Decreased SLIM1 Expression and Increased Gelsolin Expression in Failing Human Hearts Measured by High-Density Oligonucleotide Arrays

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Background—Failing human hearts are characterized by altered cytoskeletal and myofibrillar organization, impaired signal transduction, abnormal protein turnover, and impaired energy metabolism. Thus, expression of multiple classes of genes is likely to be altered in human heart failure.

Methods and Results—We used high-density oligonucleotide arrays to explore changes in expression of \( \approx 7000 \) genes in 2 nonfailing and 2 failing human hearts with diagnoses of end-stage ischemic and dilated cardiomyopathy, respectively. We report altered expression of (1) cytoskeletal and myofibrillar genes (striated muscle LIM protein-1 [SLIM1], myomesin, nonsarcomeric myosin regulatory light chain-2 [MLC2], and \( \beta \)-actin); (2) genes responsible for degradation and disassembly of myocardial proteins (\( \alpha \)-antichymotrypsin, ubiquitin, and gelsolin); (3) genes involved in metabolism (ATP synthase \( \alpha \)-subunit, succinate dehydrogenase flavoprotein [SDH Fp] subunit, aldose reductase, and TIM17 preprotein translocase); (4) genes responsible for protein synthesis (elongation factor-2 [EF-2], eukaryotic initiation factor-4AII, and transcription factor homologue-HBZ17); and (5) genes encoding stress proteins (\( \alpha \)B-crystallin and \( \mu \)-crystallin). In 5 additional failing hearts and 4 additional nonfailing controls, we then compared expression of proteins encoded by the differentially expressed genes, \( \alpha \)B-crystallin, SLIM1, gelsolin, \( \alpha \)-antichymotrypsin, and ubiquitin. In each case, changes in protein expression were consistent with changes in transcript measured by microarray analysis. Gelsolin protein expression was also increased in cardiomyopathic hearts from tropomodulin-overexpressing (TOT) mice and rac1-expressing (racET) mice.

Conclusions—Altered expression of the genes identified in this study may contribute to development of the heart failure phenotype and/or represent compensatory mechanisms to sustain cardiac function in failing human hearts. (Circulation. 2000;102:3046-3052.)

Key Words: heart failure ■ cardiomyopathy ■ gene expression

End-stage heart failure represents a clinical end point secondary to a diverse range of cardiac diseases, e.g., hypertension, coronary artery disease, left ventricular hypertrophy, valvular heart disease, idiopathic cardiomyopathy, diabetes, viral infection, and genetic mutations. All failing hearts have in common a temporary or permanent decline in contractile function. At the cellular level, the functional impairment is observed as a decreased amplitude and time course of myocyte contraction, as well as abnormal calcium cycling. Abnormal cytoskeletal remodeling, attenuated inotropic response to \( \beta \)-adrenergic stimulation, impaired metabolism, and altered protein turnover are also correlated with development of heart failure. There is an urgent need to better understand the cellular events that trigger the cascade of functional and structural changes that result in development of heart failure, as well as the compensatory changes that take place to preserve cardiac function.

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Because heart failure is a multifactorial disease, expression of multiple clusters of genes is likely to be altered.\(^1\) Large-scale sequencing studies have shown that the frequencies of several expressed sequence tags (ESTs) differ in cDNA libraries from hypertrophic versus nonfailing hearts.\(^2\) Differ-
ential patterns of gene transcription by microarray analysis have also been reported in animal models of cardiomyopathy and myocardial infarction. However, quantitative measurements of changes in expression of large numbers of genes in end-stage human heart failure have not been reported.

We used high-density oligonucleotide arrays to simultaneously quantify expression of ~7000 full-length genes in 2 nonfailing and 2 failing human hearts with diagnoses of end-stage ischemic (ICM) and dilated cardiomyopathy (DCM), respectively. Nineteen genes in 5 gene classes demonstrated altered expression levels in both failing hearts. Western blot analysis of a sample of proteins encoded by a sample of these genes showed consistent changes in protein expression. Our results point to changes in cytoskeletal and myofibrillar organization, protein turnover, and energy metabolism in the failing human heart.

**Methods**

**Human Myocardium**

ICM and DCM myocardium was obtained from 7 explanted hearts of transplant recipients at the Cleveland Clinic Foundation with diagnoses of end-stage heart failure (New York Heart Association class IV) (Table 1). Tissue from 6 nonfailing human hearts was obtained from unmatched organ donors through LifeBanc of Northeast Ohio (Table 1). All tissue was flash-frozen as previously described. The protocol for tissue procurement was approved by the Cleveland Clinic Institutional Review Board.

**Hybridization of cRNA to the High-Density Oligonucleotide Arrays**

Poly(A) RNA (1 μg) was isolated from the left ventricular free wall of 2 failing (ICM1 and DCM1) and 2 nonfailing (NF1 and NF2) hearts. Biotinylated cRNA was generated as previously described. Fragmented biotin-cRNAs (50 μg) were hybridized to the perfect match (PM) and mismatch (MM) oligonucleotides on the A, B, C, and D Hu6800 GeneChip (Affymetrix).7

**Data Analysis**

Average difference intensity (Avg Diff), equivalent to level of gene expression, was determined for each cDNA on the chips by use of GeneChip 3.1 software (Affymetrix). In addition to the default parameters of the software, we added additional criteria that a threshold of 6 positive probe pairs and >50 Avg Diff units per transcript were required for a gene to be considered "present" in our samples.

Probe sets specific for the 5', middle, and 3' region of the GAPDH and β-actin genes are present on each of the A, B, C, and D Hu6800 arrays. Thus, we performed an independent comparison of GAPDH and β-actin expression by 2-way ANOVA to determine differences in hybridization efficiency among the A-D chips of the same sample and differences in expression level of the GAPDH and β-actin genes among the 4 different hearts. The Tukey test was then performed to identify any significant differences in pairwise comparisons for β-actin expression among the 4 hearts. We used a global scaling approach for data comparison, with the average hybridization intensity on each of the A-D chips scaled to a constant value, 150, as recommended by the manufacturer.

Among the 7085 oligonucleotide probes on the Hu6800 GeneChips, 1300 can hybridize to 1 to 5 genes with high sequence homology. To eliminate results from nonspecific hybridization, we excluded these 1300 oligonucleotide probes from our analysis. Oligonucleotides for ribosomal genes were also eliminated. We were therefore able to analyze cRNA hybridization to 5708 of 7085 probes on the Hu6800 arrays.

**Northern Blot Analysis**

To confirm that genes known to be expressed in the heart were present in our samples, we performed Northern blot analysis to determine the abundance of L-type Ca²⁺ channel and Na⁺/Ca²⁺ exchanger transcripts in the ICM1, DCM1, NF1, and NF2 hearts (Table 1). Previous studies have shown differential expression of these 2 genes in failing and nonfailing hearts. cDNA probes for the human L-type Ca²⁺ channel and the Na⁺/Ca²⁺ exchanger genes, obtained from the IMAGE consortium (http://www-bio.llnl.gov), were labeled with [α-32P]dCTP by random prime labeling. A 24mer oligonucleotide for rat 18S rRNA was radiolabeled with T4 kinase. Ten micrograms of total RNA was used for Northern blot analysis.8

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**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Race</th>
<th>LVEF, %</th>
<th>Cause of Death/Diagnosis</th>
<th>Treatment</th>
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<tr>
<td>Nonfailing hearts</td>
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<td></td>
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<tr>
<td>NF1</td>
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<td>DP, CZ</td>
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<tr>
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<td>20</td>
<td>CVA</td>
<td>DP, DB, NE</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>White</td>
<td>10</td>
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<td>15</td>
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<td>CP, DG, ML</td>
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<tr>
<td>DCM2</td>
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<td>Male</td>
<td>Black</td>
<td>11</td>
<td>DCM</td>
<td>DG, EN</td>
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<tr>
<td>DCM3</td>
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<td>White</td>
<td>10</td>
<td>DCM</td>
<td>NA</td>
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<td>DCM4</td>
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<td>White</td>
<td>10–15</td>
<td>DCM</td>
<td>DG</td>
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<td>DCM5</td>
<td>33</td>
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<td>White</td>
<td>5–10</td>
<td>DCM</td>
<td>None</td>
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<tr>
<td>DCM6</td>
<td>68</td>
<td>Male</td>
<td>Other</td>
<td>15</td>
<td>DCM</td>
<td>DG, EN</td>
</tr>
</tbody>
</table>

Other race indicates race other than white, black, or Asian; LVEF, left ventricular ejection fraction; NA, not available; GSW, gun shot wound to head; S-J, suicide, jumped; CVA, cerebrovascular accident; AM, amiodarone; CP, captopril; CZ, Cardizem; DB, dobutamine; DG, digoxin; DP, dopamine; EN, enalapril; ML, milrinone; NE, norepinephrine; and VP, verapamil.
Figure 1. Expression levels of GAPDH, β-actin, and ANF genes in failing and nonfailing hearts. A, B, and C are fluorescent images of biotin-cRNAs from failing (ICM1 and DCM1) and nonfailing (NF1 and NF2) hearts, hybridized to PM and MM oligonucleotides specific for 5', middle, and 3' regions of GAPDH and β-actin cDNAs and 3' region of ANF cDNA, respectively. Hybridization intensities (Avg Diff) for these cDNA probes are shown in D, E, and F, respectively. Blue represents no hybridization, whereas white depicts maximal hybridization. In D and E, bars represent mean Avg Diff units ± SE. By 2-way ANOVA, expression levels of GAPDH gene were not significantly different among these failing and nonfailing hearts or between A-D chips of same heart. However, significant difference (P<0.05) was found for β-actin expression, indicated by *, for NF1 vs ICM1, NF1 vs DCM1, NF2 vs ICM1, and NF2 vs DCM1 but not for NF1 vs NF2 and ICM1 vs DCM1. Avg Diff units of β-actin gene determined on A-D chips from same heart were not significantly different.
Western Blot Analysis

Protein levels of striated muscle LIM protein-1 (SLIM1), gelsolin, αB-crystallin, α-antichymotrypsin, and ubiquitin were measured in 5 different failing (DCM2 to DCM6) and 4 other nonfailing (NF3 to NF6) human hearts (Table 1). The choice of the proteins that we measured by Western blot was determined exclusively by availability of specific antibodies and cross-reactivity of these antibodies with human heart tissue. Protein levels of gelsolin were also determined in cardiomyopathic hearts from rac1-expressing (racET) mice (unpublished results), tropomodulin-overexpressing (TOT) mice (2 weeks old),11 and nontransgenic controls.

Immunoblot analysis was performed as described previously.5 Briefly, cardiac tissue was homogenized in a lysis buffer. Tissue homogenate or supernatant of the homogenate was separated on 10% polyacrylamide gels by SDS-PAGE and transferred onto polyvinylidene fluoride membrane, followed by incubation with anti-SLIM1 rabbit antibody (1:10),12 anti-gelsolin goat antibody (1:1000; Santa Cruz), anti-αB-crystallin rabbit antibody (1:2000; StressGen), anti-α-antichymotrypsin monoclonal antibody (1:3000; QED Bioscience), anti-ubiquitin antibody (1:1000; Zymed), or anti-GAPDH monoclonal antibody (1:3000; Research Diagnostics).

Protein expression was normalized to GAPDH expression on the same immunoblot. Briefly, one portion of the immunoblot was incubated with antibody specific for gelsolin (21 kDa), αB-crystallin (21 kDa), α-antichymotrypsin (~70 kDa), or ubiquitin (~8.5 kDa). Immunoblotting for SLIM1 (~32 kDa) and GAPDH (~36 kDa) was performed sequentially with the same blot. After SLIM1 immunoblot analysis, antibodies were removed from the membrane.13 The stripped membrane was then used for anti-GAPDH immunoblotting. Statistical analysis was performed with Student’s t test.

Results

Sensitivity, Reproducibility, and Specificity of cRNA Hybridization

The detection limit of our GeneChip assay was 1.5 to 5 pmol/L, as previously reported.7 To assess reproducibility of the assay, we analyzed hybridization of GAPDH and β-actin probes on the A-D chips for each failing and nonfailing heart.

Three probe sets specific for the 5′, middle, and 3′ regions of the GAPDH and β-actin genes are present on each of the A-D chips. Biotin-cRNAs hybridized predominantly to PM oligonucleotides specific for the GAPDH gene (Figure 1A). By 2-way ANOVA, the difference in GAPDH hybridization between different hearts was not statistically significant, and there were no statistically significant differences between different A-D chips for each sample (Figure 1D).

Intensity and staining pattern of β-actin hybridization was similar for the 2 nonfailing hearts but reduced in failing hearts.

TABLE 3. Genes Detectable Only in Both Nonfailing Hearts or in Both Failing Hearts

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Definition</th>
<th>NF1</th>
<th>NF2</th>
<th>ICM1</th>
<th>DCM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>J03764</td>
<td>Plasminogen activator inhibitor-1</td>
<td>193</td>
<td>228</td>
<td>65</td>
<td>42</td>
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<tr>
<td>X97544</td>
<td>TIM17 preprotein translocase</td>
<td>403</td>
<td>312</td>
<td>202</td>
<td>94</td>
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<tr>
<td>D30655</td>
<td>Eukaryotic initiation factor-4All</td>
<td>&lt;0</td>
<td>155</td>
<td>791</td>
<td>975</td>
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<tr>
<td>L02950</td>
<td>μ-Crystallin</td>
<td>176</td>
<td>129</td>
<td>968</td>
<td>976</td>
</tr>
<tr>
<td>L36033</td>
<td>Pre-B cell stimulating factor homologue-SDF1β</td>
<td>&lt;0</td>
<td>&lt;0</td>
<td>307</td>
<td>496</td>
</tr>
<tr>
<td>X77366</td>
<td>Transcription factor homologue-HBZ17</td>
<td>&lt;0</td>
<td>&lt;0</td>
<td>330</td>
<td>631</td>
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<tr>
<td>M54951</td>
<td>ANF</td>
<td>198</td>
<td>28</td>
<td>9199</td>
<td>8573</td>
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</tbody>
</table>
Relative to both nonfailing hearts, β-actin expression was decreased by 47% and 49% in the ICM1 and DCM1 hearts, respectively (Figure 1E; Table 2). By 2-way ANOVA, β-actin expression was significantly different among the 4 hearts (P<0.001) but not between the A-D chips from the same heart. By Tukey test, a significant difference (P<0.05) was identified for NF1 versus ICM1, NF1 versus DCM1, NF2 versus ICM1, and NF2 versus DCM1 but not for ICM1 versus DCM1 or NF1 versus NF2. These findings are consistent with previous studies showing β-actin downregulation in failing human hearts.14 Our β-actin and GAPDH results indicate that the efficiency of cRNA hybridization to the A-D chips was comparable among the 4 human hearts assayed.

Atrial natriuretic factor (ANF) is expressed in atria of normal human hearts and in failing ventricles.15,16 We showed that cRNA hybridization to the ANF probes was more intense in both failing hearts than in either of the nonfailing hearts (Figure 1E and 1F). With GeneChip 3.1 software, ANF was found to be present in both failing hearts but absent in nonfailing hearts (Table 1). Results of these studies, together with results for GAPDH and β-actin expression, are consistent with previous findings using alternative methodologies. The GeneChip system has a reported false-positive rate of 0.1% to 2%.17 To further reduce the possibility of false-positive results, we included additional criteria in evaluation of the data. Because of cross-hybridization of the probes (eg, α- and β-myosin heavy chain genes), several genes (eg, cardiac sarcoplasmic reticulum Ca2+ −ATPase) were not present on the Hu6800 GeneChips. Hybridization of the labeled-cRNA to oligonucleotides specific for the L-type Ca2+ channel and Na+/Ca2+ exchanger did not provide a detectable signal for either the failing or nonfailing hearts. However, by Northern blot analysis, the 8-kb L-type Ca2+ channel mRNA and the 6-kb Na+/Ca2+ exchanger mRNA were identified in the same failing and nonfailing hearts that were used for the GeneChip assay. Furthermore, relative to the 18S rRNA, abundance of the L-type Ca2+ channel mRNA decreased, and the Na+/Ca2+ exchanger mRNA was increased in both failing hearts versus the 2 nonfailing controls (Figure 2), consistent with previous reports.8,9 Empirical rules were used by the manufacturer in selection of oligonucleotides to achieve optimal sensitivity and specificity.17 However, individual probes may not perform equally well in hybridization to the cRNAs.18 Results of our studies suggest that the sensitivity of the GeneChip assay may not be comparable for all of the genes on the arrays. However, results of this Northern blot analysis do demonstrate that transcripts undetected on the chips are indeed present in our samples.

**Genes Detectable in Both Nonfailing Hearts**

We next determined which genes were expressed in both NF1 and NF2 hearts. In the present study, a gene with an "undetectable" signal indicates that cRNA hybridization to the oligonucleotide probes of the gene did not meet our minimal criteria for detection. Among 701 to 714 detectable genes in 2 nonfailing hearts, expression levels of 473 genes were considered not different between these 2 hearts (Table I; see data supplement at http://www.circulationaha.org). Although various factors may affect gene expression in different individuals (Table 1), coordinated expression of these 473 genes in both nonfailing hearts suggest that this subset of genes may encode proteins required for normal physiological function of the heart. We used these 473 genes (online Table 1) as a baseline to determine altered gene expression in the 2 failing hearts.

**Decreased SLIM1 Expression in Failing Hearts**

Results of our GeneChip studies showed downregulation of mRNA for SLIM1 in both failing hearts relative to both nonfailing controls (Table 2). To validate and extend these results to the protein level, we also compared expression levels of SLIM1 protein in 4 other nonfailing (NF3 to NF6) and 5 other failing (DCM2 to DCM6) hearts (Table 1). Relative to the GAPDH control, SLIM1 protein expression was downregulated 3.7-fold (P=0.016) in DCM hearts versus nonfailing controls (Figure 3A). Thus, expression levels of SLIM1 mRNA and protein are significantly decreased in failing human hearts. SLIM1 is a recently identified skeletal muscle LIM protein that comprises 4.5 LIM domains expressed in the outflow tract of the developing heart13 and in skeletal muscle. SLIM1 localizes to focal adhesions, which suggests the protein may act to regulate cytoskeletal interactions.19

**Increased Gelsolin Expression in Failing Hearts**

We also demonstrated upregulation of gelsolin mRNA in both failing hearts (Table 2). Quantitative immunoblot analysis was also performed to compare gelsolin protein levels between the nonfailing and DCM human hearts. We showed...
a 1.6- to 2.3-fold increase ($P=0.005$) in gelsolin protein in DCM hearts ($n=5$) relative to nonfailing controls ($n=4$) (Figure 3B).

To explore altered gelsolin expression in heart failure animal models, we performed additional immunoblot analysis in cardiomyopathic hearts of the racET mice and TOT mice, as well as nontransgenic controls. Development of dilated cardiomyopathy and heart failure in the juvenile TOT mice has been described previously. The TOT mouse hearts showed a 3.0-fold increase in gelsolin expression relative to the nontransgenic controls (Figure 3B). Similarly, 3.9-fold and 2.0-fold increases in gelsolin protein levels were found in hypertrophic and dilated cardiomyopathic racET mouse hearts, respectively (Figure 3B). Thus, gelsolin expression is upregulated in failing human hearts, and these changes are correlated with similar changes in cardiomyopathic hearts from 2 different lines of transgenic mice exhibiting cardiac hypertrophy or heart failure phenotypes.

Altered Expression of 5 Clusters of Genes in the Failing Hearts

Other genes with increased or decreased expression levels in the failing human hearts were also identified. Of 14 differentially expressed genes, 12 genes encoding proteins with known functions are listed in Table 2. In addition, we identified genes that were only detectable in both nonfailing hearts and another subset of genes that were only detectable in both failing hearts (Table 3). These genes can be categorized into 5 gene clusters: (1) genes encoding cytoskeletal and contractile proteins, ie, SLIM1, myomesin MLC2, and $\beta$-actin; (2) genes encoding proteins responsible for degradation or disassembly of myocardial proteins, ie, gelsolin, $\alpha_1$-antichymotrypsin, and ubiquitin; (3) genes encoding proteins involved in metabolism, primarily mitochondrial proteins, ie, ATP synthase $\alpha$-subunit, SDH Fp, aldose reductase, and TIM17 preprotein translocase; (4) genes encoding proteins responsible for protein synthesis, ie, EF-2, eukaryotic initiation factor-4AII, and transcription factor homologue HBZ17; and (5) genes encoding stress proteins, ie, $\alpha_B$-crystallin and $\mu$-crystallin.

Consistent with our GeneChip results, we showed a 5-fold decrease in $\alpha_1$-antichymotrypsin protein ($P=0.007$) in 5 other DCM hearts versus 4 other nonfailing controls (Figure 3C). Similar to previous studies by 2D gel electrophoresis, we also showed decreased expression of $\alpha_B$-crystallin protein in failing human hearts (Table 2; Figure 3D), as well as a significant increase ($P<0.018$) in ubiquitin protein level in failing hearts (Figure 3E).

Figure 3. Altered protein expression in failing human hearts.

Either tissue homogenates (50, 25, 100, and 100 $\mu$g/lane for A, C, D, and E, respectively) or supernatant (150 $\mu$g/lane, B) from nonfailing and failing human hearts or from hypertrophic (hyp) and dilated cardiomyopathic (dil) hearts of racET mice and TOT mice and nontransgenic controls were separated on 10% SDS-PAGE gels and transferred to nitrocellulose (B) or polyvinylidene fluoride membrane (A, C, and D). Immunoblots were incubated with antibody specific for SLIM1 (A), gelsolin (B), $\alpha_1$-antichymotrypsin (C), $\alpha_B$-crystallin (D), and ubiquitin (E). Immunoblots were developed using an enhanced chemiluminescence assay. Bars represent normalized protein expression $\pm$ SE.
Discussion

The ICM1 and DCM1 hearts used in this microarray analysis of gene expression were from patients of different age, sex, race, diagnosis, and treatment (Table 1). However, despite these differences, we were able to identify similar changes in expression of 12 genes in both failing hearts, which indicates that these changes may be fundamental to development and progression of human heart failure (Table 2). We also identified an additional 5 genes expressed only in failing hearts, plus 2 genes present only in the nonfailing hearts (Table 3). Some of the changes we observed, eg, in ANF, β-actin, and αB-crystallin, are consistent with previous studies.14–16,20 Our results from the GeneChip assay (Table 2) are also validated by the results of our Western blot analyses (Figure 3).

Both SLIM1 mRNA and protein were downregulated in failing human hearts (Table 2; Figure 3A). Although the precise function of SLIM1 has not been determined, its expression in the outflow tract of the developing heart and its localization to focal adhesions suggests it may act in an analogous manner to muscle LIM protein (MLP).12,19 MLP serves as a scaffold for interaction between the thin filaments and the cytoskeleton,21 and MLP knockout mice develop dilated cardiomyopathy.22 Both MLP and SLIM1 contain LIM domains with sequence similarity.

Gelsolin mRNA was upregulated in the ICM1 and DCM1 hearts (Table 2), and gelsolin protein was also upregulated in 5 different failing human hearts versus 4 different nonfailing hearts (Figure 3B). Increased expression of gelsolin was also identified in 2 different mouse models of heart failure (Figure 3B). Gelsolin regulates assembly and turnover of thin filaments in cardiac myocytes.23 Recent studies have shown that L-type Ca$^{2+}$ channel activity is significantly increased in neonatal myocytes isolated from gelsolin knockout mouse hearts.24 Thus, increased expression of gelsolin in failing hearts could contribute to perturbation of thin filament organization and attenuation of Ca$^{2+}$-induced Ca$^{2+}$ release in failing heart muscle cells.

In the present study, conservative strategies were used to evaluate the GeneChip data; specifically, a high threshold was set for identification of differentially expressed genes in failing versus nonfailing hearts. Thus, the number of false-negative results is likely to be increased, ie, we may have underestimated the number of genes showing significant differences in expression between failing and nonfailing hearts. Nevertheless, as we have demonstrated by evidence of altered expression of novel genes and their encoded proteins, gene profiling by high-density oligonucleotide arrays represents a useful tool for identification of genes and gene families that may contribute to development and progression of heart failure.

Acknowledgments

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References

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