohorts were analyzed in the present study.

The first cohort (cohort 1) consisted of 15 mice of the FVB strain (Jackson Laboratories) from our published study (26) that had undergone transverse aortic banding surgery ($n = 3$ female and $n = 3$ male) to create LVH or sham surgery ($n = 3$ female and $n = 6$ male) at 8 wk of age. For aorta-banded animals, the transverse aorta was banded at the level of the aortic arch between the carotid arteries as described (16). Sham-operated animals underwent the identical procedure without placement of the ligature around the aorta. At 30 wk after surgery, mice were characterized by echocardiography and microarray analysis ($n = 6$ aorta banded and $n = 9$ sham-operated mice). Parameters of cardiac structure and function in LVH were determined by echocardiography and were correlated to gene expression from microarray analysis. Briefly, echocardiography was performed with spontaneous respiration under light anesthesia with intraperitoneal pentobarbital to maintain a heart rate >400 beats/min. Short-axis two-dimensional images using an 8- to 12-MHz transducer placed at the midpapillary level of the LV were stored as digital loops (26). Parameters are as follows: 1. LV mass measured by echocardiography (LVW echo/BW) increases as a compensatory response to pressure overload; 2. AWT and PWT: both increase in compensatory hypertrophy; 3. EDD: a measure of left ventricular cavity dimension relative to left ventricular wall thickness; FS: a measure of systolic function, and left ventricular wall thickness; EDD: a measure of systolic function, and left ventricular wall thickness.

The second cohort (cohort 2) consisted of 18 mice of the FVB strain (Jackson Laboratories) generated in our laboratory and studied 10 wk after transverse aortic banding surgery ($n = 4$ female and $n = 3$ male) or sham surgery ($n = 6$ female and $n = 5$ male) performed at 8 wk of age, characterized by echocardiography as described above for cohort 1 and used for real time validation of transcripts identified by cohort 1.

The third cohort (cohort 3) was an independent cohort provided by CardioGenomics/PGA/NIH (http://cardiogenomics.med.harvard.edu/public-data) and comprised 36 mice of the FVB strain (Charles River Laboratories) that underwent ascending aortic banding or sham surgery at 12 wk of age. Mice were characterized by degree of cardiac hypertrophy (by echocardiography and postmortem heart weight/body weight) at six time points after surgery (1 h, 4 h, 24 h, 48 h, 1 wk, 8 wk; $n = 3$ aorta-banded and $n = 3$ sham-operated mice/time point) and microarray analysis. Details of the surgical procedure and data analysis are provided at (http://cardiogenomics.med.harvard.edu/public-data). This cohort was used to validate our findings in an independent, publicly available data set performed by another laboratory.

In studies performed in our laboratory (cohorts 1 and 2), RNA was isolated from the same location of the left ventricle (left ventricular free wall) to avoid possible regional differences in gene expression throughout the left ventricle. The anatomic location of the aortic banding differed between cohorts generated in our laboratory (cohorts 1 and 2) and the cohort generated by CardioGenomics, but both surgical techniques produce pressure overload on the left ventricle. In all three cohorts, the FVB strain of mouse was used. The FVB mouse strain is a well-characterized, widely used inbred strain that is genetically homozygous at virtually all loci having undergone at least 20 generations of inbreeding (see http://www.jax.org). The use of the same inbred strain in all three cohorts of mice avoided variability in microarray results due to genetic background differences.

**Data analysis.** Microarray data generated from cohort 1 were used for the analysis of hypertrophy and echocardiographic parameters 30 wk after aortic banding or sham surgery. A separate analysis of these data has been published (26). We previously showed that time after aortic banding (acute, 1 day, vs. chronic, 30 wk after aortic banding) influences the genomic response to pressure overload (26). We also previously observed sex differences in the genomic response to left ventricular pressure overload that were more pronounced in samples studied 1 day (acute pressure overload) than at 30 wk (chronic pressure overload) after aortic banding (26). We did not observe sex differences in physiological parameters in samples studied 30 wk after aortic banding (chronic LVH), which comprise cohort 1 in the present study. The focus of the present analysis was to identify genes with expression levels that correlate to physiological parameters; thus we did not perform separate analyses between males and females in the present study.

Eight micrograms of RNA from individual left ventricular samples were hybridized to Affymetrix Oligonucleotide Murine Genome U74A Array version 2 (MG-U74Av2) array chips, which contain ~12,483 known genes and expressed sequence tags. Expression data were normalized using the Invariant Set normalization method as recommended on the dChip software (11) and then subjected to model-based expression during which all negative values were truncated to 0. These values were then used for t-test and the subsequent linear correlation analysis. The data derived from a microarray experiment are in arbitrary units and represent the relative fluorescence detected for the specific probe sets. Additional details of the hybridization and validation of the quality of the RNA and of the data are published (26). These data have been submitted to the Gene Expression Omnibus repository under series number GSE2459. Microarray data generated from cohort 3 were used for the analysis of hypertrophy in a time course up to 8 wk after aortic banding or sham surgery. These data are available in the public domain (http://cardiogenomics.med.harvard.edu/groups/proj1/pages/hand_phys.html) and were used to provide independent validation of our data from cohort 1.

An analysis overview is presented in Fig. 1. Student’s t-test analysis was applied to identify transcripts from cohort 1 with significantly different expression levels in aorta-banded vs. sham-operated animals 30 wk after surgery at the level of $P < 0.05$. The transcripts identified in this low stringency binary screen were then subjected to significance analysis of microarrays (SAM) software analysis, a statistical technique for finding significant genes in a set of microarray experiments (24). The input to SAM software was gene expression measurements from each of the identified transcripts across all 15 animals, as well as a response variable from each animal. The response variables were the quantitative physiological variables: LVW/BW, EDD, AWT, PWT, and FS. SAM computes a statistic, $di$, for each gene, $i$, measuring the strength of the relationship between gene expression and the response variable ($di$). It uses repeated permutations of the data to determine whether the expression of any of the genes is significantly related to the response. The cutoff for significance is determined by a parameter delta, chosen by the user based on the false positive rate (% P value). The delta for this analysis was set at 1.2 and generated data that showed false positive detection rates between 0.01 and 2.5%. The genes that passed these criteria were then subjected to Pearson’s correlation analysis to identify transcripts with a high correlation with each of the responses queried ($CC > 0.641$, $P < 0.01$). The analysis then focused on transcripts that showed high associations with at least three of the four measurements of wall thickness (LVW echo/BW, LVW wet/BW AWT, and PWT). To further ensure that our analysis was targeted only to genes that showed true significant associations with parameters of heart phys-
ology relevant to hypertrophy, the Pearson’s t-test ($P < 0.01$) was applied. Across all 15 samples the gene expression level (gene expression levels were not transformed) of each of the genes identified by SAM analysis was tested for significant correlations with LVW echo BW, LVW wet BW, AWT, PWT, and EDD measurements. This approach excluded genes that can obscure true positives by increasing false positive detection errors (18).

Real-time RT-PCR was applied to RNA extracted from left ventricular samples obtained from cohort 2 to test for validation of 10 genes.

Data from cohort 3 (CardioGenomics database) was next queried for expression of these transcripts across all the samples (1 h, 4 h, 24 h, 48 h, 1 wk, 8 wk post surgery). 80 transcripts present 59 showed significant correlation in at least one time point

Validation in an independent microarray database with samples at 1h, 4h, 24h, 48h, 1 wk, 8 wk post surgery. 80 transcripts present 59 showed significant correlation in at least one time point

Validation by real time RT-PCR of 10 transcripts in an independent data set: 10 weeks after aortic banding or sham surgery. 10 out of 10 genes showed significant correlations

Real-time RT-PCR (RT-PCR) reactions were carried out in iCycler IQ Real-Time Detection Systems (Bio-Rad). SuperScript One-step RT-PCR with Platinum Taq kits (Invitrogen) were used for all RT-PCR amplification in a total volume of 50 μL, which contained 200 ng total RNA, 5 mM MgSO4, 500 nM forward and reverse primers, and 200 nM fluorogenic probe. RT-PCR amplification for each RNA sample was performed in triplicate. One No-RT (without reverse transcriptase) control for each RNA sample and one No-RNA (substitute RNA with deionized H2O) control for each primer and probe set were also performed. The one-step RT-PCR condition is as follows: 15 min at 50°C and 5 min at 95°C, followed by a total of 45 two-temperature cycles (15 s at 95°C and 1 min at 60°C). Relative gene expression analysis was carried out using the standard curve method according to the manufacturer (Bio-Rad). Expression values from real-time RT-PCR analysis were correlated with echocardiography measurements from cohort 2.

RESULTS

Summary statistics for characterization of LVH for cohort 1 are shown in Table 1. Individual values have been published (26). At 30 wk after surgery, aorta-banded mice developed significant LVH. All parameters of LVH used for correlations of gene expression were significantly increased in aortic banded vs. sham-operated mice. Of note, FS was similar between aorta-banded vs. sham-operated mice, indicating that systolic function was preserved.

Initial t-test analysis of microarray gene expression from cohort 1 identified 548 load-responsive transcripts that were differentially expressed between aorta-banded vs. sham-operated mice at the level of significance of $P < 0.05$ (Fig. 1). This filtering step identified hypertrophy-related genes, and only these genes were used in further analysis. To identify hypertrophy-related genes that showed significant correlations with quantitative

**Table 1. Summary statistics for aorta-banded and sham-operated mice studied 30 wk after surgery (cohort 1)**

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>LV Wet Weight, g</th>
<th>LV Wet Weight/BW, mg/g</th>
<th>Echo LV Weight, g</th>
<th>Echo LV Wt/BW, mg/g</th>
<th>PWT, mm</th>
<th>AWT, mm</th>
<th>EDD, mm</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta-banded ($n = 6$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>28.8</td>
<td>0.135</td>
<td>4.7</td>
<td>0.150</td>
<td>5.2</td>
<td>1.173</td>
<td>1.285</td>
<td>3.218</td>
<td>56.9</td>
</tr>
<tr>
<td>SE</td>
<td>0.9</td>
<td>0.006</td>
<td>0.3</td>
<td>0.010</td>
<td>0.4</td>
<td>0.040</td>
<td>0.030</td>
<td>0.056</td>
<td>3.9</td>
</tr>
<tr>
<td>Sham-operated ($n = 9$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>29.3</td>
<td>0.093</td>
<td>3.2</td>
<td>0.097</td>
<td>3.3</td>
<td>0.983</td>
<td>1.098</td>
<td>2.833</td>
<td>60.0</td>
</tr>
<tr>
<td>SE</td>
<td>0.9</td>
<td>0.003</td>
<td>0.1</td>
<td>0.004</td>
<td>0.1</td>
<td>0.026</td>
<td>0.039</td>
<td>0.056</td>
<td>2.3</td>
</tr>
<tr>
<td>$P$</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>0.004</td>
<td>0.004</td>
<td>0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean and SE values are given for aorta-banded and sham-operated mice. Data were analyzed by Student’s t-test and a $P$ value $<0.05$ was considered significant. BW, body weight; LV, left ventricle; PWT, posterior wall thickness; AWT, anterior wall thickness; EDD, end-diastolic dimension; FS, fractional shortening; NS, not significant. Echo LV weight is by echocardiography. Data were summarized from individual values in Table 1 in Ref. 25.
physiological measurements, both the quantitative response function in SAM software (24) and Pearson’s correlation analyses were used. The input to SAM was gene expression measurements from each of the 548 transcripts across all 15 animals, and each of the quantitative physiological variables (LVW echo/BW, LVW wet/BW, EDD, AWT, PWT, and FS). For FS, we were unable to detect any correlated genes using these criteria; therefore, fractional shortening was not analyzed further. This was expected since fractional shortening was similar between aorta-banded and sham-operated mice.

Overall, 176 transcripts showed high correlations with LVW wet/BW, 142 transcripts with LVW echo/BW, 116 transcripts with PWT, 92 transcripts with AWT, 65 transcripts with EDD and 0 with FS. As stated above, the analysis focused on transcripts that showed high associations with at least three of the five measurements of cardiac hypertrophy (LVW echo/BW, LVW wet/BW AWT, PWT, and EDD) and therefore had high probability of being functionally associated with the hypertrophic phenotype. Ninety-five transcripts passed this criterion (Supplemental Table 1; the online version of this paper contains supplemental material). Of these 95 genes, 73 were directly (positive Pearson’s r) correlated, whereas 22 were inversely (negative Pearson’s r) correlated with parameters of LVH. Genes with functions related to cell growth, morphology, differentiation, and extracellular matrix activity dominated the group of positively correlated genes, whereas genes with inverse correlations to parameters of hypertrophy were largely genes with functions related to fatty acid metabolism and mitochondria.

The 95 transcripts in Supplemental Table 1 were sorted in descending order of their correlations to LVW wet/BW (highest correlation to lowest correlation) and are shown with their corresponding correlations for the other parameters. Furthermore, Supplemental Table 1 is annotated to highlight the physiological parameter with the highest correlation for each gene (pink) and the lowest correlation for each gene (blue). With a few exceptions, LVW wet/BW, LVW echo/BW, and PWT showed the highest correlations for the most number of genes compared with AWT and EDD.

Genes encoding secreted proteins are highlighted in bold text. Transcripts for 17 secreted proteins, many of which are novel for an association with cardiac hypertrophy, showed significant, high positive correlations with parameters of LVH, which has clinical relevance for investigation of their use as biomarkers in cardiovascular diseases such as cardiac hypertrophy and failure. One gene encoding a secreted protein, amyloid β-preursor protein-binding-1 (Apbb1), showed high negative correlation for LVH.

The genes with the highest correlation for each of the five physiological (LVW wet/BW, LVW echo/BW, PWT, AWT, EDD) are highlighted in dark pink. The gene with the highest correlation for LVW wet/BW and LV echo mass/BW is the well-established clinical hypertrophic marker, atrial natriuretic peptide (ANP), a protein secreted from the heart in response to an increase in load. Apbb1 had expression levels with the highest negative correlation to LVW wet/BW, which, like ANP, may have clinical usefulness as a biomarker.

EDD, when elevated in pressure-overloaded ventricles in the absence of systolic dysfunction, may herald the progression to heart failure; thus it is informative to identify genes with the highest correlation to it. The gene with the highest correlation to EDD was peristin (osteoblast specific factor, r = 0.861), and the gene encoding a secreted protein with the highest correlation to EDD was biglycan (r = 0.847, highlighted in orange) followed by ANP (r = 0.826), both of which also showed the highest correlations to LV wet weight/BW and LV echo mass/BW. The gene with the highest negative correlation to EDD was the mitochondrial gene, isocitrate dehydrogenase-2 (r = −0.861).

Representative correlation patterns for nine of the 95 transcripts (four with positive correlation and five with negative correlation) are shown in Fig. 2. These genes are highlighted in purple in Supplemental Table 1.

Validation by relative quantitative real-time RT-PCR. To validate our observations in an independent cohort of animals, we performed real-time RT-PCR for 10 of the genes listed in Supplemental Table 1 using RNA extracted from cohort 2. We selected genes for validation by real-time RT-PCR from functional classes that were highly represented in the positively correlated genes (extracellular matrix, adhesion, cell growth) and negatively correlated genes (fatty acid metabolism, mitochondrial). Three of the genes selected for validation by RT-PCR encode secreted proteins (fibulin, biglycan, follastatin-like 1). In cohort 2, stable concentric hypertrophy was evident in aorta-banded mice compared with sham-operated mice (Table 2). All parameters of hypertrophy were significantly higher in aorta-banded mice compared with controls with the exception of EDD. The finding that EDD was not elevated in this cohort likely reflects the earlier time point at which cohort 2 was studied: the first sign of a transition to heart failure was not yet present in the measurement of EDD (increased LV cavity size). Expression levels of all 10 genes, six with positive correlations and four with negative correlations, were significantly correlated to LVW wet/BW; eight genes were significantly correlated to three out of four parameters, and two genes showed a significant association with two measurements (AWT and LVW wet/BW). Real-time RT-PCR validation is shown in Table 3. These genes are highlighted in yellow in Supplemental Table 1. These quantitative RT-PCR results in cohort 2 validate our filtering criteria applied to cohort 1.

Meta-analysis using the CardioGenomics database. To validate our findings in a cohort of aorta-banded and sham-operated mice generated in another laboratory and to follow the natural history of correlations of the identified genes with cardiac remodeling in a time-dependent manner following pressure overload, we used the PGA CardioGenomics database (cohort 3). Gene expression of was analyzed for an association with postmortem heart weight/body weight for each sample and each time point (n = 6/group at 1 h, 4 h, 24 h, 48 h, 1 wk, and 8 wk after surgery). Heart weight/body weight was significantly greater 24 h, 48 h, and 1 wk after aortic banding, and there was a trend for an increase at 8 wk compared with sham-operated mice (Fig. 3, left; data available at website). Body weight was similar in both groups with the exception of 24 h when it was slightly, but significantly, lower in aorta-banded mice compared with controls (Fig. 3, right).

Of the 95 transcripts that we identified, 80 were found in the CardioGenomics database, which were then subjected to correlation analysis as described above. The SAM delta parameter, set at 0.5, generated data that showed high Pearson’s CC for each time point (CC > 0.811, P < 0.05). In detail, 59
transcripts showed CC = 0.811, \( P < 0.05 \) and 41 showed CC = 0.917, \( P < 0.01 \). In an effort to determine the patterns of correlation during the progression of hypertrophy, the CC values for each gene and each time point were subjected to clustering analysis (Fig. 4). At 1 and 4 h postaortic banding, none of the genes were correlated with heart weight/body weight based on the above filtering criteria. From 24 h and later, a large number of genes were highly associated with heart weight/body weight. Overall, 59 out of the 80 transcripts were highly correlated with at least one of the time points (24 h, 48 h, 1 wk, and 8 wk, \( P < 0.05 \)), 35 of which showed CC values of \( >0.9 \) (\( P < 0.01 \)).

In an effort to determine the patterns of correlation and expression during the progression of the hypertrophic phenotype in the 35 genes that passed through the filtering steps, the correlation coefficient values for each gene of the 35 transcripts were subjected to clustering. As shown in Fig. 4, two major clusters consisted of genes that were positively (cluster A) and negatively (cluster B) correlated with heart weight/body weight from 24 h onward. Cluster A was enriched for extracellular matrix, cell adhesion genes with \( >50\% \) encoding secreted proteins, whereas cluster B was enriched for energy metabolism/mitochondria genes. Total concordance was observed between the CardioGenomics data vs. our data from \textit{cohort 1} for genes with positive correlations. For genes with negative correlations, we identified four genes to be positively correlated to hypertrophy (UDP-glucose pyrophosphorylase 2, decorin, carboxypeptidase E, carboxypeptidase X2), whereas CardioGenomics data observed them to be negatively correlated to hypertrophy. This discrepancy may be due to the small sample size of CardioGenomics \( (n = 3 \text{ aorta-banded, } n = 3 \text{ sham mice/time point}) \), with higher variability in the degree of hypertrophy at 1 and 8 wk (Fig. 3, \textit{left}), and due to the fact that heart weight/body weight missed being significant at 8 wk (\( P = 0.13 \)). Expression levels of these four genes appeared to be decreased in the cluster at 24 and 48 h (Fig. 4) but were increased or unchanged at later time points.

**DISCUSSION**

In the present study, genomic profiles of heart tissue isolated from individual mice with pressure overload LVH due to transverse aortic banding were analyzed quantitatively with cardiac physiological measurements. First, we identified by low-stringency binary screening a subset of chronic pressure overload-responsive genes consisting of 548 out of 12,483 genes (4.4%). These 548 genes were tested for significant correlations to parameters of LVH. This quantitative approach identified 95 transcripts (<1% of represented genes) that significantly correlated positively or negatively with measurements of LV geometry and size relevant to LVH. Of those, we validated 70% by evaluating their expression in an indepen-
dent, publicly available database generated in a study funded by NIH/National Heart, Lung, and Blood Institute (NHLBI) (9) that provided gene expression profiles at various time points in the progression of LVH due to ascending aortic banding. In addition, we validated 10 of these transcripts using quantitative real-time RT-PCR in a separate cohort of mice with pressure overload hypertrophy performed in our laboratory as an additional validation of our observations. By employing these different methodologies, we reduced some of the pitfalls of microarray analysis such as high false positive detection rates, and sample heterogeneity. Moreover, by correlating levels of expression of a subset of transcripts most sensitive to cardiac hypertrophic growth, we demonstrated that microarray analysis such as high false negative detection rates, and sample heterogeneity.

The approach taken in the present study contrasts with the approach in our previous study (26) in which we distinguished the molecular phenotype of acute vs. chronic left ventricular pressure overload and determined sex differences in these responses. The genomic response to acute pressure overload involved a greater number of regulated genes compared with chronic pressure overload. Furthermore, sex differences in the genomic response to acute pressure overload were marked comparing with during chronic pressure overload. The approach taken in the present study, numerical correlation of gene expression to physiological phenotypic variables, revealed transcripts most sensitive to cardiac hypertrophic growth, many of which are novel transcripts that were not identified in our prior study. This demonstrates that microarray analysis performed with validated DNA microarrays combined with detailed physiological measurements can be a rich data mine to address more than one question or thesis.

Table 3. Real-time validation of transcripts with strong correlation to cardiac hypertrophy at 10 wk

<table>
<thead>
<tr>
<th>Matrix Genes</th>
<th>Fbn2</th>
<th>Bgn</th>
<th>Osf2</th>
<th>Enah</th>
<th>Fhl1</th>
<th>Fstl</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>14</td>
<td>16</td>
<td>17</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>AWT</td>
<td>0.526</td>
<td>0.56</td>
<td>0.696</td>
<td>0.551</td>
<td>0.618</td>
<td>0.476</td>
</tr>
<tr>
<td>PWT</td>
<td>0.506</td>
<td>0.528</td>
<td>0.416</td>
<td>0.516</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>LVW Echo/BW</td>
<td>0.407</td>
<td>0.588</td>
<td>0.437</td>
<td>0.605</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVW wet/BW</td>
<td>0.632</td>
<td>0.59</td>
<td>0.689</td>
<td>0.53</td>
<td>0.651</td>
<td>0.612</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolic Genes</th>
<th>Ech1</th>
<th>Dec1</th>
<th>Decr1</th>
<th>Acca2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>12</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>AWT</td>
<td>0.665</td>
<td>-0.723</td>
<td>0.452</td>
<td>0.509</td>
</tr>
<tr>
<td>PWT</td>
<td>0.530</td>
<td>0.38*</td>
<td>0.35*</td>
<td>0.37*</td>
</tr>
<tr>
<td>LVW Echo/BW</td>
<td>0.876</td>
<td>-0.560</td>
<td>0.35*</td>
<td>0.37*</td>
</tr>
<tr>
<td>LVW wet/BW</td>
<td>-0.686</td>
<td>0.723</td>
<td>0.452</td>
<td>0.509</td>
</tr>
</tbody>
</table>

Only significant CC values are shown (*P < 0.05). LVW Echo/BW, left ventricular mass measured by echocardiography; LVW wet/BW, left ventricular wet weight/body weight. *P < 0.1.
is responsible for the prion neurodegenerative diseases (4). Although the physiological function of prion protein remains unclear, accumulating evidence suggests that it may be involved in the cellular response to oxidative stress (2, 22); this function going awry in neurodegenerative diseases. In a paper by Miele et al. (17), prion protein ablation resulted in reduced mitochondrial numbers, unusual mitochondrial morphology, and elevated levels of mitochondrial manganese-dependent superoxide dismutase antioxidant enzyme. Prion protein may have mitochondrial functions in the heart, a mitochondria-rich organ, during the process of adaptation to pressure overload.

Candidate heart failure/hypertrophy biomarkers. Cardiac biomarkers are invaluable for the diagnosis and prognosis of heart disease. The natriuretic peptides ANP and B-type natriuretic peptide (BNP) are examples of cardiac biomarkers that are widely used clinically for the diagnosis and prognosis of heart failure, acute coronary syndrome, and myocardial infarction (15). They are secreted from the heart in response to hemodynamic overload or injury and can be detected and measured in the systemic circulation (15). A successful biomarker must correlate to one or many physiological parameters in vivo. One of us (E. O. Weinberg) recently evaluated ST2, a secreted protein of the interleukin-1 receptor family that was identified by genomic screening, as a biomarker for heart failure (27). In the present study we identified additional candidate biomarkers in transcripts that fit the criteria of encoding secreted proteins and that correlate in a continuous manner to physiological parameters of LVH. We found the expected upregulation of ANP and BNP gene expression in hearts with compensated pressure overload LVH, with highly
significant correlations in a continuous manner to structural markers of cardiac hypertrophy. ANP and BNP are widely used biomarkers because of correlations between serum/plasma levels and prognostic indicators. We identified additional genes encoding secreted proteins including biglycan, procollagen type VIII, fibulin-2, matrix Gla protein, lumican, connective tissue growth factor, microfibrillar associated protein-5, lectin galactose binding soluble 1, follistatin-like-1, and decorin. All of these candidates, shown in Fig. 4, showed similar correlation profiles as ANP and BNP to the level of LVH from 24 h onward after the imposition of hemodynamic overload. The proteins of these genes are candidate novel markers of hemodynamic stress that can be evaluated in serum samples from patients with LVH who are at high risk of developing heart failure.

**Extracellular matrix genes.** Turnover of extracellular matrix components is an active process during cardiac remodeling to provide mechanical support, cell communication, and proliferation. Interestingly, many of the extracellular proteins, identified in this study by their strong correlation with cardiac remodeling, are also involved in bone remodeling. Biglycan is an extracellular proteoglycan involved in cell adhesion, collagen fibril assembly, and growth factor interactions (28). Biglycan-deficient mice show low bone mass that becomes more obvious with age (28). Also, Matrix Gla protein (Mglap) is a vitamin K-dependent protein. Mglap is also expressed at high levels in the heart, kidney, and lung and is upregulated by vitamin D in bone cells. Nonfunctional Mglap in humans is related to Keutel syndrome (abnormal cartilage calcification) (20), whereas Mglap-targeted mice showed problems in the calcification of both cartilage and the arteries (13). Of note, in a human study, polymorphisms of the mglap gene were associated with increased risk of plaque calcification and myocardial infarction (7). Periostin, or osteoblast-speciﬁc factor (Osf2), is also a well-known marker of osteoblastic differentiation. Many different reports have mentioned the upregulation of Osf2 in cardiac remodeling; however, its role is still unknown (8, 26). The observation that common genetic pathways are involved in bone formation and LVH may reflect a basic common remodeling mechanism and may explain secondary phenotypes in patients with cardiac disease. For instance cardiac transplant patients show a higher prevalence of osteoporosis, which has been attributed to disturbed turnover of bone formation biochemical markers (6).

Another interesting upregulated extracellular matrix protein identified in our analysis is fibulin-2. Fibulin-2 plays an essential role in organogenesis, particularly in embryonic heart development. In a recent paper, Tsuda and colleagues (23) reported that during development fibulin-2 expression is robustly upregulated when the transformed mesenchymal cells migrate into the existing extracellular matrix. In the epicardium, epicardial cells produce fibulin-2 upon their migration over the myocardial surface, and its expression persists throughout coronary vasculogenesis and angiogenesis (23). Fibulin-2 is also expressed in adult heart where it plays a role in maintain tensile strength of the cardiac valves (29).

**Metabolic genes.** Many metabolic genes, especially those involved in fatty acid oxidation, showed negative correlations with LVH measurements. Enolase 3, an enzyme accounting for 90% of human muscle enolase activity that is associated with metabolic myopathies and distal glycolysis, was reduced with the increase in left ventricular size (3). A similar pattern was observed for other metabolic genes involved in various mitochondrial energy production mechanisms [mitochondrial iso-citrate dehydrogenase 2 (NADP*)-Idh2, NADH dehydrogenase (ubiquinone) flavoprotein 1-Nduv1, and peroxisomal enoyl coenzyme A hydratase 1-Ech1]. Finally, mitochondrial 3-oxoacyl-Coenzyme A thiolase, which catalyzes the last step of mitochondrial fatty acid beta-oxidation, 3,2-trans-enoyl-Coenzyme A isomerase, which is involved in modification of intermediates of beta-oxidation of unsaturated fatty acids, and mitochondrial 2,4-dienoyl CoA reductase 1, which is an auxiliary enzyme of beta-oxidation, were negatively correlated with the increase in wall thickness. Fatty acids are the primary energy source for the normal heart. Free fatty acid oxidation is depressed in severe heart failure due to reduced activity of mitochondria fatty acid oxidation enzymes (21).

Our data indicate that expression of metabolic genes and in particular those involved in fatty acid metabolism is disturbed at a very early stage of cardiac disease. These changes are taking place in correlation with the levels of myocardial hypertrophy, and functional studies are needed to determine whether they have a causative pathogenic role in the subsequent development of heart failure.

A gene with the third highest negative correlation to cardiac growth was phosphatidylinositol 3-kinase catalytic alpha (Pik3ca). Pik3ca, a member of the PI3-kinase pathway, is well known to be involved in compensatory cardiac hypertrophy and myocyte survival (14, 25). PI3-kinase is upstream of Akt, a key regulator of myocardial survival (25), and this down-regulation of expression may indicate a diminution of this pathway during the progression of hypertrophy.

Expression of the voltage-gated potassium channel subfamily H2 (Kcnh2), which encodes a protein involved in long QT syndrome 2, was downregulated with the development of hypertrophy. Aberration of Kcnh2 in adult mice leads to episodic sinus brachycardia (10). Downregulation of pathways regulating cardiac conductivity is an additional risk to patients with cardiac hypertrophy.

In conclusion, our novel filtering strategy enabled us to isolate genes with expression levels that were strongly correlated with LVH in a continuous manner. Some of these genes have been validated by two different methods (microarray and quantitative RT-PCR), two independent studies, and various time points (data from our lab and the CardioGenomics/PGA database). The finding that the majority of the genes belonged to extracellular matrix and energy metabolism categories highlights the importance of these processes from the early stages onward in the progression of cardiac hypertrophy. In particular, the observation that potential disturbances in fatty acid oxidation occur as early as 24 h after the imposition of hemodynamic overload is novel since these changes were thought to occur much later and may indicate that they are involved in the transition to failure. Novel candidate biomarkers of LVH/heart failure were also identified as genes encoding secreted proteins that correlated linearly with cardiac structure and function. Functional analyses of the identified genes including overexpression and/or knockdown in vivo and in vitro are required to validate these findings.
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