Gene expression analysis of ischemic and nonischemic cardiomyopathy: shared and distinct genes in the development of heart failure

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DILATED CARDIOMYOPATHY is a common cause of congestive heart failure and the leading cause of cardiovascular morbidity and mortality in the United States (27). Dilated cardiomyopathy can be initiated by a variety of factors, such as ischemia, pressure or volume overload, myocardial inflammation or infiltration, and inherited mutations (14). A prevailing hypothesis is that, despite the varied inciting mechanisms that initiate the heart failure syndrome, there is a final common pathway that drives heart failure progression (47). Because of this, there is limited research into specific molecular events that are unique to the underlying process. This issue is especially relevant in the two major forms of dilated cardiomyopathy, nonischemic (NICM) and ischemic (ICM). Although NICM and ICM have similar presentations (26), they are characterized by different pathophysiology, prognosis, and response to therapy (19, 21, 23, 24, 32, 42), and understanding their different pathophysiological mechanisms is essential in guiding future therapies.

The emergence of microarray technology to simultaneously assess mRNA levels of tens of thousands of genes offers a novel approach to compare and contrast the myocardial transcriptome of NICM and ICM. Although previous studies have examined changes in gene expression in failing vs. nonfailing (NF) hearts (1, 5, 44, 45, 51), they have focused only on NICM. The goal of this study was to simultaneously examine the differences in transcriptomes between either NICM or ICM and normal hearts to establish a set of shared and unique genes differentially expressed in the two major causes of heart failure. The present approach is distinct, but complementary, to our previous study (33) in which we used the method of nearest shrunken centroids (46) to determine a clinical prediction algorithm (i.e., a gene expression-based biomarker) that differentiates between NICM and ICM. The current analysis offers insight into both disease-specific pathogenesis and therapeutics. Furthermore, an understanding of the distinctions with potential pathophysiological underpinnings between these two conditions supports and complements ongoing biomarker development efforts to differentiate heart failure of different etiologies (33).

METHODS

Patient population. The study sample was composed of 31 end-stage cardiomyopathy and 6 NF hearts. Myocardial tissue from end-stage cardiomyopathy patients was obtained at the time of left ventricular assist device (LVAD) placement or cardiac transplantation from two institutions: 1) Johns Hopkins Hospital in Baltimore, MD (n = 24 NICM and ICM samples and 6 NF samples), and 2) University of Minnesota in Minneapolis, MN (n = 7 NICM samples). Samples from the latter institution were collected and prepared independently (11), and gene expression data files were kindly provided. Discorded myocardial tissue from the left ventricular free wall or apex obtained during surgery was immediately frozen in liquid nitrogen and stored at −80°C. There is no evidence that differences in left ventricular sampling sites contribute to sample variability, and, in our previous experience, the sampling of tissue from these two sites did not contribute to variability in gene expression (33). The dissecting pathologist selectively excluded areas of visible fibrosis from the portion stored for analysis. Because myocardial tissue obtained at LVAD placement and unused donor hearts are considered discarded tissue, we obtained an exemption from the Johns Hopkins Institutional Review Board for this study.

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Nonischemic cardiomyopathy.

In two comparisons:

Cardiac index. The NF hearts (fraction, higher pulmonary capillary wedge pressure, and lower less often on intravenous inotropic therapy. Compared with elevated pulmonary arterial and wedge pressures, and reduced severely reduced ejection fraction, left ventricular dilation, and oxysterol binding protein-like-8). In cell growth and maintenance (acyl-CoA synthetase long chain family member-3 and oxysterol binding protein-like-8). In cell growth and maintenance, upregulated genes included cyclin-dependent kinase and oxysterol binding protein-like-8). In cell growth and maintenance, upregulated genes included cyclin-dependent kinase

<table>
<thead>
<tr>
<th></th>
<th>No-LVAD* (n = 7)</th>
<th>Pre-LVAD* (n = 3)</th>
<th>No-LVAD* (n = 8)</th>
<th>Pre-LVAD* (n = 13)</th>
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<tr>
<td>Age, yr</td>
<td>54 (49 and 60)</td>
<td>60 (59–60)</td>
<td>51 (48 and 53)</td>
<td>46 (37 and 52)</td>
</tr>
<tr>
<td>Male</td>
<td>100%</td>
<td>100%</td>
<td>86%</td>
<td>62%</td>
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<tr>
<td>Ejection fraction, %</td>
<td>20 (15 and 25)</td>
<td>17.5 (10–25)</td>
<td>17.5 (7.5 and 27.5)</td>
<td>15 (12.5 and 20)</td>
</tr>
<tr>
<td>LVIDd, cm</td>
<td>6.8 (6.7 and 7.6)</td>
<td>6.5 (6–7)</td>
<td>8.5 (7.5 and 9.3)</td>
<td>7.3 (6.8 and 8.1)</td>
</tr>
<tr>
<td>PCWP, mmHg</td>
<td>15 (12 and 23)</td>
<td>30 (30–32)</td>
<td>13.5 (13 and 14)</td>
<td>27 (21 and 31)</td>
</tr>
<tr>
<td>Cardiac index, l/m²</td>
<td>2.4 (2.3 and 2.4)</td>
<td>1.4 (1.3–1.5)</td>
<td>2.4 (1.9 and 2.8)</td>
<td>1.5 (1.3 and 1.6)</td>
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<tr>
<td>β-Adrenergic antagonists</td>
<td>71%</td>
<td>67%</td>
<td>38%</td>
<td>36%</td>
</tr>
<tr>
<td>ACE inhibitors or ARBs</td>
<td>100%</td>
<td>100%</td>
<td>88%</td>
<td>55%</td>
</tr>
<tr>
<td>Diuretics</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>64%</td>
</tr>
<tr>
<td>Inotropic therapy</td>
<td>100%</td>
<td>33%</td>
<td>13%</td>
<td>73%</td>
</tr>
</tbody>
</table>

Values are medians or percentages. *Median (25th and 75th percentiles). †Median (range). ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; LVAD, left ventricular assist device; LVIDd, left ventricular end-diastolic diameter; PCWP, pulmonary capillary wedge pressure. Inotropic therapy includes dopamine, dobutamine, and milrinone. ‡P < 0.05, difference between no-LVAD and pre-LVAD groups. §P < 0.05, difference between ischemic and nonischemic cardiomyopathy.

Review Board for sample collection and medical chart abstraction without written informed consent.

Differential gene expression. Details of sample preparation, microarray hybridization, data normalization, and validation with quantitative PCR are available as Supplemental Material (see the Physiological Genomics web site).1 We identified differentially expressed genes in two comparisons: 1) NICM vs. NF hearts and 2) ICM vs. NF hearts. Statistically significant changes in gene expression were identified using significance analysis of microarrays (SAM) (49). SAM identifies genes with statistically significant changes in expression by identifying a set of gene-specific statistics (similar to the t-test) and a corresponding false discovery rate (FDR; similar to a P value adjusted for multiple comparisons). Using the “one class” option, we identified genes with an FDR of <5% (corresponding to a P value adjusted for multiple comparisons <0.05) and an absolute fold change of ≥2.0. This threshold has been used in other similar studies (44) and may maximize specificity (20). These differentially expressed genes were visualized by hierarchical clustering (2) and heat mapping (22), using Euclidean distance with complete linkage.

RESULTS

Clinical specimens. Subjects with ischemic (n = 10) or nonischemic (n = 21) end-stage cardiomyopathy exhibited severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and reduced cardiac index (Table 1). ICM subjects were older, all male, more often on angiotensin-converting enzyme inhibitors, and less often on intravenous inotropic therapy. Compared with no-LVAD patients, pre-LVAD patients had lower ejection fraction, higher pulmonary capillary wedge pressure, and lower cardiac index. The NF hearts (n = 6) were from unused cardiac transplant donors. The unused donor subjects were younger (median age 42 yr with interquartile range 24–50 yr) and predominantly male, and echocardiographic and hemodynamic information and medications were not available.

Differential gene expression: NICM vs. NF and ICM vs. NF. There were 257 genes differentially expressed between NICM and NF samples and 72 genes differentially expressed between ICM and NF samples, with a FDR of <5% and an absolute fold change of ≥2.0. Of the differentially expressed genes, only 41 were common to both NICM and NF and ICM and NF comparisons. As a measure of variability of gene expression, the coefficient of variation for these differentially expressed genes is depicted in Supplementary Fig. S1. The coefficient of variation is low and comparable for both NICM and ICM.

Differentially expressed genes common to both NICM-NF and ICM-NF comparisons. The majority of the 41 shared genes fell into functional classes of cell growth, maintenance, and signal transduction (Fig. 1). Genes implicated in the fetal gene program induction were among those differentially expressed, including downregulation of α-myosin heavy chain polypeptide-6 (36) and upregulation of atrionatriuretic peptide receptor C (18). In the cell growth and maintenance class, there were multiple probes corresponding to hemoglobin α- and β-chains. There were also genes involved in signal transduction, including endothelin receptor type A and monococyte chemotactic protein-1. In addition, there were genes encoding components of the sarcomere (α-myosin heavy chain noted above), the cytoskeleton (collagen type-21α and ficolin), and the extracellular matrix (asporin). The majority of the genes were upregulated in NICM and ICM hearts compared with NF hearts, and for all 41 shared genes, fold changes were remarkably similar in direction and magnitude between NICM-NF and ICM-NF comparisons (Table 2).

Differentially expressed genes unique to the NICM-NF comparison. Of the 216 genes that were uniquely differentially expressed in NICM hearts, the majority fell into metabolism, cell growth, maintenance, signal transduction, and binding (Fig. 1 and Supplementary Table S1). The genes involved in metabolism included angiotensin I-converting enzyme-2 (ACE2) and genes involved in fatty acid and cholesterol metabolism (acyl-CoA synthetase long chain family member-3 and oxysterol binding protein-like-8). In cell growth and maintenance, upregulated genes included cyclin-dependent kinase inhibitor-1B and delta sleep-inducing peptide, a vagal-potentiating peptide with influences on cardiac rhythm (39). Genes involved in signaling pathways were upregulated, including signal transducer and activator of transcription-1 and -4 (members of the JAK/STAT signaling pathway), as well as receptors for leptin, growth hormone, transforming growth factor-β, and platelet-derived growth factor. Several genes implicated in inflammation and the immune response showed increased ex-

1The Supplemental Material for this article (Supplemental Figs. S1–S3, Supplemental Tables S1–S3) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00255.2004/DC1.
pression in NICM hearts, including interleukin-27, a major
histocompatibility complex molecule, and a component of the
complement pathway, H factor-1. There were also several
genes related to cell adhesion, apoptosis, and development. All
genes were upregulated in NICM hearts except one: a zinc
transporter that was downregulated twofold.

Differentially expressed genes unique to the ICM-NF com-
parison. The 31 genes uniquely differentially expressed be-
tween ICM and NF hearts were predominantly in functional
classes of cell growth, maintenance, catalytic activity, and
signal transduction (Fig. 1 and Supplementary Table S2). They
also included genes implicated in the fetal gene program
induction, including upregulation of natriuretic peptide precur-
sor B, atrial natriuretic factor, and an embryonic atrial myosin
light chain polypeptide (14).

Differentially expressed genes and functional categories.
As shown in Fig. 1, the majority of genes on the array (>50%)
belonged to functional classes of binding and metabolism; a
moderate number of genes (15–40%) were in the classes of
catalytic activity, cell growth/maintenance, development, nu-
cleus, signal transduction, and transcription; and few genes
(<10%) belonged to classes of apoptosis, cell adhesion, cyto-
skeleton, and inflammatory response (the combined percent-
ages total over 100%, since genes can belong to more than one
functional category). This pattern does not match that of our
data ($P < 0.001$ in a $\chi^2$-test). This suggests that the differences
in functional categories identified were not solely a function
of their representation on the microarray.

Clustering. The heat maps with clustering algorithms for the
two comparisons, ICM-NF and NICM-NF, are shown in Fig. 2.
The NF samples formed a distinct cluster from the ICM
samples. For the NICM-NF comparison, there were two domi-
nant clusters. One dominant cluster contained only NICM
samples obtained from patients at the time of LVAD implan-
tation (NICM/pre-LVAD). The other dominant cluster con-
tained two subgroups: 1) predominantly NF samples and 2) the
remaining portion of NICM samples, which were all obtained
from patients who did not have an LVAD before cardiac
transplantation (NICM/no-LVAD). Thus there was a clear
discrimination among the NICM samples obtained from 1) pa-
patients who required LVADs before cardiac transplantation
and 2) patients who survived to cardiac transplantation without
LVAD support.

To determine the specificity of the profiles, we also created
a heat map with clustering algorithm for all 288 genes that
were identified as differentially expressed in at least one of the
two comparisons (Supplementary Fig. S2). Samples formed
three distinct etiology clusters, NF, ICM, and NICM, but this
was likely due to the presence of shared differentially ex-
pressed genes. To confirm the specificity of the differentially
expressed genes, we performed two additional heat maps with
clustering (Supplementary Fig. S3, A and B): first, NF and ICM
samples using only those genes identified as differentially
expressed between NF and NICM samples, and second, NF
and NICM samples using only those genes identified as dif-
ferentially expressed between NF and ICM samples. If, as we
assumed, the genes uniquely identified as differentially ex-
pressed in ICM relative to NF hearts were truly unique to the
ICM-NF comparison, then a heat map of these genes in NICM
and NF hearts should demonstrate no clustering by etiology,
and vice versa for NICM genes in ICM hearts. This was the
case: as expected, in both heat maps, the samples did not
cluster by etiology, indicating that the unique differentially
expressed genes were specific to the given comparison.

Validation. We selected 16 genes of potential biological
interest and validated the microarray findings in NICM, ICM,
and NF hearts using quantitative PCR (qPCR). As shown in
Fig. 3, qPCR confirmed 27 of the 32 microarray predictions
with regard to fold change; 11 of these agreed completely in
fold change and significance. Of the five that did not agree on
fold change, three were nonsignificantly changed in both com-
parisons (the leptin receptor in ICM, serine protease inhibi-
tor, clade E, member-1 in NICM, and the acyl-CoA synthetase
long chain family member-3 in ICM), leaving only two clear
disagreements: S100 calcium binding protein-A8 was signifi-
cantly downregulated by qPCR but nonsignificantly upregu-
lated by microarray, and lumican was significantly upregulated
in ICM by microarray and nonsignificantly downregulated by
qPCR. Notably, of the 10 genes significantly expressed only in
one comparison, NICM or ICM, relative to NF hearts, 17 of the
20 comparisons were confirmed by fold change and/or signif-
The principal finding of this investigation is that cardiomyopathies of different etiologies exhibit both shared and distinct changes in gene expression compared with NF hearts. Remarkably, of the >22,000 transcripts present on the Affymetrix microarray platform, only a total of 288 genes (1–2%) are differentially expressed in NICM and ICM relative to NF hearts, and 41 of these genes are common to both comparisons with comparable fold changes. This suggests that there are both

<table>
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<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>ICM-NF</th>
<th>FDR</th>
<th>NICM-NF</th>
<th>FDR</th>
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<td>HBA2</td>
<td>hemoglobin, alpha-2</td>
<td>4.3</td>
<td>0.50</td>
<td>2.7</td>
<td>0.18</td>
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<td>HSAGL2</td>
<td>human alpha-globin gene</td>
<td>3.5</td>
<td>0.50</td>
<td>2.4</td>
<td>0.18</td>
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<tr>
<td>HBB</td>
<td>hemoglobin, beta</td>
<td>3.4</td>
<td>0.50</td>
<td>2.6</td>
<td>0.18</td>
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<td>HBA2</td>
<td>hemoglobin, alpha-2</td>
<td>3.4</td>
<td>0.50</td>
<td>2.2</td>
<td>0.18</td>
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<td>HBA1</td>
<td>hemoglobin, alpha-1</td>
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<td>2.1</td>
<td>0.18</td>
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<td>AF059180</td>
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<td>0.50</td>
<td>2.4</td>
<td>0.18</td>
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<tr>
<td>HBB</td>
<td>hemoglobin, beta</td>
<td>3.0</td>
<td>0.50</td>
<td>2.6</td>
<td>0.18</td>
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<td>DUT</td>
<td>dUTP pyrophosphatase</td>
<td>2.2</td>
<td>0.50</td>
<td>2.2</td>
<td>0.18</td>
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<td>RARRES1</td>
<td>retinoic acid receptor responder-1</td>
<td>-3.0</td>
<td>0.90</td>
<td>-2.2</td>
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<td>PIK3R1</td>
<td>phosphoinositide 3-kinase, reg subunit, polypeptide-1</td>
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<td>0.50</td>
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<td>0.18</td>
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<td>NPR3</td>
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<td>0.18</td>
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<td>CBLB</td>
<td>Cas-Br-M ectropic retroviral transforming sequence b</td>
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<td>0.50</td>
<td>2.3</td>
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<td>EDNRA</td>
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<td>2.76</td>
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<td>DKFZp564I1922</td>
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<td>TNFRSF11B</td>
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<td>SCYA2</td>
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<td>0.50</td>
<td>5.1</td>
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<td>pleckstrin homology-like domain, family A, member-1</td>
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<td>0.50</td>
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<td>ASPN</td>
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<td>P311</td>
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<td>0.18</td>
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<td>0.50</td>
<td>3.1</td>
<td>0.18</td>
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<td>SERPINA3</td>
<td>serine (or cysteine) proteinase inhibitor clade A, member-3</td>
<td>-2.5</td>
<td>0.50</td>
<td>-2.0</td>
<td>0.18</td>
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</table>

Differentially expressed genes (n = 41) shared between ischemic cardiomyopathy (ICM) vs. nonfailing (NF) heart and nonischemic cardiomyopathy (NICM) vs. NF heart comparisons. “Fold change” describes the mean gene expression for ICM and NICM samples relative to NF samples. False discovery rate (FDR) is analogous to a P value (as a percentage) adjusted for multiple comparisons. NICM-NF, comparison between NICM and NF samples; ICM-NF, comparison between ICM and NF samples.
shared and distinct mechanisms that contribute to the development of heart failure of different etiologies, which supports the recent identification of a gene expression-based diagnostic biomarker that differentiates between ICM and NICM cardiomyopathy (33). In addition, a better understanding of these distinctions encourages ongoing efforts to develop cause-specific therapies specifically targeted at NICM and ICM (7).

These results complement our recent identification of a gene expression profile that differentiates between ICM and NICM cardiomyopathy (33). In that analysis, we used prediction analysis of microarrays (PAM) (46) to identify and validate a 90-gene profile that could differentiate between NICM and ICM. Unlike the current analysis, PAM identifies the smallest number of genes that succinctly characterizes a class. These genes do not necessarily have biologic significance, since they are chosen based on the stability of their expression rather than a combination of magnitude and stability (46). This prior study demonstrated that gene expression profiles correlated with clinical parameters in heart failure patients and supported ongoing efforts to incorporate expression profiling-based biomarkers in determining prognosis and response to therapy in heart failure.

The current study has a distinctly different purpose and uses different samples and statistical methods. Instead of identifying and validating a gene expression profile as a diagnostic biomarker, the current study focuses on novel gene discovery:
identifying differentially expressed genes to better understand the similarities and differences between the two major forms of cardiomyopathy, ICM and NICM. In addition, because we were interested in the genesis of cardiomyopathy, we compared both ICM and NICM with NF hearts (the prior study did not involve NF hearts). Finally, in the current study, we used SAM (49) to identify differentially expressed genes and validated our findings with qPCR, as opposed to using PAM (46) and validating our findings by testing the gene expression prediction profile in an independent set of samples.

Thus the two studies target two different goals of microarray analysis, using a pattern of gene expression as a biomarker vs. examining gene expression for novel gene discovery (7, 15). These findings of the unique and shared genes expressed in NICM and ICM relative to NF hearts complement those of the prior study. Both demonstrate that unique gene expression exists in the two major forms of cardiomyopathy. On one hand, this allows a pattern of gene expression to function as a diagnostic biomarker. On the other hand, the unique patterns of gene expression can be further investigated to better define cause-specific therapies for heart failure. These two analyses are clearly not redundant, since they used different sets of samples and different statistical methods and, most importantly, had different purposes. Furthermore, given the complementary nature of the two analyses, it is not surprising that only four of the genes in the current study were observed in our prior identification of a gene expression profile that differentiated between ICM and NICM (33). The current analysis also focused on differential gene expression and thus targeted different genes than the one investigating prediction (46).

The current study is unique for a number of reasons. First, we have studied 37 samples, which is a large number relative to gene expression studies in cardiomyopathy to date (1, 3, 5, 10, 11, 25, 28, 44, 45, 51). There are no accepted means of calculating sample size and power in microarray experiments, but because our study examines a larger number of samples than prior studies, we have increased power to detect significant changes in gene expression. Furthermore, we have the added advantage of uniformity among samples: all NICM hearts were from individuals with idiopathic cardiomyopathy, and the clinical characteristics were reasonably similar within groups.

The second unique feature of this study is that we have not compared only failing and NF hearts, as in many previous studies (1, 5, 45, 51), but extended this analysis to compare the differential gene expression of NICM and ICM relative to NF hearts. This offers further insight into the mechanisms involved in the development of heart failure of varying etiologies. Many genes are shared between NICM and ICM relative to NF hearts, and this is consistent with clinical experience: the presentation and standard treatment for cardiomyopathy of both etiologies are similar (27). However, despite similar presentations and therapies, NICM and ICM are distinct diseases; patients with ICM have decreased survival compared with their NICM counterparts (21, 24) and respond differently to therapies (19, 23, 32, 42). Thus an understanding of the

Fig. 3. Independent assessment of gene expression levels. To validate selected microarray findings using a complementary methodology, we quantified transcript abundance of 16 genes using quantitative PCR (qPCR). Fold change is expression in NICM and ICM hearts compared with NF hearts according to qPCR (black bars) and microarrays (gray bars). ACE2, angiotensin-converting enzyme-2; ATP1B3, ATPase, Na+/H+ transporting, beta 3 polypeptide; FACL3, acyl-CoA synthetase long chain family member-3; HBA2, hemoglobin A2; LEPR, leptin receptor; LUM, lumican; MYH6, myosin heavy chain 6; NAP1L3, nucleosome assembly protein 1-like-3; NPR3, atrionatriuretic peptide receptor C; PHLD1A, pleckstrin homology-like domain family A member-1; RPS4Y, ribosomal protein S4, Y-linked; S100A8, S100 calcium binding protein A8; SERPINE1, serine (or cysteine) proteinase inhibitor, clade E, member-1; SLC39A8, solute carrier family-39, member-8; TNFRSF11B, tumor necrosis factor receptor superfamily member-11b; TXNIP, thioredoxin interaction protein. *P < 0.05 compared with NF hearts by Wilcoxon rank sum test. †P < 0.05 by significance analysis of microarrays.
distinctions between the two conditions at the level of gene expression may guide future efforts to design etiology-based therapies.

The predominance of metabolism genes in NICM hearts suggests that the derangements involved in the genesis and maintenance of NICM may be metabolic in nature. This is supported by an early trial of beta-blockers in heart failure that demonstrated a greater mortality benefit in NICM than ICM (13). Beta-blockers improve myocardial efficiency by shifting myocardial metabolism from free fatty acids to glucose. The increase in fatty acid metabolism genes specifically in NICM in our analysis would explain why beta-blockers may be particularly beneficial in NICM. Furthermore, our results suggest that future etiology-specific therapies in NICM could target metabolic pathways, including those of fatty acid or cholesterol synthesis. One particular relevant example is ranolazine. This investigational compound shifts myocardial cells from fatty acid to glucose metabolism and is currently being investigated as a treatment for myocardial ischemia (9). On the basis of our results, this drug could also be helpful in patients with NICM.

In ICM, on the other hand, our results suggest that abnormalities in catalytic activity may predominate, and an anti-ischemic protective effect of the specific catalytic enzymes identified, serine proteinase inhibitors, has been previously observed in pigs subject to experimentally induced myocardial ischemia (31). Given our results, it may be possible that such enzymes could also be beneficial in patients with ICM.

Our work agrees to an extent with the findings of a similar analysis of differential gene expression by Steenman et al. (44), in which pooled samples of NICM and ICM were compared with one NF sample, and 95 differentially expressed genes were identified between failing and NF hearts. Compared with our list of 288 genes, we found 8 genes in common (Supplementary Table S3). There are a number of reasons why our results differed from those of the prior study. The prior study had only one NF heart, and it was from a patient with cystic fibrosis. This heart is likely very different, not only in age but also in hemodynamic parameters, from a heart from an unused cardiac transplant donor. In addition, we used different statistical algorithms for normalization and identification of differentially expressed genes. We normalized with robust multivariate algorithm for normalization and identification of differentially expressed genes. We normalized with robust multivariate algorithm (29). We identified differentially expressed genes with SAM, which has been validated in a number of studies (6, 41, 49, 50) and may be more accurate than other commonly used methods for identifying differentially expressed genes, such as t-tests (43). In addition, our analysis may have more external validity because we studied more samples (37 vs. 7 patients) with individually hybridized, as opposed to pooled, data. Individual hybridization may be more accurate than pooling because it allows the estimation of the within-group variance for each gene (38).

Some of the genes shown to be differentially expressed in our study have been previously identified as differentially expressed in studies of NICM vs. NF hearts, with remarkably similar fold changes between studies (Supplementary Table S3). Commonly identified genes include those involved in the fetal gene program (14), including natriuretic peptide precursor B, atrial natriuretic factor, cardiac muscle myosin heavy chain, and atrial alkali myosin light chain. The majority of genes are upregulated in NICM and ICM hearts vs. NF hearts, and this has also been noted in prior studies (1, 5, 44, 45, 51). This is likely due to biological differences, since prior studies all used different methods to normalize data and identify differentially expressed genes. Furthermore, because the expression of many of these genes was confirmed with qPCR in these prior studies, this offers indirect further confirmation of the validity of our differentially expressed genes. This highlights the critical point in microarray analysis used for gene discovery: the results should be considered hypothesis generating, and the gene expression should be confirmed with other quantitative techniques, such as qPCR (15).

Through qPCR, we confirmed the expression of 27 of the 32 comparisons with 16 genes of interest in heart failure. Of greatest interest are the novel genes from our analysis, including ACE2 and a member of the tumor necrosis factor (TNF) receptor superfamily (TNFRSF11B, also known as osteoprotegerin). We show that, in subjects with end-stage cardiomyopathy, ACE2 is significantly upregulated in NICM but not ICM. ACE2 is expressed predominantly in vascular endothelial cells of the heart and kidney, and ACE and ACE2 have different biochemical activities. Angiotensin I is converted to angiotensin I-9 (with 9 amino acids) by ACE2 but is converted to angiotensin II, which has eight amino acids, by ACE. Whereas angiotensin II is a potent blood vessel constrictor, angiotensin I-9 has no known effect on blood vessels but can be converted by ACE to a shorter peptide, angiotensin I-7, which is a blood vessel dilator (4). Interestingly, loss of ACE2 was associated with upregulation of hypoxia-inducible genes, suggesting a role for ACE2 in mediating the response to cardiac ischemia, although ACE2 was not significantly changed in ICM cardiomyopathy in our study (17). The upregulation of ACE2 in NICM but not ICM cardiomyopathy cannot be ascribed to the increased prescription of ACE inhibitors in ICM cardiomyopathy subjects, because, unlike ACE, ACE2 is insensitive to inhibition by ACE inhibitors (48). Thus our results offer insight into a possible new etiology-specific therapeutic target in heart failure.

Another novel finding of interest is the significant downregulation of a member of the TNF receptor subfamily, TNFRSF11B, in both NICM and ICM. Levels of TNF have been shown to be upregulated in chronic heart failure (34), and increasing levels of TNF have been correlated with disease severity (40). However, in clinical trials, soluble TNF-α antagonists did not reduce mortality or heart failure hospitalizations (12, 37). One might speculate that this lack of benefit may relate somehow to the downregulation of the TNF receptor in chronic heart failure.

The results of the unsupervised hierarchical clustering algorithm suggest that hearts of patients with NICM who do not undergo LVAD implantation resemble NF hearts more than those of NICM patients who require an LVAD before cardiac transplantation. An examination of their baseline characteristics confirms this: NICM-LVAD patients are a sicker subset, with higher pulmonary capillary wedge pressure and increased need for intravenous inotropes, two known markers of poor prognosis in chronic heart failure patients (8, 16). Although there are documented changes in gene expression between hearts before and after LVAD support (3, 10, 11, 25), there is no evidence that differential gene expression exists between
end-stage cardiomyopathy samples obtained before LVAD placement and at the time of cardiac transplantation in patients without LVADs or between patients with different clinical presentations. Because this result was obtained with an unsupervised clustering algorithm, it is free of bias of predefined categories (35). Although it is possible that the differences were due, in part, to the use of seven NICM-LVAD samples from an outside institution, this is less likely based on our prior results with these samples, which indicated that the institution of origin did not contribute to variability in gene expression (33), and because the outside institution samples themselves did not form a distinct cluster. This unanticipated difference between end-stage NICM patients could offer insight into the differential gene expression of different stages of heart failure. This requires further study, and lends credence to the notion that gene expression can be correlated with clinically relevant parameters in heart failure patients to aid in determining prognosis and response to therapy.

Although the analysis of gene expression using oligonucleotide microarrays is a powerful technique, limitations warrant mention. Not all genes are represented on the Affymetrix U133A arrays used in this study, and therefore the knowledge that can be acquired from these experiments remains incomplete. In addition, an NF unused donor heart is not the same as a normal heart, because circumstances causing a donor heart to be ineligible for cardiac transplantation, such as infection or prolonged hypotension, can also affect gene expression. One study suggested that the differential gene expression identified between failing and NF hearts may have been due to age and gender differences rather than differences in ventricular function (5). However, normal age- and sex-matched hearts are impossible to obtain, and other researchers have used comparable unused donor hearts in their experiments (1, 5, 45, 51).

Another limitation of this study is that microarray analysis is essentially hypothesis generating. However, in the tradition of such studies in the microarray literature (1, 3, 5, 10, 11, 25, 30, 44, 45, 51), this is a hypothesis-generating analysis with biological validation of select genes confirmed by qPCR. We have followed the practice of other studies in the field and extended the analysis to include more samples with different etiologies of heart failure and a careful comparison with the results of prior studies (in Supplementary Table S4), which is unprecedented in the literature thus far. For this reason, we believe that these analyses, while mainly hypothesis generating, do have significant value and should be made available to other individuals interested in microarray analysis of ICM and NICM.

In conclusion, we offer a novel addition to the analysis of differential gene expression between failing and NF hearts by providing new insight into the genetic pathways involved in the genesis of cardiomyopathy. By comparing differential gene expression in NICM and ICM relative to NF hearts, we have shown that there are a number of common and unique genes involved in the development of heart failure of differing etiologies. This analysis will provide valuable insight into the pathophysiology of heart failure and offers a basis for future studies of cause-specific therapies in the complex management of heart failure patients.

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