Cardiac Overexpression of Myotrophin Triggers Myocardial Hypertrophy and Heart Failure in Transgenic Mice*

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Cardiac hypertrophy and heart failure remain leading causes of death in the United States. Many studies have suggested that, under stress, myocardium releases factor(s) triggering protein synthesis and stimulating myocyte growth. We identified and cloned myotrophin, a 12-kDa protein from hypertrophied human and rat hearts. Myotrophin (whose gene is localized on human chromosome 7q33) stimulates myocyte growth and participates in cellular interaction that initiates cardiac hypertrophy in vitro. In this report, we present data on the pathophysiological significance of myotrophin in vivo, showing the effects of overexpression of cardiосpecific myotrophin in transgenic mice in which cardiac hypertrophy occurred by 4 weeks of age and progressed to heart failure by 9–12 months. This hypertrophy was associated with increased expression of proto-oncogenes, hypertrophy marker genes, growth factors, and cytokines, with symptoms that mimicked those of human cardiomyopathy, functionally and morphologically. This model provided a unique opportunity to analyze gene clusters that are differentially upregulated during initiation of hypertrophy versus transition of hypertrophy to heart failure. Importantly, changes in gene expression observed during initiation of hypertrophy were significantly different from those seen during its transition to heart failure. Our data show that overexpression of myotrophin results in initiation of cardiac hypertrophy that progresses to heart failure, similar to changes in human heart failure. Knowledge of the changes that take place as a result of overexpression of myotrophin at both the cellular and molecular levels will suggest novel strategies for treatment to prevent hypertrophy and its progression to heart failure.

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EXPERIMENTAL PROCEDURES

Animals—Animal studies were approved by the institutional Animal Research Committee according to internal policies and guidelines of the National Institutes of Health for the humane care and use of animals in research.

Generation of Transgenic Mice—An α-MHC-myoctrophin transgene was constructed using a recombinant myotrophin (myo) in pcDNA3-myo-vector. We designed a 72-base oligomer carrying the 5′ skeletal α-actin untranslated region and used it to generate a chimera myo/myo placed adjacent to the κ-cytomegalovirus promoter of pcDNA3. The α-MHC promoter-containing vector was provided by Dr. Jeffrey Robbins (University of Cincinnati). This vector has an α-MHC promoter and a heterologous 3′-untranslated region containing the human growth hormone poly(A) site. The α-MHC-myocardin recombinant vector was digested using the NotI restriction enzyme. The resulting linear DNA fragment containing the α-MHC promoter-myo-coding sequence and human growth hormone poly(A) was isolated and purified using Qiagen gel extraction kit (Qiagen, Valencia, CA). Pronuclear injection was performed at the University of Cincinnati transgenic animal facility, using standard techniques.

Determination of the Ratio of Heart Weight to Body Weight—Mice were euthanized by CO2, and autopsied immediately for gross signs of heart failure, inflammatory lesions, or congenital defects. The hearts were then removed, washed with 1× phosphate-buffered saline, blotted dry, and weighed in a Mettler precision balance (Mettler-Toledo, Inc., Columbus, OH). Hypertrophy was measured using the heart weight (HW)/body weight (BW) in mg/g (3).

Hybridization Analysis—Four founder mice were identified by Southern analysis of genomic DNA (10 μg) prepared from tail biopsies. Northern blots were performed with ~20 μg of total cardiac RNA. The labeled myotrophin cDNA probe was generated using random primers, as described previously (9).

Quantitation of Myotrophin Protein—Western blots were performed using 10 μg of total protein from 9-month-old wild-type (WT) and transgenic (Tg) hearts via standard techniques using monoclonal anti-myotrophin antibody and was normalized using glyceraldehyde-3-phosphate dehydrogenase antibody (Novus Biologicals Inc., Littleton, CO). Myotrophin from the hearts of Tg and WT mice was quantitated as described previously (8).

RNase Protection Assay—Total RNA from WT and Tg mice hearts (4 weeks and 9 months old; n = 5) was extracted as described previously (10), and 15 μg was used in an RNase protection assay using templates specific for growth factors and cytokines according to the manufacturer’s protocol (RiboQuant; BD PharMingen; MCK-3B template set). After RNase digestion, protected fragments were resolved on 6% denaturing polyacrylamide gels and quantified using a PhosphorImager. We normalized the data using the human phosphoglycerate kinase (PGK) gene (11). Myotrophin gene expression was normalized to the PGK for comparison with expression in the heart. Statistical analysis was performed using a Student t test.

Statistical Analysis—Results were expressed as mean ± S.E. Data was analyzed by two-way analysis of variance, and differences between groups were determined by a least-square means test (SUPEROVA). A value of p < 0.05 was considered significant.

Gene Array Studies—Gene array analyses utilized RNA from heart tissue isolated from WT and Tg mice aged 4 weeks (initiation of hypertrophy) or 36 weeks (end-stage transition from hypertrophy to heart failure). Five WT and five Tg animals from each age group were examined. Total cellular RNA (10 μg) was reverse transcribed, and double-stranded cDNA (1 μg) was transcribed into cRNA (Enzo Bioarray RNA transcript labeling kit; Affymetrix, Inc., Santa Clara, CA). Biotinylated and fluor-labeled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips (Affymetrix, Inc., Santa Clara, CA) and measured using the GeneChip Reader (Affymetrix, Inc.). A value of p < 0.05 was considered significant.

Gene Array Analysis—Gene expression data were analyzed using the GeneChip Operating System (Affymetrix, Inc.). Experimental data were filtered to allow only genes showing significant expression differences with a minimum fold change of 2.0. Gene expression data were normalized using a proprietary algorithm, Significance Analysis of Microarrays (SAM), which is available as part of the Bioconductor project in the R statistical software package. Statistical analysis was performed using a Student t test.

Gene clusters with similar expression patterns were identified using the self-organizing map (SOM)-clustering algorithm (10) of the Data Mining Tool software package (Affymetrix, Inc.; version 3.0; Affymetrix, Inc.). Gene clusters for each probe set were imported into the Data Mining Tool to identify those with "significant" or "absent" presence of a gene in any of the pertinent samples.

Detailed tables for the changes in gene expression between failing and nonfailing hearts are provided as Supplemental Material (Supplemental Table I).

RESULTS

Generation of Tg Mice

Four founders were identified from the live births resulting from pronuclear injection (42 mice) with the myotrophin transgene. All four lines were expanded by crossing with non-Tg mice. The transmission rate of α-MHC myotrophin transgenic mice showed a 45% transmission frequency, determined by Southern analysis, typical of a Mendelian inheritance (line 1, 81/156 (52%); line 2, 7/32 (22%); line 3, 20/51 (39%); line 4, 20/51 (39%). The ventricles and atria were enlarged significantly in all four lines of Tg mice by 4 weeks of age compared to non-Tg mice.
with the age-matched WT mice. The heart weight/body weight (HW/BW) ratio also increased significantly in all Tg mice during the progression to hypertrophy (Fig. 1, A–B). All four lines of mice displayed myotrophin overexpression and developed significant hypertrophy, which eventually led to heart failure.

At ~36 weeks of age, the Tg mice, which overexpress cardiospecific myotrophin, developed symptoms of heart failure, including lethargy, edema, pulmonary effusion, and lack of alertness. The kidneys of the Tg mice did not differ from those of WT mice (Table I).
Cardiac Hypertrophy in Tg Mice Overexpressing Myotrophin

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Myotrophin Overexpression Was Associated with Histologic Lesions in Heart Tissues of Tg Mice

The LV heart walls of Tg mice (18 weeks old) were severely thickened compared with those of WT mice (Fig. 1C-I) and showed concentric hypertrophy (Fig. 1C-I). Both right and left ventricles of the Tg mice were enlarged and displayed increased septal thickness compared with those of the WT mice. Histology examination showed typical large nuclei in the Tg group, confirming myocyte hypertrophy (Fig. 1C-II). Multiple foci of classic myocyte disarray were observed in the Tg mouse tissue, a change not present in the WT hearts (Fig. 1C-II). Fibrotic foci accompanied by dystrophic calcification were also observed in the Tg mice but were absent in WT hearts (Fig. 1C-III). Small foci of apparent myocyte slippage surrounded the coronary vessels.

Myotrophin Is Overexpressed in Heart Tissue of Tg Mice

Myotrophin mRNA (Fig. 2A-I) and protein (Fig. 2B-I and 2B) were increased in the mycardium of all four Tg lines through four generations compared with WT mice. When regression analysis was done between mRNA expression and HW/BW in WT and Tg mice (age varying from 18 to 24 weeks) from all four lines, a linear correlation between myophen gene expression and HW/BW was observed (Fig. 2A-2); y = 14.916x + 8.9615 and $r^2 = 0.9691$ for Tg; $y = 15.209x - 15.744$ and $r^2 = 0.9227$ for WT). Fluorescein isothiocyanate-tagged myotrophin was abundant and distinctly visible in the myocytes from 24-week-old Tg mice from all lines, compared with age-matched WT (Fig. 2B-3). However, myotrophin mRNA expression in the kidneys, livers, and lungs of Tg mice did not differ from that in WT mice (data not shown). As a consequence of myotrophin gene overexpression, expression of both hypertrophy marker genes (ANF and β-MHC) and proto-oncogenes (c-fos, c-jun, and c-myc) were also up-regulated in all four generations of the Tg mice lines (Fig. 2, C and D).

Myotrophin Overexpression Leads to Myocyte Hypertrophy in Tg Mice

To document myotrophin overexpression-induced changes in myocytes, we quantitated myocyte dimension by hematoxylin/eosin staining of heart tissue and image scanning using the Image Pro Plus software program. The cross-sectional area of myocytes in both the 4-week-old and 9-month-old mice was significantly increased (Fig. 3A). The myocyte cross-section increased from 350 to 781 per $\mu m^2$ ($p < 0.01$) in the 4-week-old transgenic mice. The increase was even larger in the 9-month-old mice (534–2164) per $\mu m^2$ ($p < 0.001$).

We also quantitated myocyte dimension by isolating myocytes from WT and Tg mice from 9-month-old mice heart. The cross-sectional area of myocytes in 9-month-old mice was significantly increased (Fig. 3B) from 2431 ± 712 per $\mu m^2$ in WT to 6297 ± 1280 per $\mu m^2$ in Tg mice ($p < 0.001$). All myocytes were hypertrophied, and no atrophy was observed. These data provide evidence that cardiac hypertrophy was present in the transgenic animals in all four lines and four generations as early as 4 weeks of age and that this hypertrophy worsened in the older Tg animals (Fig. 3). The cross-sectional areas were quantified in 30 myocytes from each mouse (WT $n = 5$; Tg $n = 8$, representing all four lines).

Cytokine and Growth Factor Gene Up-regulation Is Associated with Disease Stage in Tg Mice

We examined the relative expression of growth factors and cytokines in the Tg mice representing all four lines, using RPA (Fig. 4, $n = 5$), compared with age-matched WT mice. We studied two age groups of animals: 4-week-old mice, which represented the onset of hypertrophy, and 36-week-old mice, which represented the chronic phase of hypertrophy, during its transition to heart failure. A novel finding was the age-associated changes in expression of different cytokines. As shown in Fig. 4A, at 4 weeks of age, some of the cytokine transcripts were induced in Tg hearts, compared with age-matched WT. Expression of LT-β, TGF-β2, and TGF-β3 were significantly up-regulated in 4-week-old Tg mice compared with age-matched WT ($p < 0.05$). In the 36-week-old Tg mice, interleukin-6, macrophage migration inhibitory factor, tumor necrosis factor-α, interferon-γ, and different isoforms of the transforming growth factor-β family were significantly elevated, compared with the age-matched WT mice ($p < 0.01$). However, the percentage increase in cytokine transcripts was comparatively higher in 36-week-old Tg than in 4-week-old Tg mice. Interestingly, expression levels of interleukin-6, tumor necrosis factor-α, interferon-γ, TGF-β2, and macrophage migration inhibitory factor did not change in the young Tg animals during initiation of hypertrophy compared with the age-matched WT mice (Fig. 4B). These data suggest that the cytokine/growth factor-mediated hypertrophic process is different in young and old Tg mice, especially during transition to heart failure.

### Table 1

| Age     | WT ($n = 10$) | Tg ($n = 12^a$) | P-value |
|---------|---------------|----------------|---------|
| 4 weeks |               |                |         |
| HW/BW (mg/g) | 4.8 ± 0.54   | 5.9 ± 0.8⁸     | 0.05    |
| Kidney weight/BW (mg/g) | 7.7 ± 0.1    | 7.6 ± 0.33     | 0.88    |
| Myocyte cross-sectional area ($\mu m^2$) | 350.2 ± 73.8 | 781 ± 217.3⁹   | 0.01    |
| M-mode echocardiographic analysis |          |                |         |
| Left atrial chamber diameter (mm) | 0.16 ± 0.005 | 0.20 ± 0.02⁵   | 0.03    |
| Interventricular septal wall thickness (mm) | 0.1002 ± 0.03 | 0.1140 ± 0.005⁰ | 0.01    |
| Left ventricular posterior wall thickness (mm) | 0.1066 ± 0.004 | 0.1222 ± 0.007⁶ | 0.001   |
| Left ventricular chamber dimension (systolic; mm) | NA⁶ | NA⁶ |         |
| Left ventricular chamber dimension (diastolic; mm) | NA⁶ | NA⁶ |         |
| Fractional shortening (%) | 50 ± 0.01 | 48 ± 0.03 | 0.09    |

* Representing all four lines; 2–4 mice from each line.

⁸ NA, not measurable accurately.

⁵ $p < 0.05$.

D - M-mode echocardiographic data are displayed, showing several parameters in both WT and Tg mice.

### References

For a detailed explanation of the methods and results, please refer to the original publication. The data presented here are intended to highlight the key findings and implications of the study. Further research is recommended to validate and expand upon these observations.
**Fig. 2.** A (1), Northern blot analysis of myotrophin gene expression in transgenic mice from four founders (Fn1–Fn4, 24 weeks old) compared with age-matched WT. A (2), correlation between myotrophin gene expression ($y$ axis) and HW/BW in Tg and WT mice (between 16 and 24 weeks of age, representing all four lines). A significant correlation was observed between myotrophin gene expression and HW/BW ($r^2 = 0.9227$ for WT mice, and $r^2 = 0.9691$ for Tg mice). B (1), Western blot analysis showing myotrophin protein expression in 24-week-old WT and Tg mice from all four lines.
M-mode Echocardiographic Analysis of 9-Month-old Tg Mice Revealed Progression to Heart Failure

M-mode echocardiographic data from the 4-week-old and 9-month-old Tg mice from all four lines are shown in Fig. 5 and Table I. In 4-week-old Tg mice, left atrial diameter (0.20 ± 0.02 mm), interventricular septal wall thickness (0.114 ± 0.004 mm), and left ventricular posterior wall thickness (0.122 ± 0.007 mm) were significantly elevated, compared with their age-matched WT. Importantly, however, the functional parameter, (Fn1–Fn4). B (2), graph showing quantification of myotrophin protein from WT and Tg mice. B (3) shows fluorescein isothiocyanate staining to localize myotrophin in 24-week-old WT and Tg (Fn2) myocytes (magnification, × 63). C shows increased expression of ANF and β-MHC transcripts from four generations (Gn1–Gn4), representing all four Tg lines (24 weeks old), compared with age-matched WT mice. Increased expression of ANF and β-MHC transcripts in all four generations confirmed the presence of hypertrophy in Tg mice. D shows increased expression of proto-oncogenes in the hearts of young and old Tg mice compared with WT mice.
fractional shortening (FS), was not changed in the 4-week-old Tg mice, compared with WT (FS = 50 ± 0.01% in WT versus 48 ± 0.04% in the Tg group (p = not significant)).

Echocardiographic data from 9-month-old Tg mice revealed statistically significant changes compared with the age-matched control mice: hypertrophied septum (0.105 ± 0.004 versus 0.086 ± 0.003 mm, p < 0.01), enlarged LV diastolic dimensions (0.375 ± 0.008 versus 0.334 ± 0.016 mm, p < 0.01), enlarged LV systolic dimensions (0.262 ± 0.037 versus 0.150 ± 0.001 mm, p < 0.02), and lower FS (26 ± 0.09 versus 55 ± 0.03% in WT, p < 0.01). We noted a trend toward left atrial diameter enlargement (0.285 ± 0.05 mm versus 0.214 ± 0.022 mm, p < 0.01) and increased left ventricular posterior wall thickness (0.090 ± 0.007 versus 0.075 ± 0.016 mm, p < 0.05) in the Tg mice. Furthermore, we found a large amount of pleural effusion in the Tg mice, which suggested that hypertrophy had already advanced to heart failure.

These data suggest that in the young Tg mice, cardiac function was not compromised, despite the presence of hypertrophy, whereas in the 9-month-old Tg mice, cardiac function was significantly compromised.

**DNA Microarray Results**

**Changes in Gene Expression at the Initiation of Hypertrophy**—To identify candidate genes that mediate physiological responses to myotrophin overexpression, oligonucleotide gene array analyses were performed on heart samples from Tg and age-matched WT controls. Cardiac RNAs from five transgenic and five WT animals at each age (4 weeks and 9 months) were used in the gene profiling studies. To identify genes with expression patterns that correlated with initiation of hypertrophy (4 weeks) or transition to heart failure (9 months), two strategies were used. Pairwise comparisons between the experimental animals of interest and all other animal samples were used to identify genes with consistent up- or down-regulation at a particular developmental time. In addition, SOM clustering was used to identify gene clusters with similar expression patterns that might reflect similar modes of regulation within the pertinent samples. Genes up-regulated by more than 1.8-fold are included in Tables II–IV. A detailed list of up-regulated and down-regulated genes is included as Supplemental Material (Supplemental Table I).

Eighty genes were consistently up-regulated in all pairwise combinations when the 4-week-old Tg mice were compared with all other mice (9-month-old Tg and WT and 4-week-old WT) (Table II). When just one of the pairwise combinations was varied with the others, 179 genes were induced. Of those, 39 genes were clustered in three major functional categories: extracellular matrix and cytoskeleton, cell signaling, and growth factors/transcriptional regulators. Eleven of 30 up-regulated expressed sequence tags (ESTs) had some assigned function. Among these, sarcosommal protein SLAP, actin-cross-linking protein 7, talin, glycogenin 1, and Cdc 5-like protein were elevated during the initiation of cardiac hypertrophy.

When 4-week-old Tg animals were compared with their age-matched WT animals only, a slightly different picture emerged (Table III). Seventy-four genes were up-regulated in all pairwise comparisons between 4-week-old Tg versus WT. These genes were clustered into six functional categories: extracellular matrix, myofibrillar and cytoskeletal protein, cellular signaling factors, growth and transcription factors, cell defense, and protein expression regulators. Forty-five known genes were down-regulated in 4-week-old Tg animals compared with the WT animals. Down-regulated genes were clustered primarily as cell signaling or mitochondrial proteins (Table III).

**Changes in Gene Expression during Transition to Heart Failure**—Pairwise comparisons were also used to identify genes that decreased in expression when comparing 9-month-old Tg hearts with all other samples (Table II). One hundred thirty-three genes were consistently elevated in failing hearts compared with nonfailing WT or younger Tg hearts. Fifty-one of these genes were functionally clustered into six different categories: cell signaling; growth and transcription factors; extracellular matrix and cytoskeletal protein, cell defense; apoptosis; and protein expression regulators and metabolic enzymes. Of 82 ESTs, only 11 had unknown functions.

Approximately 50 genes were down-regulated in Tg hearts compared with all other samples. Most of these genes were
are tabulated in Table I.

mice from all four lines, showing similar changes. Several echocardiogram parameters from 4-week-old and 9-month-old mice hearts (WT and Tg) versus changed genes in 9-month-old Tg ESTs, 16 had known functions (Table III). Of 159 down-regulated functional groups, including cell signaling, matrix and cytoskeleton, or mitochondrial enzymes. Of 159 down-regulated genes, 206 genes were consistently down-regulated across the pairwise comparisons. Forty-seven were clustered into several functional categories: extracellular matrix and cytoskeleton or protein expression. Apart from these groups, some genes were categorized as transcriptional and growth factors, cell defense proteins, apoptosis-related proteins, or proteins involved in cell division. Twenty-eight genes were identified as known ESTs among 113 ESTs that showed increased expression in failing mouse hearts. In addition to the up-regulated genes, 206 genes were consistently down-regulated across the pairwise comparisons. Forty-seven were clustered into several functional groups, including cell signaling, matrix and cytoskeleton, or mitochondrial enzymes. Of 159 down-regulated ESTs, 16 had known functions (Table III).

Fig. 6 summarizes SOM clustering analysis of the maximally changed genes in 9-month-old Tg versus all as well as 4-week-old Tg versus all. When SOM clustering was performed using absolute gene expression values from all samples from 9-month-old Tg animals compared with either age-matched WT or 4-week-old WT or Tg mice, definitive clusters of candidate genes up- or down-regulated during the transition from hypertrophy to heart failure emerged (Fig. 6, a and b). Those maximally up-regulated include fibronectin, VCAM1, slow myosin heavy chain, matrix metalloproteinase 3, ceruloplasmin, apolipoprotein D, and MRPs. Approximately 80 genes were expressed at a higher level in three 4-week-old Tg animals when compared with all other animals (Fig. 6c). Included within this group were skeletal muscle actin, MLC3F, calsequestrin, immediate early genes, SLAP, glycogenin, skeletal muscle tropomyosin, talin, and disintegrin. All genes from this cluster were identified in the pairwise comparisons noted above. Interestingly, SOM analysis did not identify clusters of genes consistently down-regulated at this early developmental time point (data not shown).

Comparison of Gene Expression between Heart Failure and Initiation Stage: Old Tg (9 Months Old) Versus Young Tg (4 Weeks Old)—The genes expressed more highly in 9-month-old Tg animals included a subset of genes that specifically increased in expression between 4 weeks and 9 months, as the Tg animals progressed from hypertrophy to heart failure. Pairwise comparison of 9-month-old Tg and 4-week-old Tg samples identified 276 genes that were specifically up-regulated in Tg comparisons but not in WT animals (thereby excluding age-regulated genes) (Table IV). Of these up-regulated genes, 44 genes were classified as extracellular matrix and cytoskeleton, growth and transcription factors, cell signaling factors, cell defense, apoptotic, protein expression regulators, or mitochondrial proteins. Eleven of the ESTs with known functions included calcium-binding protein A15, casein kinase I, insulin-like growth factor 3, and Rab-6 (a ras oncogene family protein).

Similar pairwise comparisons identified 30 cardiac genes down-regulated in 9-month-old Tg animals compared with 4-week-old Tg animals. These genes clustered into several functional groups: extracellular matrix and cytoskeleton proteins, mitochondrial enzymes, cell signaling factors, or cell cycle regulators. Fifteen known ESTs in this group included cyclophilin D, tropomyosin 5, exportin 1, and Ras-related protein RAL1.

**DISCUSSION**

This study reinforces the proofs we have previously presented that myotrophin is a significant causal factor in the hypertrophy/heart failure continuum. Data presented here document the effects of myotrophin protein overexpression at molecular, cellular, morphological, and functional levels in a specially developed line of Tg mice. These data indicate that myotrophin overexpression initiates cardiac hypertrophy, eventually progressing to heart failure, a process associated with changes in expression of proto-oncogenes, ANF, β-MHC, and cytokines. Importantly, using this model and the new tools of state-of-the-art DNA microarray analysis (Fig. 6 and Tables II and III), we have elucidated patterns of gene up-regulation and down-regulation that may be involved during initiation of cardiac hypertrophy and progression to heart failure in humans.
We documented several novel mechanistic changes that occur during the transition from hypertrophy to heart failure. We confirmed that myotrophin overexpression resulted from increased myotrophin mRNA and protein levels in all lines and generations of Tg mice (Fig. 2). Importantly, the increase in myotrophin triggered a significant increase in cytokines and growth factors such as LT-α, tumor necrosis factor-α, interferon-γ, interleukin-6, TGF-β1, TGF-β2, TGF-β3, and macrophage migration inhibitory factor, in 9-month-old Tg mice, when chronic hypertrophy advanced to heart failure (Fig. 4), whereas, at 4 weeks of age, three genes (LT-α, TGF-β2, and TGF-β3) were up-regulated in Tg mice, compared with the age-matched WT. Echocardiographic data showed significant hypertrophy of left ventricle and septum (asymmetric hyper-
| Accession no. | 9 Tg versus 9 WT (n = 5) Mean -fold change | Accession no. | 4 Tg versus 4 WT (n = 5) Mean -fold change |
|--------------|------------------------------------------|--------------|------------------------------------------|
| **Up-regulated genes** | | **Up-regulated genes** | |
| **Cell signaling** | | **Extracellular matrix and cytoskeletal proteins** | |
| D16497 | Natriuretic peptide precursor B 2.3 | X12973 | MLC3F gene for myosin alkali light chain 2.0 |
| K02781 | Natriuretic peptide precursor A 5.1 | U93291 | Skeletal muscle calsequestrin 1.8 |
| M84487 | Vascular cell adhesion molecule 1 3.1 | U03419 | Precolagen α1 type 1 2.5 |
| X66449 | Calcyclin 2.7 | X69767 | Collagen 8α1 2.3 |
| Z68618 | Transgelin 3.2 | X13986 | Minopontin 12.3 |
| U07882 | Endothelin 1 2.3 | M28729 | Tubulin α1 1.8 |
| **Extracellular matrix and cytoskeletal proteins** | | **Cell signaling** | |
| X13986 | Minopontin 70.0 | AF020185 | Protein inhibitor of nitric-oxide synthase 2.3 |
| X58251 | Procollagen type 1 alpha 2 3.1 | D16497 | Natriuretic peptide precursor type B 2.4 |
| M18194 | Fibronectin 3.5 | M84487 | VCAN 1 2.3 |
| X70854 | Fibulin 2.6 | X77952 | Endoglin 1.9 |
| AA22622 | Slow myosin heavy chain β 3.1 | M92680 | Lipocortin 1 1.9 |
| X66492 | Matrix metalloproteinase 4.1 | Cell defense | |
| **Protein expression** | | **Growth factors and transcription factors** | |
| X82648 | Apolipoprotein D 7.3 | AF020185 | Protein inhibitor of nitric-oxide synthase 2.3 |
| M83218 | Calcium binding protein, MRP-8 8.1 | M18194 | Procollagen type 1 alpha 2 3.1 |
| Z11911 | Glucose-6-phosphate dehydrogenase 6.2 | M19681 | Small inducible cytokine A2 4.1 |
| **Growth factors and transcription factors** | | **Apoptosis and cell division** | |
| X61007 | Inosine-like growth factor protein 8.9 | X94127 | SRY box containing gene 2 5.9 |
| M32745 | Transforming growth factor- beta-5 4.2 | AF035717 | Transforming growth factor β 2.8 |
| **Apoptosis and cell division** | | **Extracellular matrix and cytoskeletal proteins** | |
| AB019600 | Caspase-9 2.3 | U49430 | Ceruloplasmin 2.5 |
| AP041054 | Nip3 1.9 | M12481 | Cytoplasmic α1 actin 0.6 |
| X50846 | GAS6 2.3 | M70642 | Fibroblast-inducible secreted protein 4.2 |
| AP055886 | Cyclin 1 2.1 | M16405 | Calpain 1 light chain 2.1 |
| **Cell defense** | | **Cysteine-rich glycoprotein SPARC** 2.3 | |
| AF022110 | Tumor necrosis factor family 3.1 | Down-regulated genes | |
| M33960 | Plasminogen activator inhibitor-1 2.7 | Extracellular matrix and cytoskeletal proteins | |
| U49430 | Ceruloplasmin 2.9 | M12481 | Cytoplasmic β actin 0.6 |
| V00385 | Metallothionein 3 3.2 | U09181 | Cardiac troponin I 0.8 |
| RSTs | AF058281 | AF093624 | Nsp1 0.6 |
| AA698838 | BeKg 1.9 | U94423 | Mouse MEF2A mRNA 0.4 |
| AW125874 | Cdk5 2.4 | L0343 | Calcium channel β2 0.6 |
| A843106 | p53 homologue 3.1 | M31331 | Cadherin 2 0.6 |
| A849615 | Gas 5 2.4 | M63801 | Connexin 43 0.4 |
| AW124175 | Sarcolemma-associated protein 1.5 | U05590 | Angiotensin I mRNA 0.5 |
| **Down-regulated genes** | | **Cell signaling** | |
| U09181 | Cardiac troponin I 0.43 | AF029892 | SERCA 2 0.6 |
| M91602 | Myosin light chain 2 0.6 | AF029892 | Mitochondrial enzymes 0.6 |
| M29793 | Slow cardiac troponin C 0.6 | Z49204 | Nicotinamide nucleotide transhydrogenase 0.7 |
| **Mitochondrial enzymes** | | **Extracellular matrix and cytoskeletal proteins** | |
| X53157 | Mitochondrial cytochrome c oxidase 0.6 | U12961 | NAD/FH oxidoreductase 1 0.7 |
| AF058955 | ATP-specific succinyl-CoA synthetase β 0.6 | M76727 | Pyruvate dehydrogenase E1 α subunit 0.6 |
| **Cell signaling** | | **Cell signaling** | |
| M28723 | Antioxidant protein 1 0.7 | X17069 | Transition protein TP2 0.7 |
| AP029892 | SERCA 2 0.6 | M28723 | Potassium channel Kv4.2 mRNA 0.3 |
| U06924 | STAT1 0.7 | AF0107780 | Fumarate hydratase 0.5 |
| RSTs | AA870575 | ATP synthase | |
| AW12564 | Global ischemia-induced protein 0.4 | | |
| A1836740 | Aconitase 2 0.6 | | |
| A1852862 | Fumarate hydratase 0.5 | | |
| A118132 | Creatine kinase 0.5 | | |
trophy) in 36-week-old Tg mice hearts, a typical change observed in human hypertrophy. Mice afflicted with hypertrophy also had severely compromised cardiac function associated with pleural effusion, a common occurrence during human heart failure. However, this compromised function did not occur in the hypertrophied hearts of young 4-week-old Tg mice despite presence of hypertrophy. Our data also suggest that atrial enlargement arises from mitral and tricuspid valve regurgitation, which occurs because the ventricular cavity enlarges, causing an incomplete sealing in these valves. This cluster of symptoms mimics human cardiomyopathic hypertrophy with end-stage heart failure. Although other Tg models have been reported (14, 15), none have studied the progression of hypertrophy that advances to heart failure in the manner we have described. Previously, using isolated myocytes, we have shown that the mode of action of myotrophin protein is mediated through protein kinase C and NF-κB signaling pathways (16). This in vivo model, overexpressing myotrophin, provided us with the opportunity to dissect out the role of myotrophin-induced signaling pathways for the initiation process of cardiac hypertrophy and its progression to heart failure. Work is in progress to determine protein kinase C and NF-κB cascade in Tg hearts at 4 weeks, 16 weeks, and 9 months compared with their age-matched WT.

This model provided the opportunity to further the genome-wide screening of cardiac tissue as a tool to identify new genes

### Table IV

Comparison of gene expression between heart failure and initiation of hypertrophy: Tg (9 months old) versus Tg (4 weeks old) (n = 5)

| Accession no. | Gene description | Mean-fold change |
|---------------|------------------|-----------------|
| **Up-regulated genes** | | |
| **Extracellular matrix and cytoskeletal proteins** | | |
| AF061372 | C-type lectin | 20.3 |
| M18194 | Fibronectin mRNA | 2.5 |
| X66402 | Matrix metalloproteinase 3 | 4.1 |
| AB007848 | Bone matrix protein osteomodulin | 2.7 |
| U04541 | α-Tropomyosin, slow | 2.5 |
| X67348 | Procollagen type X, α | 2.3 |
| **Growth and transcription factors** | Insulin-like growth factor-binding protein 2 | 2.7 |
| U17291 | Transcription factor AP2 | 3.1 |
| **Cell signaling** | Vascular cell adhesion molecule 1 | 2.9 |
| U12684 | p53 variant mRNA | 1.8 |
| U28423 | Protein kinase inhibitor p58 | 4.6 |
| M21856 | Cytochrome P450 | 1.6 |
| U40930 | Oxidative stress-induced protein | 2.6 |
| AF047838 | Calcium-sensitive chloride conductance protein 1 | 4.4 |
| **Cell defense** | Tumor necrosis factor superfamily member | 3.1 |
| AF019048 | Lymphototoxin A | 6.4 |
| M33960 | Plasminogen activator inhibitor (PAI-1) | |
| M17015 | | |
| **Protein expression** | Intracellular calcium-binding protein 8 | 1.8 |
| M83219 | Calmegin (Ca<sup>2+</sup>-binding protein) | 2.3 |
| U08373 | Fibroblast-inducible secreted protein | 1.8 |
| M70642 | Nip3 (Bcl2-binding protein) | 2.4 |
| AF041054 | Caspase-9 | 1.9 |
| AB019600 | | |
| **ESTs** | Insulin-like growth factor-binding protein 3 | 3.1 |
| AI842277 | Casein kinase I | 2.2 |
| AI846289 | Myosin heavy chain, nonmuscle type B | 2.1 |
| AI505453 | Calcium-binding protein A 15 | 2.3 |
| AA612146 | | |
| **Down-regulated genes** | Skeletal muscle actin α1 | 0.4 |
| M12347 | Cytoskeletal γ-actin mRNA | 0.5 |
| M21495 | Microtubule-associated protein Tau | 0.5 |
| M18775 | | |
| **Mitochondrial enzymes** | Mitochondrial ATP synthase coupling factor 6 | 0.6 |
| U77728 | Pyruvate dehydrogenase E1 α | 0.7 |
| M76727 | ATP synthase E subunit | 0.5 |
| U59282 | Cytochrome c oxidase | 0.7 |
| X53157 | TIM 23 | 0.55 |
| AB021122 | | |
| **Cell signaling** | Connexin 43 | 0.3 |
| M63801 | Protein kinase C inhibitor γ | 0.6 |
| U97170 | Mitogen-activated protein kinase kinase | 0.7 |
| U02536 | Protein inhibitor of nitric-oxide synthase | 0.3 |
| AP620185 | HSP60 | 0.4 |
| X55384 | HMG 14 | 0.6 |
| X53576 | Cell division cycle 42 | 0.6 |
| L78075 | Cell division cycle 42 | 0.6 |
| **ESTs** | Cyclophilin D | 0.5 |
| AW122022 | Mitochondrial import inner membrane translocase | 0.5 |
| AW124594 | Mitochondrial ribosomal protein L36 | 0.4 |
| AI848416 | NAPD:ubiquinin oxidoreductase | 0.6 |
| AI835547 | | |
| AI849767 | H<sup>+</sup>-transporting ATP synthase | 0.6 |
| AW125336 | Pyruvate dehydrogenase β | 0.5 |
During initiation, progression, and transition from hypertrophy to heart failure, several hypertrophy-associated genes were up-regulated. This study is unique because it is not possible in humans. The alteration of several hypertrophy-associated genes reported in recent gene array studies in human failing heart (17) were found to be similar to the murine heart failure model overexpressing myotrophin, reported in this study. For the first time, we documented the alterations of gene clusters that participate during the initiation of hypertrophy and during the transition from hypertrophy to heart failure (Table II). Our data also suggest that the initiation of hypertrophy utilizes a transcriptional program involving specific sets of genes, which are distinct from those that operate during the transition phase. Characterization of the expression of these novel genes during initiation and transition phases could provide new insights into cardiac remodeling.

In failing hearts, natriuretic peptide precursors type A and B were ranked as the top candidate genes when compared with either age-matched WT or with the total of all young WT and Tg samples. SOM clustering analysis identified the maximally changed gene expressions during both initiation and progression of hypertrophic process. The functions of many of them are yet to be defined during hypertrophy to heart failure. This gene cluster continues to be expressed during the progressive deterioration of cardiac function (18). In addition, significantly increased expression of extracellular matrix proteins, like collagen type I and type VIII, fibronectin, C-type lectin, and matrix metalloproteinase, was observed in failing hearts (19). Growth factors like TGF-β2, TGF-β1, tumor necrosis factor-α, insulin-like growth factor, and hypoxia-inducing factor 1α can be important during the transition from hypertrophy to heart failure. Genes involved in fatty acid metabolism (e.g., apolipoprotein-D and -E) and glucose metabolisms (glucose-6-phosphate dehydrogenase) were up-regulated, whereas lactate dehydrogenase and fatty acid-binding proteins were down-regulated. Several mitochondrial enzymes were consistently and significantly down-regulated in failing hearts compared with either WT or 4-week-old Tg hearts, a finding that may explain the reduced cardiac energy production during heart failure. Induction of apoptotic proteins such as CIDE-A, Bcl2-binding protein NIP3, and caspase-9 in failing hearts signifies active programmed cell death. Metallothionein 1 and 2 are stress-inducible, metal-binding proteins whose antioxidant function and regulation of apoptosis in the heart were reported previously (20). Unlike Tan et al. (17), we found increased expression of metallothionein proteins as well as such other cell cycle regulators as GAS 6, cyclin 1, and histone H1. This seeming discrepancy indicates that cell death and cell regeneration can occur simultaneously in the failing heart. No such genes were found when 36-week-old Tg mice were compared with 4-week-old Tg mice, although NIP3 and caspase-9 were up-regulated in old Tg animals. Apoptosis was not evident during the initiation phase of hypertrophy, yet induction of cyclin and cdk genes started as early as 4 weeks of age in Tg mice heart.

Our data showed that initiation of hypertrophy was associated with induction of fewer genes. Compared with genes from failing hearts, interestingly, no genes were down-regulated during the initiation of hypertrophy (4-week-old Tg mice). Among the cytoskeletal proteins, myosin alkali light chain, skeletal muscle calsequestrin, β-tropomyosin, and transcriptional activators like mitogen-activated protein kinase/extracellular signal-regulated kinase/ extracellular signal-regulated kinase kinase kinase 3, STAT6, Pod1, and DP1 were up-regulated in the hearts of 4-week-old Tg mice. Combining all of these findings, the data are expected to provide information for changes in cardiac metabolism during onset of hypertrophy and its transition to heart failure.

In conclusion, this new Tg mouse model of hypertrophy resulting from myotrophin overexpression, leading to heart failure, is important because of its similarity to end-stage heart failure in human beings in both molecular (gene expression of hypertrophy marker genes, proto-oncogenes, cytokines, and growth factors) and physiological parameters (pleural effusion and lethargy). Heart failure occurred in all of our Tg mice sacrificed to date (n = 150), and the average life span was 8–11 months in the F1, F2, F3, and F4 generations. Several growth factors and cytokines were increased during the initiation

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phase and during the transition of hypertrophy to heart failure. Comparison of the gene array data between the initiation of hypertrophy and its transition to heart failure involves differential activation of functional gene clusters. Up-regulation of growth factors, calcium-binding proteins, proteins regulating programmed cell death, and extracellular/cytoskeletal proteins as well as down-regulation of mitochondrial proteins and cytoskeletal/myofibrillar proteins mark the transition phase, which is associated with severely compromised heart function, thereby differing from the mechanisms of the initiation process of hypertrophy as well as those operating in nonfailing WT hearts.

The intricate, multifaceted process of heart failure, especially its transition from longstanding hypertrophy to heart failure, involves many factors. Eventual heart failure is probably the result of cross-talk between neurohumoral mechanisms and growth factors. Our new genetic data, added to our prior findings based on molecular and biochemical data, convincingly demonstrate that myotrophin is a factor that not only initiates hypertrophy but is also associated with the progression to heart failure. We expect that this new mouse model will provide the key to elucidate further molecular mechanisms that occur during advancement of hypertrophy to heart failure and will facilitate the design of effective therapies.

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REFERENCES

1. Chien, K. R., Zhu, H., Knowlton, K. U., Miller-Hance, W., van-Bilsen, W., O’Brien, T. X., and Evans, S. M. (1993) Annu. Rev. Physiol. 55, 77–95
2. Sadoshima, J., and Izumo, S. (1997) Annu. Rev. Physiol. 59, 551–571
3. Sen, S., Tarazi, R. C., Khairallah, P. A., and Bumpus, F. M. (1974) Circ. Res. 35, 775–781
4. Sen, S. (1987) Circulation 75, 181–184
5. Sen, S., Kundu, G., Mekhail, N., Castel, J., Misono, K., and Healy, B. (1990) J. Biol. Chem. 265, 16635–16643
6. Mitra, S., Timor, A., Gupta, S., Wang, Q., and Sen, S. (2001) Cytogenet. Cell Genet. 93, 151–152
7. Mukherjee, D. P., McTiernan, C. F., and Sen, S. (1993) Hypertension 21, 142–148
8. Sil, P., Mukherjee, D. P., and Sen, S. (1995) Circ. Res. 76, 1020–1027
9. Sivasuramanian, N., Adhikary, G., Sil, P. C., and Sen, S. (1996) J. Biol. Chem. 271, 2913–2916
10. Iwaki, K., Sukhatme, V. P., Shubeita, H. E., and Chien, K. R. (1990) J. Biol. Chem. 265, 13809–13817
11. Sil, P., Kandaswamy, V., and Sen, S. (1998) Circ. Res. 82, 1173–1188
12. Bialkowska, K., Kulkarni, S., Du, X., Goll, D. E., Sado, T. C., and Fox, J. E. (2000) J. Cell Biol. 151, 685–696
13. Tamayo, P., Slonim, D., Mesirov, J. P., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E. S., and Golub, T. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2907–2912
14. Vikstrom, K. L., Bohlmeyer, T., Factor, S. M., and Leinwand, L. A. (1998) Circ. Res. 82, 773–778
15. James, J. F., Hewett, T. E., and Robbins, J. (1998) Circ. Res. 82, 407–415
16. Gupta, S., Purcell, N. H., Lin, A., and Sen, S. (2002) J. Cell Biol. 159, 1019–1028
17. Tan, F. L., Moravec, C. S., Li, J., Apperson-Hansen, C., McCarthy, P. M., Young, J. B., and Bond, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11387–11392
18. Masutomo, K., Makino, N., Sugano, M., Miyamoto, S., Hata, T., and Yanaga, T. (1999) J. Mol. Cell. Cardiol. 31, 1607–1615
19. Schaper, J., Froede, R., Hein, S., Bueck, A., Hashizume, H., Speiser, B., Friedl, A., and Heese, N. (1991) Circulation 83, 504–514
20. Kang, Y. J. (1999) Proc. Soc. Exp. Biol. Med. 222, 263–273
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