

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Circ. Res. 2005;96:592-599; originally published online Feb 17, 2005;

DOI: 10.1161/01.RES.0000159390.03503.c3

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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Mixed Messages

Transcription Patterns in Failing and Recovering Human Myocardium

Kenneth B. Margulies, Sunil Matiwala, Carla Cornejo, Henrik Olsen,
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Abstract—In previous studies, mechanical support of medically refractory hearts with a left ventricular assist device (LVAD) has induced regression of many morphological and functional abnormalities characteristic of failing human hearts. To identify transcriptional adaptations in failing and LVAD-supported hearts, we performed a comprehensive transcription analysis using the Affymetrix microarray platform and 199 human myocardial samples from nonfailing, failing, and LVAD-supported human hearts. We also used a novel analytical strategy that defines patterns of interest based on multiple intergroup comparisons. Although over 3088 transcripts exhibited significantly altered abundance in heart failure, most of these did not exhibit a consistent response to LVAD support based on our analysis. Of those 238 with a consistent response to LVAD support, more than 75% exhibited persistence or exacerbation of HF-associated transcriptional abnormalities whereas only 11%, 5%, and 2% exhibited partial recovery, normalization, and overcorrection responses, respectively. Even among genes implicated by previous reports of LVAD-associated myocardial improvements, partial or complete normalization of transcription did not predominate. The magnitude of differences in transcript abundance between nonfailing and failing hearts, and between failing and LVAD-supported hearts, tended to be low with changes greater than or equal to 2-fold infrequently observed. Our results indicate that morphological or functional myocardial improvements may occur without widespread normalization of pathological transcriptional patterns. These observations also suggest that many failure-associated transcriptional changes have only a limited role in regulating cardiac structure and function and may represent epiphenomena and/or nonspecific myocardial plasticity responses. Differences in mRNA localization, translation efficiency, and posttranslational protein modifications or interactions may be more pivotal in regulating myocardial structure and function. (*Circ Res.* 2005;96:592-599.)

Key Words: remodeling ■ genes ■ heart failure ■ recovery ■ circulatory assistance

In recent years, several lines of research have demonstrated that severely failing human hearts retain plasticity including a potential for normalization that was previously unappreciated. In particular, tissue-based studies enabled by the use of a left ventricular assist device (LVAD) in some patients awaiting cardiac transplantation have provided a strong proof of concept that myocardial failure is not irreversible. Specifically, LVAD support induces a regression of pathological hypertrophy, improvements in contractile performance and contractile reserve, regression of pathological electrophysiological markers, and reductions in myocardial cytokines and apoptosis.¹⁻⁸ Moreover, changes observed during LVAD support demonstrate that reverse remodeling can occur even in the most advanced cases of dilated cardiomyopathy.

These observations of multilevel regression of the pathological phenotype of the failing human myocardium would

tend to support a hypothesis that mechanical unloading of failing hearts promotes normalization of myocardial gene expression. Indeed, two recent studies using microarray technology to compare tissues obtained before and after LVAD support identified large numbers of genes with altered transcript abundance.^{9,10} However, in normal rat hearts, Depre et al¹¹ demonstrated that hemodynamic loading and unloading produce similar transcriptional shifts, suggesting that at least some changes in gene expression during unloading are nonspecific and reflective of a general program of plasticity.

The purpose of this research was to examine broad transcriptional patterns associated with heart failure and LVAD support with the intention of distinguishing transitions that represent normalization of transcript abundance from transitions that do not. A related goal was to define the balance between genes showing transcriptional recovery versus genes

Original received December 8, 2003; resubmission received December 6, 2004; revised resubmission received February 1, 2005; accepted February 3, 2005.

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Dr Margulies has received research grant support from Gene Logic, Inc. Grant support includes salary support for both Drs Margulies and Matiwala. Dr Bednarik is a former employee of Gene Logic, Inc.

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Circulation Research is available at <http://www.circresaha.org>

DOI: 10.1161/01.RES.0000159390.03503.c3

demonstrating persistence or exacerbation of pathological gene expression. To address these goals, we applied microarray technology to characterize myocardial tissues from a relatively large number of nonfailing, failing, and LVAD-supported patients. This inquiry was facilitated by application of a new analytical framework in which multiple intergroup comparisons are made and genes are categorized based on their adherence to predefined response templates.

Materials and Methods

Human Myocardial Samples

Human myocardium was obtained from patients undergoing cardiac transplantation (Failing and LVAD-supported) or from donor hearts deemed unsuitable for transplantation (Nonfailing) as previously described.³ The Temple University Institutional Review Board approved these studies, and prospective or retrospective informed consent was obtained from all living patients. Fourteen tissues were obtained from nonfailing hearts (NF), 157 tissues were obtained from failing hearts (HF), and 28 tissues were obtained from LVAD-supported failing hearts (LVAD). Patients with less than 30 days of LVAD support were excluded from these studies. Among these tissues, there were 11 cases where HF and LVAD tissues were from the same patient. There were no intergroup differences in the mean age of patients providing heart tissue (NF 55 ± 4 years, HF 54 ± 2 years, LVAD 51 ± 2 years), but there were significant differences in the mean left ventricular ejection fraction (NF $53 \pm 2\%$, HF $14 \pm 1\%$, and LVAD $15 \pm 2\%$, before device placement) measured via echocardiography. The duration of heart failure was 51 ± 8 months in the HF Group and 41 ± 8 months in the LVAD Group.

Structural and Functional Phenotyping

To characterize cardiac myocyte morphology, we used a perfusion-based myocyte disaggregation procedure, iso-osmotic fixation with 1.5% glutaraldehyde and Ficoll gradient enrichment.² Median cell volume was determined by Coulter Channelyzer analysis of $>10\,000$ myocytes, and mean cell length was measured in at least 30 myocytes using an image analysis technique.² We examined changes in cardiac repolarization using the QT interval on the surface ECGs of patients receiving LVAD support. Using Bazett's formula, we evaluated the heart rate corrected QT interval (QTc) before and after a period of sustained LVAD support using a paired analysis as previously described.⁴

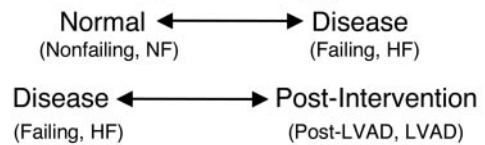
Sample Preparation and Chip Hybridization

The sample preparation and processing procedure was performed as described in the Affymetrix GeneChip Expression Analysis manual and recent publications.¹² For these studies, we used the Affymetrix HG_U133 Human Chipset which includes 22 242 sequences (including 2484 ESTs) on the A chip and 22 577 sequences (including 13 921 ESTs) on the B chip. In accordance with MIAME guidelines, the annotation for the Affymetrix microarray is available in the public domain at http://www.mged.org/Workgroups/MIAME/miame_1.1.html.

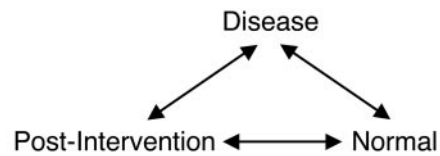
Normalization of Microarray Data

For the microarray experiments reported in this study, we used a combination of Affymetrix normalization and GeneExpress MAS 5.0 (Gene Logic, Inc) as previously described.¹³ Affymetrix normalization is a global scaling method, where the overall intensity of the chip affects the scaling factor. The top and bottom 2% of all expression intensity values on the chip are discarded, and the remaining 96% of values are used to compute the "trimmed mean." The scaling factor (SF) is then calculated using this adjusted mean ($SF = 100/\text{trimmed mean}$), and this single scale factor is applied to the expression values for every fragment on a given chip to produce normalized expression intensity. The MAS 5.0 normalization was designed by Affymetrix to more accurately reflect the distribution of data from microarray experiments. Expression values are calculated

A. Usual Analysis Strategy



B. Alternative Analysis Strategy



C. Possible Effects of Intervention (LVAD) on Dysregulated Transcripts

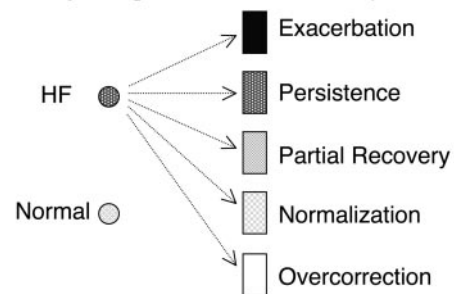


Figure 1. Alternative analytical strategies for assessing transcriptional changes. A, Simple contrasts usually involve a comparison between normal and disease or comparisons with or without an intervention. In the former case, any difference from normal is assumed to be pathological. However, in the latter case, differences after the intervention could represent recovery, exacerbation of the pathological pattern, or an alternative adaptation. B, An alternative analytical strategy involves considering each of three states simultaneously and viewing the response to an intervention in the context of the contrast between normal and disease. C, Specific case of HF and LVAD support is illustrated. For genes showing a significant difference between HF and NF, five general responses to LVAD support are possible.

based on the hybridization intensities of each probe pair within a probe set representing a single transcript.

Identification of Alternative LVAD-Induced Changes

As highlighted in Figure 1, during comparisons between a normal and an abnormal state, it is generally assumed that the normal is "ideal" and differences observed represent pathological changes. However, when the starting point is itself a pathological state (such as HF), the changes induced by an intervention (such as LVAD support) may or may not represent a return to a nonpathological state. One fairly simple approach for clarifying this uncertainty is to perform a contrast analysis between the normal, disease, and postintervention expression states for every transcript represented on the microarray. With this approach, the nature of the postintervention expression pattern (if consistent) for each gene can be defined by where it resides compared with the average expression values observed in the normal and disease states. Using this approach, it is possible to assign genes with consistent postintervention expression changes into one of five general response zones that might be labeled exacerbation, persistence, partial recovery, normalization, or overcorrection as illustrated in Figure 1C.

TABLE 1. Pattern Definitions

Phenomenon	Pattern	HF	LVAD	NF
Exacerbation	HF up	1	4	-5
	HF down	-1	-4	5
Normalization	HF up	2	-1	-1
	HF down	-2	1	1
Partial Normalization	NF<LVAD<HF	1	0	-1
	NF>LVAD>HF	-1	0	1
Persistence	NF up	-1	-1	2
	NF down	1	1	-2
Overcorrection	HF up	5	-4	-1
	HF down	-5	4	1

Phenomenology column labels gene expression corresponding to normalization, partial normalization, persistence, exacerbation, and overcorrection patterns based on the relative position of the LVAD expression intensity. Values in each column for the three sample groups are the coefficients for the Pearson correlation and contrast calculations. For the pattern-matching calculation, normalization matches regulation in the NF samples, partial normalization matches LVAD expression lying between the HF and NF samples, persistence matches regulation in the HF samples, and exacerbation and overcorrection match values more extreme than HF and NF, respectively.

Pattern Recognition Analytical Strategy and Statistical Analysis

Of the genes and ESTs on the Affymetrix HG_U133 microarray, only those that were classified as present in at least 60% of the samples in at least one of the three groups (NF, HF, and LVAD) were included in further analyses. For genes represented by multiple probe fragments on the HG_U133 microarray, only fragments lacking any warnings and representing the highest mean expression value across the entire myocardial sample set were selected. When appropriate, three separate intergroup fold-change analyses (NF versus LVAD, HF versus LVAD, and HF versus NF) were performed for each of the selected genes and ESTs. Gene profiles of interest were defined a priori based on the hypothetical results of the three fold-change analyses as shown in Figure 1. The MAS 5.0 expression values were subsequently base 10 log-transformed before any calculations were made, and the distribution of the transformed data was near normal. Ten possible expression patterns were defined to classify each observed gene expression, as shown in Table 1. In this table, the patterns are defined by coefficients for the three sample groups.

Similarity versus rank plots were generated in which genes and ESTs in the final Gene Set were ranked based on their degree of similarity to each gene profile. This analysis defines a Pearson similarity coefficient for each gene with respect to each gene profile. The Pearson similarity is defined as the Pearson correlation between the individual group means and the individual contrast weights for the patterns. In this analysis, we required a greater than 0.8 Pearson correlation coefficient. Each gene's expression was analyzed using two measures of match to an expression pattern: (1) the Pearson correlation of the gene's mean expression to the pattern coefficients, and (2) a t statistic for the contrast defined by the specified pattern coefficients. For each gene, the pattern with maximum Pearson correlation and maximum contrast t was determined, and a bootstrap procedure was used to estimate a probability value for the t statistic. If the best matching patterns from the two procedures agreed, and the bootstrap-corrected P for the contrast t was less than 0.05, the gene was identified as displaying that pattern of expression.

The dual-match condition ensures that the pattern of mean expression closely matches the desired pattern. Note that the t calculation for the partial recovery pattern includes the standard deviation contribution from the LVAD group, while excluding the LVAD group from the estimate of contrast. This can lead to a high t for partial normalization, whereas the Pearson correlation shows a

match to a normalization or persistence pattern. The probability value condition finds genes whose contrasts are the most significant.

For each gene, we let x_{ij} represent the logarithm of the expression data, where $i=1, 2, 3$ labels HF, LVAD, and NF groups, respectively, and $j=1..N$ labels the overall number of expression measurements, and N is the total number of samples. Each sample set has n_i samples, and its members are denoted by X_i .

The sample set mean M_i is defined by the following:

$$(1) \quad M_i = \frac{1}{n_i} \sum_{j \in X_i} x_{ij}$$

where the sum is over js belonging to sample set X_i .

For each pattern, defined by contrast coefficients c_i appearing in table X, the test statistic t is the ratio:

$$(2) \quad t = \frac{\hat{C}}{\hat{\delta}},$$

where

$$(3) \quad \hat{C} = \sum_{i=1}^3 c_i M_i$$

is the estimated contrast, and

$$(4) \quad \hat{\delta} = \hat{\sigma} \sqrt{\sum_{i=1}^3 \frac{C_i^2}{n_i}}$$

is the estimate of the deviation for each contrast. The mean square error $\hat{\sigma}$ is defined:

$$(5) \quad \hat{\sigma} = \frac{1}{N-3} \sum_{i=1}^3 \sum_{j \in X_i} (x_{ij} M_i)^2.$$

The t statistic is used to rank the relative significance of each contrast for pattern matching. A probability value corrected for multiple comparisons was estimated using a bootstrap method. First, samples are randomly reassigned without replacement to different groups, and the maximum contrast t over all genes for the rearranged groups calculated. This process is repeated n times, and an estimated distribution is built of all the maximum ts calculated for each permutation. These bootstrap ts (t_1, t_2, \dots, t_n) are ordered from least to greatest, with t_1 being the least and t_n the greatest. Finally, each observed maximum t for the individual genes was compared with the bootstrap distribution, and a probability value estimated: for observed t greater than any bootstrap t , $P=0$; otherwise, the nearest bootstrap t_i was found, and $P=1-i/n$. For the results presented in this article, $n=100$ was used.

Microarray Validation

Using GeneExpress expression analysis software, raw expression values of genes were determined for the NF, HF, and LVAD samples. The DecisionSite Functional Genomics analytical software was used to perform principal component analysis and samplewise-3D cluster plots were generated for each experimental group. The sample set for validation was derived from samples clustered within two standard deviations along the three principal component axes for the each group. The final validation sample set included 13 NF, 29 HF, and 21 LVAD samples. Quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR) was performed using TaqMan chemistry (Applied Biosystems) and the ABI Prism 7900 Sequence Detection System. Relative target mRNA was normalized to the 18S ribosomal control in the same sample; each sample was run in triplicate; and the results were averaged. For intergroup analysis, Q-RT-PCR data were analyzed by one-way ANOVA with Fishers PLSD post-tests when appropriate.

Assessment of Protein Abundance

In a subset of the samples used for the Q-RT-PCR analyses, we performed Western blot analysis to examine the abundance of two

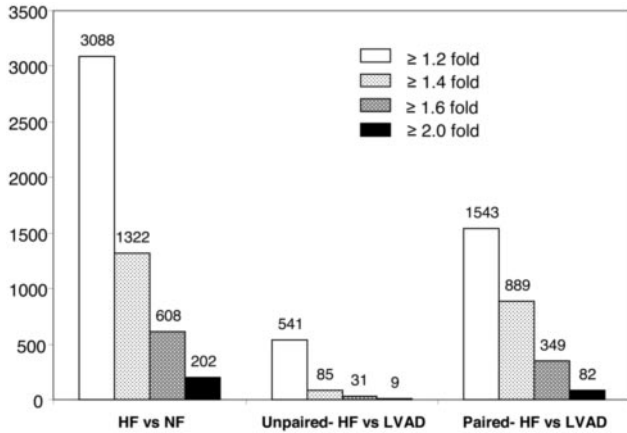


Figure 2. Number of genes exhibiting significant differences in expression based on simple contrasts between experimental groups. Results are shown for HF vs NF; HF vs LVAD based on an analysis of all samples; and HF vs LVAD based on analysis of only the 11 paired pre- and post-LVAD samples from the same patient. The majority of statistically significant intergroup differences involve less than a 2-fold difference in gene expression.

proteins encoded by mRNA species that matched our predefined “normalization” templates. Because a variety of factors other than mRNA abundance can affect protein abundance, our purpose here was simply to illustrate the possible relationships between protein and mRNA abundance rather than validate the mRNA measurements per se. Protein extraction and Western blot analysis were performed as previously described¹⁴ For these experiments, we used commercially available antibodies for the following proteins: PDK1_{301 to 312} (Abcam) and SOCS3_{209 to 225} (Abcam). Rabbit and goat IgGHRP were obtained from Abcam.

Results

Reverse Remodeling Phenotype in LVAD-Supported Hearts

Isolated myocyte median cell volume was derived by Coulter analysis in 113 HF, 6 NF, and 12 LVAD-supported hearts. Average values were 46 308±1227 μm³ in HF, 34 384±7096

μm³ in NF, and 38 125±4121 μm³ in LVAD hearts (*P*<0.05 HF versus NF; *P*<0.05 HF versus LVAD). Isolated myocyte mean cell length was derived by image analysis in 103 HF, 5 NF and 12 LVAD-supported hearts. Average values were 204±3 μm in HF, 136±9 μm in NF and 170±8 μm in LVAD hearts (*P*<0.001 HF versus NF; *P*<0.001 HF versus LVAD; *P*<0.05 NF versus LVAD). In a paired analysis of 11 LVAD-supported hearts, the QTc interval before LVAD support was 456±12 ms and the QTc after LVAD support was 427±13 ms (*P*<0.005). Together, these findings indicate that the hearts used for our transcriptional analysis exhibited typical morphological and functional responses to LVAD support.

Simple Intergroup Contrasts

The results of separate contrasts between NF versus HF, HF versus post-LVAD (unpaired samples), and pre-LVAD versus post-LVAD (paired samples) are summarized in Figure 2. All genes exhibiting statistically significant differences in these three contrasts are listed in online Tables 1 through 3 in the online data supplement available at <http://circres.ahajournals.org>. When a fold-change inclusion criterion of ≥1.2 was used, there were 3088 statistically significant differences observed between HF and NF and 541 differences observed between the HF and LVAD groups. The comparison of 11 paired pre- and post-LVAD samples from the same patient yielded 1543 significant differences. For all comparisons, the significant differences in transcription between HF and LVAD samples usually involve small changes in gene expression, with few genes exhibiting a greater than 1.6-fold difference.

Balance Among Alternative Transcriptional Patterns After LVAD Support

From the 3088 genes showing significant dysregulation in failing hearts, we used our multicomparison, pattern-matching approach to examine how many demonstrate each of the five general types of transcriptional shifts after LVAD support (Figure 1C). As shown in Figure 3, 132 genes

Frequency of Alternative Expression Patterns Among Genes That Are Abnormal in HF

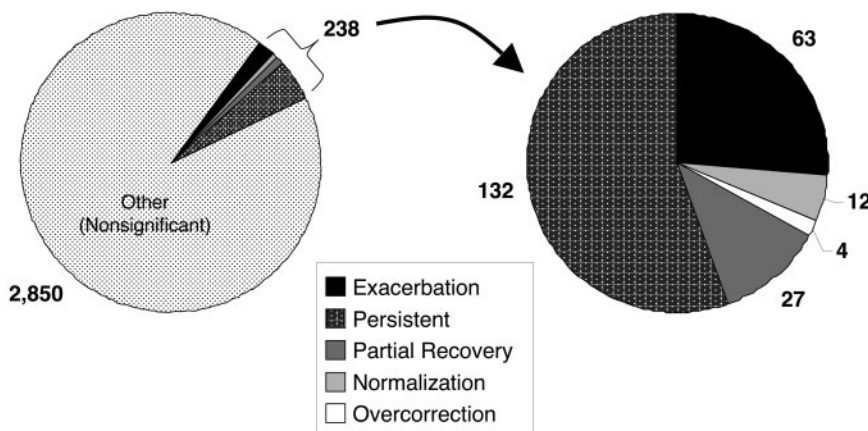


Figure 3. Frequency of alternative transcriptional responses to LVAD support among genes dysregulated in heart failure. For the 3088 genes exhibiting significant ≥1.2-fold differences in expression between NF and HF groups, responses to LVAD support are categorized based on where post-LVAD responses fall along the continuum of expression intensities defined by the NF and HF groups as illustrated in Figure 1. Left, Only 238 of these genes demonstrated a statistically significant match to one of the predefined patterns, whereas the responses for other genes were too inconsistent to achieve a statistically significant match to any of the five patterns. Right, When transcriptional shifts were consistent, LVAD-associated persistence and exacerbation of pathological dysregulation were much more common than partial recovery, normalization, or overcorrection.

exhibited a clear pattern of persistent dysregulation, whereas only 12 genes exhibited a pattern of normalization with another 27 genes exhibiting partial recovery. Interestingly, 63 genes fit the pattern of exacerbation of the dysregulation associated with HF, whereas only four demonstrated overcorrection of HF-associated abnormalities. The majority of genes dysregulated in HF (versus NF) exhibit LVAD-associated changes that are not consistent enough to match the predetermined patterns. Online Table 4 includes the full expression statistics for each the specific genes matching each of the 10 predefined pattern templates with the α value set at $P < 0.05$.

Phenomenology-Guided Pathway Exploration

During the past decade, many investigators have reported myocardial adaptations during LVAD support that suggest improvements in specific aspects of the heart failure phenotype. Among the phenomenology reported to date are LVAD-associated regression of pathological hypertrophy,² improvements in basal and rate-dependent contractility,^{3,5} improved β -adrenergic responsiveness,^{3,6,16} shortening of action potential duration,⁴ reduced myocardial apoptosis,⁷ and changes in cardiac metabolism.¹⁵ Within the pathways implicated by these previous reports, we hypothesized there would be an increased frequency of genes displaying normalization or partial recovery patterns. Accordingly, we examined the expression patterns of 473 separate fragments implicated by previous reports of LVAD-associated myocardial adaptations including calcium-handling proteins, sarcolemmal ion channels, β -adrenergic signaling molecules, TNF- α -associated genes, sarcomeric proteins, and regulators of protein translation, growth, apoptosis, extracellular matrix remodeling, and lipid or carbohydrate metabolism. After eliminating fragments that were not present in at least 20% of samples from at least one of the sample groups, there were 203 separate fragments that fit one or more of these phenomenology-related categories.

Online Table 5 includes the full expression statistics for each of these 203 specific fragments. In many cases, a given gene is represented by more than one fragment. From this list of fragments, we identified those in which the pattern of maximum Pearson correlation coefficient matched the pattern with the maximal t value, and then identified those gene fragments with a ≥ 1.2 -fold change difference based on microarray data and a probability value of at least $P < 0.05$ (without correction for multiple comparisons). Using these liberal inclusion criteria, we identified 57 fragments with a statistically significant match to one of our predefined templates. Of these, the distribution of fragments significantly matching the five patterns was as follows: 20 exacerbation, 18 persistence, 6 partial recovery, 4 normalization, and 9 overcorrection. Thus, even among a group of fragments implicated by reported recovery phenomena, partial or complete transcriptional recovery is infrequent compared with persistence or exacerbation of the HF transcriptional pattern.

Q-RT-PCR Validation of Microarray Results

We used quantitative reverse transcriptase PCR (Q-RT-PCR) as a means of validating a number of the genes found to exhibit either normalization or persistence patterns based on

the microarray analysis. The results of this analysis are presented in Table 2. Q-RT-PCR confirmed a normalization pattern in two of eight genes and indicated partial normalization in one case. Among the 14 genes exhibiting a persistence pattern based on the microarray analysis, 11 were confirmed to have a persistence pattern, two exhibited partial recovery, and one showed normalization by Q-RT-PCR. The higher validation rate for genes exhibiting persistent dysregulation is probably because of their greater magnitudes of dysregulation (by fold-change analysis).

Assessment of Protein Abundance

To illustrate the possible relationships between protein and mRNA abundance, we performed Western analysis to assess the abundance of two proteins, PDK1 and SOCS3, that demonstrated transcriptional normalization based on the microarray analysis. As shown in Figure 4, Western analysis indicated a normalization of protein abundance after LVAD support for both molecules. The reduced abundance of PDK1 protein observed after LVAD support was well-correlated with the changes in transcript abundance based on microarray analysis. However, the reduced expression of SOCS3 protein observed after LVAD support was the opposite of the increase in expression suggested by the microarray analysis.

Discussion

By using an unusually large number of high-quality human myocardial samples, microarray technology, and an analytical strategy that a priori defines patterns of interest based on the results of multiple comparisons, the present study has generated several novel observations about broad patterns of transcription in human myocardium. Over 3000 genes exhibit statistically significant dysregulation in heart failure, and 202 genes exhibit a 2-fold or greater difference between NF and HF samples. However, among these dysregulated genes, a relatively small number exhibit a pattern of normalization, partial recovery, or overshoot after LVAD support despite numerous previous reports demonstrating multilevel myocardial improvements with this intervention. Rather, the majority of genes that are dysregulated in HF do not exhibit consistent changes after LVAD supported hearts whereas a sizeable number demonstrate persistence or exacerbation of the transcriptional phenotype associated with HF. Moreover, of the transcriptional adaptations that do occur after LVAD support, most do not represent a return to pattern associated with the nonfailing heart (ie, normalization). Thus, it appears that there is transcriptional "hysteresis" in that many transcriptional changes during LVAD support are distinct from a return toward normalcy and could represent processes unique to myocardial unloading or recovery pathways that are not the simple inverse of changes observed during disease progression.

The fact that a sizeable number of failure-associated transcriptional changes persist or worsen as myocardial phenotype is changing (during LVAD support) raises doubts about the degree to which many transcription changes observed in the end-stage heart contribute to the development of pathological phenotype. In this regard, studies in transgenic mice and animal models of acquired disease suggest that etiology-

TABLE 2. Comparative Microarray and Q-RTPCR Analysis

Gene Symbol	NF vs HF Fold-Change		Statistical Significance by Q-RTPCR			Pattern	
	Microarray	Q-RTPCR	NF vs HF	HF vs LVAD	NF vs LVAD	Microarray	Q-RTPCR
<i>SOCS3</i>	3.13	4.99	0.0005	0.03		Normalization	Normalization
<i>NPPA</i>	2.64	6.70	0.05			Normalization	Partial recovery
<i>C14orf27</i>	1.48	1.85				Normalization	No change
<i>SH3GL2</i>	1.48	1.02				Normalization	No change
<i>RGS5</i>	1.88	1.11				Normalization	No change
<i>SAMSN1</i>	1.73	2.6	0.01	0.003		Normalization	Normalization
<i>SPTBN1</i>	1.68	1.17				Normalization	No change
<i>PDK1</i>	1.58	1.05		0.01		Normalization	Alternative†
<i>NPTX2</i>	4.07	5.22	<0.0001		<0.0001	Persistence	Persistence
<i>ASPEN</i>	6.14	5.17	0.03		0.003	Persistence	Persistence
<i>TTID</i>	5.93	3.12	<0.0001		0.001	Persistence	Persistence
<i>PLEC1</i>	3.34	1.61	0.02			Persistence	Partial recovery
<i>NAV2</i>	4.59	4.07	<0.0001		0.006	Persistence	Persistence
<i>LUM</i>	3.84	2.60	0.002		0.03	Persistence	Persistence
<i>ANKRD2</i>	2.80	5.28	<0.0001		<0.0001	Persistence	Persistence
<i>OGN</i>	3.63	3.46	0.03		0.003	Persistence	Persistence
<i>PTN</i>	3.04	2.75	0.02		0.02	Persistence	Persistence
<i>CCT2</i>	2.74	2.53	<0.0001		<0.0001	Persistence	Persistence
<i>PLA2G2</i>	4.22	12.2	<0.0001	0.04	0.003	Persistence	Partial recovery
<i>XLKD1</i>	2.26	3.32	<0.0001	0.003		Persistence	Normalization
<i>ADFP</i>	3.48	5.72	0.003		0.008	Persistence	Persistence
<i>MGST1</i>	3.28	7.84	<0.0001		<0.0001	Persistence	Persistence

†Similar in NF and HF; LVAD significantly different from both.

Twenty-two genes for which the microarray analysis indicated either normalization or persistence of pathological gene expression after LVAD support were examined by Q-RTPCR in a set of 63 samples as described in the Materials and Methods. For the 16 genes where microarray analysis indicated greater than 2-fold expression difference between NF and HF groups, a difference was confirmed by Q-RTPCR, but validation rates were lower for smaller differences in expression. As a result, the pattern of persistent dysregulation after LVAD support was usually validated by Q-RTPCR, but this was often not the case for genes with post-LVAD normalization based on the microarray analysis.

specific transcriptional changes occur relatively early in the course of disease and that most transcriptional changes observed in late disease are responses to, rather than causes of, advanced hypertrophy and failure.¹⁶ It is also possible that the lumping of all HF or post-LVAD samples together tends to obscure important etiology-specific, host-specific, and/or time-dependent changes in transcriptional regulation as sug-

gested by Boheler et al.¹⁷ Another caveat is that even validated microarray analysis cannot define possible functional distinctions between stable “pools” of mRNA, de novo synthesized mRNA, and posttranscriptional mRNA processing. Thus, discordance between microarray findings and structural and functional adaptations could reflect differences in mRNA localization, processing and stability, translation

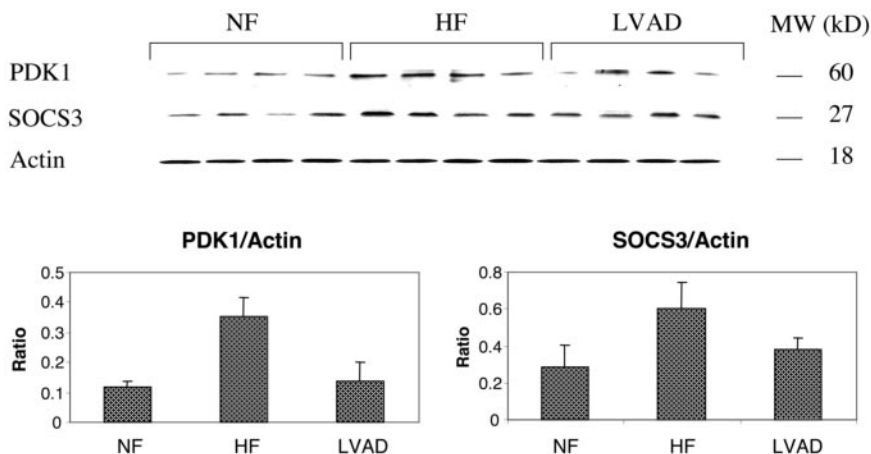


Figure 4. Correlation of microarray findings with protein abundance. Top portion of the figure shows the Western blots for PDK1 and SOCS3, two molecules demonstrating transcriptional normalization based on the microarray analysis. Mean protein abundance results (corrected for actin abundance) are shown in the bottom panels. Although LVAD-associated normalization of protein abundance was observed for both molecules, the reduced abundance of PDK1 was correlated with the changes in transcript abundance, but the reduced expression of SOCS3 protein observed after LVAD support was the opposite of the transcriptional increase observed by the microarray analysis.

efficiency and capacity, and/or posttranslational protein modifications or interactions that may be more pivotal in regulating myocardial function and recovery. The discordance we observed between the mRNA and protein abundance assessments for SOCS3 in the same samples illustrate the potential impact of posttranscriptional processes.

Similarly, the scarcity of genes showing partial recovery or normalization of transcription coupled with the many reports of myocardial improvements after LVAD support, suggests new insights into the biology of myocardial reverse remodeling. Irrespective of whether paired or unpaired samples are used, many transcriptional changes that follow LVAD support are distinct from reversions to or toward the normal phenotype and could represent processes unique to myocardial unloading or perhaps specific pathways that are only altered during myocardial recovery. More generally, it is possible that the molecular adaptations (transcriptional or otherwise) leading to myocardial recovery may not simply be the inverse of those associated with the development of heart failure. Finally, it is possible that some important processes involved with reverse remodeling are not primarily regulated via transcriptional control. For example, although myocyte size decreases during LVAD support, sarcomeric protein transcripts do not. This discordance is consistent with previous studies indicating that sarcomeric protein abundance is largely regulated via posttranscriptional, rather than transcriptional, control.^{18,19}

One explanation for the surprisingly large number of genes that exhibited persistence or exacerbations of dysregulation associated with HF is that some changes during myocardial unloading are simply plasticity responses that may be observed with myocardial remodeling irrespective of the direction. Indeed, this explanation is consistent with the studies of Depre et al¹¹ who observed similar changes in transcription during either surgically-induced loading or unloading of normal rat hearts. It is also possible that some of the genes exhibiting either persistence or exacerbation patterns might be functionally important in limiting the degree of myocardial recovery that occurs during LVAD support. If this were true, such genes might provide clues for developing adjuvant therapeutic strategies designed to promote more complete or durable recovery.

Limitations

Although LVAD-supported myocardium has provided the substrate for nearly all of the tissue-based studies of reverse remodeling in humans, it is possible that the infrequency of transcriptional normalization or partial recovery observed in this study may not be representative of the myocardial recovery process in other settings. Other therapeutic interventions sometimes associated with significant myocardial improvements, including beta-blocker therapy,²⁰ revascularization²¹ or multisite pacing,²² are nearly always applied in situations with less advanced myocardial failure. Consequently, myocardial improvements in other settings might involve greater transcriptional plasticity. Finally, it is also possible that the timing of tissue removal after LVAD support (at the time of transplantation) may not coincide with the time at which changes in gene transcription have their main

impact. Further studies will be needed to more carefully explore the extent to which the duration of mechanical support affects the balance between alternative transcriptional patterns associated with LVAD support or relevant models of reverse remodeling.

Conclusion

Increasingly, transcriptional or proteomic analysis is being used in data-driven discovery strategies designed to identify new mechanistic hypotheses. In many of these inquiries, pathological states are used as a starting point, and responses to pharmacological, molecular, or surgical interventions are examined using high throughput techniques. In these settings, we believe that our multicomparison pattern-matching analytical strategy is both powerful and broadly applicable. Specifically, incorporating a benchmark of normalcy into a simple pre- versus postintervention analysis permits a biologically intuitive sorting of responses to complement pure statistical techniques. By providing a biological context (ie, moving toward normal versus moving away from normal), our analytical strategy can help prioritize subsequent functional and mechanistic inquiries without arbitrarily increasing the stringency of fold-change or consistency thresholds.

Acknowledgments

The transcriptional analyses reported in this manuscript were performed and funded by Gene Logic, Inc (Gaithersburg, Md). This work was also supported by the NIH AG17022 (to K.B.M.). The authors acknowledge the contributions of Temple University cardiothoracic surgeons, operating room staff, and Cardiovascular Research Center personnel for their assistance with tissue procurement.

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