Silencing the myotrophin gene by RNA interference leads to the regression of cardiac hypertrophy

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Gupta S, Maitra R, Young D, Gupta A, Sen S. Silencing the myotrophin gene by RNA interference leads to the regression of cardiac hypertrophy. Am J Physiol Heart Circ Physiol 297: H627–H636, 2009. First published June 5, 2009; doi:10.1152/ajpheart.00294.2009.—Myotrophin-induced activation of NF-κB has been shown to be associated with cardiac hypertrophy (CH) that progresses to heart failure (HF). In the present study, we examined the cause-and-effect relationship between myotrophin and NF-κB activation using small hairpin RNA (shRNA) against myotrophin both in vitro (using neonatal rat myocytes) and in vivo [using myotrophin transgenic (Myo-Tg) mice, which overexpress myotrophin in the heart, develop CH, and gradually progress to HF]. Among several lentiviral vectors expressing myotrophin shRNAs, L-sh-109 showed the best silencing effect at both the mRNA (155.3 ± 5.9 vs. 32.5 ± 5.5, P < 0.001) and protein levels associated with a significant reduction of atrial natriuretic factor (ANF) and NF-κB. In vivo, when L-sh-109 was delivered directly into the hearts of 10-wk-old Myo-Tg mice, we observed a significant reduction of cardiac mass (8.0 vs. 5.7 mg/g, P < 0.001) and myotrophin gene expression (54.5% over untreated Myo-Tg mice, P < 0.001) associated with a reduction in ANF and NF-κB signaling components. Our data suggest that using RNA interference to silence the myotrophin gene prevents NF-κB activation, associated with an attenuation of CH. This strategy could be an excellent therapeutic means for the treatment of CH and HF.

nuclear factor-κB; heart failure

CARDIAC HYPERTROPHY (CH) and heart failure (HF) are the major causes of morbidity and mortality in humans. Over the past several decades, various factors have been identified and reported to be responsible for CH both in vitro (cultured cells) and in animal model systems (5, 30), but the underlying mechanisms are not well understood. Among such factors, myotrophin, a 12-kDa soluble protein identified from spontaneously hypertensive rat hearts and dilated cardiomyopathic human hearts, has been shown to induce myocyte growth (25, 32, 34). Previously, using neonatal rat myocytes, we observed that the signaling mechanism for myotrophin-induced myocyte growth is associated with the activation of NF-κB signaling pathways (11). In addition, using an α-myosin heavy chain (MHC) promoter, we developed a transgenic mouse model [the myotrophin transgenic (Myo-Tg) mouse] that overexpresses myotrophin specifically in the heart and has a gradual progression of CH to HF over a 9-mo period of time (31). Myo-Tg mice exhibited left ventricular hypertrophy, atrial and ventricular dilation, myocyte disarray, multiple areas of focal fibrosis, pleural effusion, and compromised cardiac function, closely mimicking the symptoms of human HF (31). In this Myo-Tg model, we have recently shown that signaling cascades of NF-κB are activated during the initiation and progression of CH and its transition to HF (12). Recently, we have shown that a partial regression or prevention of CH was achieved when small interfering (si)RNA of NF-κB was directly delivered into Myo-Tg mouse hearts (12). In this study, we evaluated the effect of knockdown of myotrophin gene expression on cardiac mass in the Myo-Tg mouse model. We designed siRNA against myotrophin (si-109) and administered it directly to the heart. The Myo-Tg model is an excellent model to examine the use of siRNA against myotrophin in the pathology of CH. In this study, we report the effects of silencing the myotrophin gene both in vitro (using neonatal rat myocytes) and in vivo (using Myo-Tg mice).

NF-κB is a pleiotropic transcription factor that regulates a variety of cellular responses, including inflammation, the immune response, atherosclerosis, autoimmune arthritis, septic shock, and apoptosis (4, 10, 23, 37). Since several lines of evidence over the past few years have indicated the importance of NF-κB in the hypertrophic process (16, 28) as well as in HF (17, 29, 39), we extended our study to evaluate the effect of silencing of the myotrophin gene and assessing the consequences in the CH process including its effect on NF-κB signaling pathways.

Recent studies have shown that RNA interference (RNAi) is a powerful genetic approach for efficiently silencing target genes. RNAi provides a mechanism for sequence-specific post-transcriptional inhibition of gene expression via double-stranded RNA molecules by the dicer enzyme (2, 8, 15, 33). Recently, it has been demonstrated that RNAi-mediated gene silencing can be obtained in cultured mammalian cells by the delivery of chemically synthesized short (<30 nt) double-stranded siRNA molecules (8) or by the endogenous expression of short hairpin (sh)RNA species bearing a fold-back stem-loop structure (9, 27, 35). RNAi-based gene knockdown can be achieved by a lentivirus-mediated expression system, allowing the production of shRNAs under the control U6 or H1 promoters (1, 3, 7).

In the present study, we report the efficacy of myotrophin siRNA in suppressing myotrophin gene expression and its effect on cardiac mass and on NF-κB signaling pathways. The RNAi inhibitor of myotrophin is a shRNA that is complementary to myotrophin delivered directly into the myocardium of Myo-Tg mice using a lentiviral vector. L-sh-Myo was directly delivered into the myocardium of Myo-Tg mice to elucidate the cause-and-effect relationship of myotrophin, NF-κB activation, and cardiac remodeling.

MATERIALS AND METHODS

All animal experiments were done with the approval of the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation and following National Institutes of Health guidelines.
Design and Construction of shRNA Against Myotrophin

The siRNA technique was designed to target specific region of myotrophin mRNA using the siRNA target finder program from Ambion (Foster City, CA). We identified four 21-nt stretches within the coding region of the myotrophin gene that were <50% GC rich located at the 55, 109, 196, and 267 nt positions and unique in the genome. The sense sequence was followed by the loop sequence 5'TTC AGA AGA-3' and then by the antisense sequence to form a hairpin. In addition, each sense oligonucleotide harbored a stretch of T as a PstI transcription termination signal. These small oligonucleotides had EcoRI and SalI overhangs to allow for ligation into the SalI/EcoRI sites immediately after the U6 promoter of the lentiviral plasmid LRV-U6-CMV-EGFP (L). Clones were verified by sequencing. The empty vector was used as a control plasmid in this study. A scrambled sequence of the same content: 38.1%); and a control plasmid in this study. A scrambled sequence.

Production of Lentivirus and Purification

Vesicular stomatitis virus glycoprotein-pseudotyped lentiviral particles were generated by cotransfection of 293T cells, and viral supernatants were harvested and concentrated by ultracentrifugation as previously described (21). In brief, the lentivirus particles were typically made by transfecting 293T cells with the LRV-U6-CMV-EGFP plasmid containing either sh-109 (L-sh-109) or the scrambled insert. Viral supernatants were harvested at 48 and 72 h posttransfection and filter sterilized through 0.45 μm. Using this lentiviral system, we achieved optimal yields of vector of a high transducing efficiency [10^8-10^9 transducing units (TUs)/ml and close to 10^4 TUs/ng of phospho-24 core protein]. The virus particles were further concentrated by spinning 150 ml of viral supernatant at 30,000 rpm for 2 h in an ultracentrifuge and resuspending the pellet in 150 μl of serum-free DMEM-F-12 media. The final concentration of viral particles was 10^8–10^10 viral particles/ml.

Cell Culture and Lentiviral Transduction

Neonatal rat myocytes were isolated and maintained in laminin-coated plates as previously described (11, 12, 32). In brief, 24 h after being plated, neonatal cardiomyocytes were transduced (in triplicate) separately in six-well laminin-coated plates with L-sh-109 and LRV-U6-CMV-EGFP (control) viral particles at a multiplicity of infection (MOI) of 30 in the presence of 8 μg/ml polybrene (12). After 48 h, the efficiency of the transduction was measured by monitoring enhanced green fluorescent protein (EGFP) expression under a fluorescence microscope. The transduction efficiency was found to be 80–90% after two successive infections.

RNA Extraction and Northern Blot Analysis

Isolated cardiac myocytes. Neonatal rat myocytes were treated with 40 nmol myotrophin in L-sh-109-transduced or nontransduced myocytes for 48 h. Cells were washed with PBS, and total RNA was prepared as previously described (11). Atrial natriuretic factor (ANF) and β-MHC oligonucleotide probes were used to perform Northern blot analysis as previously described (11, 13). For normalization, filters were stripped off and rehybridized using 18S rRNA as a probe.

EMSA

Nuclear extracts were made from both LRV-U6-CMV-EGFP-transduced and L-sh-Myo-transduced neonatal myocytes. For in vivo

Fig. 1. Effects of various myotrophin small hairpin (shRNA) expressions. A: Northern blot analysis of various constructs of myotrophin shRNA. Neonatal rat myocytes were transduced with L-sh-55, L-sh-109, L-sh-196, and L-sh-267 at a multiplicity of infection (MOI) of 30 for 48 h. In addition, cells were also transduced with a scrambled sequence. B: quantification of myotrophin mRNA status from the results shown in A. Values are means ± SE of the mRNA expression of myotrophin for all constructs transduced into myocytes; n = 5, *P < 0.001 compared with control (Cont; LRV-U6-CMV-EGFP transduced) vs L-sh-109. C: Western blot analysis of various constructs of myotrophin shRNA as described in A. Actin antibody was used as an internal protein loading control. D: quantification of myotrophin protein levels from the results shown in C. Values are means ± SE of protein expression of myotrophin for all constructs transduced into myocytes; n = 5, *P < 0.001 compared with control (LRV-U6-CMV-EGFP transduced) vs. L-sh-109. E: expression of enhanced green fluorescent protein (EGFP) into myocytes after transduction with control and L-sh-109 constructs.
experiments, nuclear extracts were also made from WT, Myo-Tg, and sh-Myo-Tg mouse hearts as previously described (13). EMSAs were performed using double-stranded NF-κB binding site oligonucleotides as previously described (11, 13).

**Western Blot Analysis**

Cytoplasmic protein extracts were made from LRV-U6-CMV-EGFP-transduced and L-sh-109-transduced neonatal rat myocytes. Western blot analysis was performed with an IκB-α antibody as a probe, as previously described (11). For in vivo experiments, Western blots were performed using WT, Myo-Tg, and L-sh-109-Tg mouse hearts as previously described (13). Actin antibody was used as an internal loading control.

**Viral Gene Delivery**

The lentiviral-mediated gene delivery was as previously described (12). WT and Myo-Tg mice were anesthetized using 0.1 ml of a cocktail of ketamine and xylene (1 ml ketamine, 0.9 ml xylene, and 1.5 ml PBS), ventilated, and subjected to a lateral thoracotomy for direct visualization. We gave three injections using a 30-gauge 1/2 needle: two injections (40 μl each) containing the sh-109 gene (4 × 10^4-10^7) into each of the anterior-septal and posterior-lateral walls and one injection near the apex of the heart. There were 5–8 mice/treatment group. At the end of the 6-wk treatment period, animals were killed, their hearts were removed, and the following parameters were determined: cardiac mass, body weight, levels of myotrophin gene and protein expression, and extent of NF-κB activation.

**Cytokines and Growth Factor-Targeted Oligo Gene Array Analysis**

Total RNA was isolated from Myo-Tg and L-sh-109-transduced Myo-Tg mice using the TRIzol extraction method. After the isolation, total RNA was cleaned up and subjected to DNase treatment using the Qiagen RNeasy Kit. Samples were analyzed both spectrophotometrically and in formaldehyde-agarose gel to check the quality of the RNA samples. The oligo gene array (oligo GE assay) was performed and analyzed using the kit and protocol from SuperArray Biosciences following the manufacturer’s protocol. This array is designed on 60-mer oligonucleotide probes on 3’-biased gene-specific sequences printed on each array filter. The value of individual gene expression was expressed the percent change over untreated Myo-Tg mice.

**Statistical Analysis**

Data are expressed as means ± SE. Differences between experimental groups were evaluated for statistical significance using Student’s t-test. Differences with values of P < 0.001 were considered significant. Data were also analyzed by two-way

![Fig. 2](http://www.ajpheart.org/)

A: atrial natriuretic factor (ANF) and β-myosin heavy chain (MHC) expression in L-sh-109-transduced neonatal myocytes. ANF expression was measured using ANF cDNA as a probe in L-sh-55-, L-sh-109-, L-sh-196-, and L-sh-267-transduced neonatal myocytes in the presence or absence of myotrophin. 18S rRNA was used as an internal loading control. B: quantification of mRNA expression from the results shown in A. Results are means ± SE and represent three separate experiments. *P < 0.001 compared with Cont. C: effect of β-MHC in L-sh-109-transduced myocytes. 18S rRNA was used as an internal loading control. D: quantification of β-MHC mRNA expression from the results shown in C. Results are means ± SE and represent three separate experiments; n = 3. *P < 0.001 compared with Cont; †P < 0.001 compared with myotrophin-stimulated transduced cells.
ANOVA using GraphPad Prism software (GraphPad Software, San Diego, CA) in sh-p65 lentivirus-mediated gene delivery.

RESULTS

Myotrophin shRNA Significantly Reduced Myotrophin mRNA and Protein Levels

All shRNAs made against the myotrophin gene (L-sh-55, L-sh-109, L-sh-196, and L-sh-267) were transduced into neonatal myocytes, and Northern and Western blot analyses were performed to check the effects of silencing the myotrophin gene. Transduction with L-sh-109 resulted in an inhibition of 79.2% and 83.6%, respectively, of myotrophin mRNA (155.3 ± 5.9 vs. 32.5 ± 5.5, P < 0.001) and protein levels (76.33 ± 2.32 vs. 12.45 ± 0.86) after 72 h after transduction compared with the other clones. Most importantly, we found that the scrambled shRNA had no effect (Fig. 1, A and B).

To determine transduction efficiency, L-sh-109 was transduced at 30 MOI into neonatal rat myocytes. LRV-U6-CMV-EGFP was also transduced at 30 MOI and served as a control. The transduction efficiency was calculated based on EGFP expression in neonatal myocytes. After 48 h of transduction, it appeared that 80–90% of the myocytes showed EGFP expression (Fig. 1C). Our data suggest that both constructs transduced the gene efficiently into neonatal myocytes, as evidenced by EGFP expression, and neither construct showed any detrimental effects to the cells.

L-sh-109 Significantly Inhibited CH Marker Gene Expression in Neonatal Rat Myocytes

To evaluate the extent of CH marker gene expression, we chose ANF and β-MHC gene expression in the presence or absence of treatment with myotrophin. We transduced all four L-sh-Myo constructs into neonatal rat myocytes and determined the extent of ANF expression after stimulation with myotrophin. L-sh-109 showed a 62.2% inhibition of ANF expression (181.8 ± 6.8 vs. 68.6 ± 5.3, P < 0.01) compared with other constructs (Fig. 2A and B). Moreover, we determined β-MHC gene expression using L-sh-109 and observed a 66.2% inhibitory effect (228.37 ± 4.23 vs. 77.01 ± 2.39, P < 0.01; Fig. 2C). We chose L-sh-109 only because it gave the best silencing effect. 18S rRNA was used as an internal loading control. Our data indicated that silence or knockdown of the myotrophin gene significantly inhibited ANF and β-MHC gene expression.

Fig. 3. Effect of various constructs of myotrophin on NF-κB activation in neonatal myocytes. A: cells were transduced with L-sh-55, L-sh-109, L-sh-196, and L-sh-267 at a MOI of 30 for 48 h and then stimulated with myotrophin. NF-κB oligonucleotide labeled with [32P]dATP. The complex formation was eliminated with excess unlabeled NF-κB oligonucleotide. The complex formation was confirmed by supershift analysis using p65 antibody. B: quantification of NF-κB activation from the results shown in A. Values are means ± SE for all constructs stimulated with myotrophin; n = 5. *P < 0.001 compared with untreated myocytes (lane 1); †P < 0.001 compared with myotrophin-stimulated myocytes (lane 2) and L-sh-109-transduced cells stimulated with myotrophin (lane 8). Transductions of other L-sh constructs stimulated with myotrophin were not significant compared with LRV-U6-CMV-EGFP-transduced myotrophin (lane 2), NE, nuclear extract. C: Western blot analysis using NF-κB-p65 antibody as a probe in all L-sh-Myo-transduced neonatal myocytes. Histone antibody (HA) was used as an internal nuclear protein loading control.
Effect of Knockdown of the Myotrophin Gene on NF-κB Activation in Neonatal Rat Myocytes

To study the relationship of myotrophin and NF-κB activity, we analyzed NF-κB activation by all L-sh-Myo constructs that were transduced into myocytes and stimulated with myotrophin. Our data showed that NF-κB activation was significantly inhibited in L-sh-109-transduced cells stimulated by myotrophin (60.8% over myotrophin-stimulated cells, \( P < 0.001 \)) but not by the other lentiviral constructs, suggesting that L-sh-109 is responsible for the inhibition of NF-κB activation (Fig. 3A). We further confirmed our observation by Western blot analysis, which showed that NF-κB-p65 translocation was significantly inhibited in myotrophin-stimulated L-sh-109 constructs (Fig. 3B). LRV-U6-CMV-EGFP was transduced into myocytes and used as a control. Our data suggest that a direct relationship exists between myotrophin and NF-κB and that both are required for increased myocyte growth.

Effect of Knockdown of the Myotrophin Gene on Cardiac Mass in Myo-Tg Mice

To determine the effect of L-sh-109 in vivo, we delivered L-sh-109 directly into the myocardium of 10-wk-old Myo-Tg mice. Other Myo-Tg mice, which were injected with PBS, were used as sham controls. After 6 wk, both sham and L-sh-109-Myo-Tg mice were killed. L-sh-109-treated Myo-Tg mice showed a significant reduction in the heart weight-to-body weight ratio (8.0 vs. 5.7, \( P = 0.002 \), \( n = 7 \); Fig. 4A) compared with sham controls, which showed no effect. To confirm the expression of L-sh-109 in the myocardium of Myo-Tg mice, we checked the level of in vivo expression of EGFP in the myocardium by immunohistochemistry using GFP antibody as a probe. Our data showed a robust expression of EGFP in cardiac sections from L-sh-109-Myo-Tg mice, as evidenced by green fluorescence throughout the myocardium (Fig. 4C). The red stain in Fig. 4C shows cardiac myocytes detected by α-actinin. Our data suggest that L-sh-109 was expressed in the heart only and that use of this lentiviral construct significantly inhibited myotrophin gene expression.

Effect of Transduction of L-sh-109 on Myotrophin, ANF, and β-MHC Gene Expression

To determine the effect of L-sh-109 on the gene expression of myotrophin, ANF, and β-MHC, both sham control and L-sh-109-transduced Myo-Tg mice were killed, and their myotrophin, ANF, and β-MHC expression levels were analyzed. Our data showed that significant inhibition of myotrophin gene (55.4% over untreated Myo-Tg mice, \( P < 0.001 \)) and protein expression (49.2% over untreated Myo-Tg mice, \( P = 0.002 \); Fig. 5, A and B). Our data also showed a significant reduction in both ANF (61.6% over untreated Myo-Tg mice, \( P > 0.001 \)) and β-MHC (42% over untreated Myo-Tg mice, \( P = 0.002 \)) expression (Fig. 5, C and D). GAPDH was used as an internal loading control for RNA. Actin was used as a protein loading control.

Effect of Knockdown of the Myotrophin Gene in the NF-κB Activation Cascade

To determine the effect of L-sh-109 on the NF-κB activation cascade, we analyzed NF-κB activity, IkB-α protein levels, and p65 mRNA and protein levels. The results are shown in Fig. 6. We observed significant inhibition of NF-κB activity (56.2% over untreated Myo-Tg mice, \( P < 0.001 \)). Furthermore, we observed that p65 translocation was significantly inhibited in L-sh-109-transduced Myo-Tg mice. Histone was used as an internal nuclear protein loading control. In addition, our data showed a significant reduction in IkB-α protein levels (49.2% over untreated Myo-Tg mice, \( P < 0.001 \)) and p65 mRNA (52.8% over untreated Myo-Tg mice, \( P < 0.001 \)) in L-sh-109-transduced Myo-Tg mice. The 18S rRNA oligonucleotide was used as an internal loading control for RNA, histone antibody was used as an internal nuclear protein loading control, and GAPDH antibody was used as an internal cytoplasmic protein loading control for IkB-α protein expression.

Effect of Knockdown of the Myotrophin Gene on Cytokines and Growth Factors

To evaluate the effect of L-sh-109 on cytokines and growth factor gene expression in Myo-Tg mice, we used the oligo GE microarray system from Super Array Bioscience. The GE microarray filter was set up for 82 genes. We calculated the changes of gene expression (up or down) in terms of percent over untreated Myo-Tg mice. We used a cut off limit of 40% down over Myo-Tg gene expression. Compared with Myo-Tg mice, 36 genes were found to be
downregulated in L-sh-109-treated mice, suggesting their potential role in myotrophin-induced CH. These included mostly bone morphogenetic protein (BMP), IL, and TNF superfamily genes. Additionally, we found that the growth differentiation factor 3, growth differentiation factor 5, IFN-13, and IFN-2 genes were significantly downregulated in L-sh-109-transduced Myo-Tg mice compared with age-matched Myo-Tg mice. The remainder of the genes on the array were not significantly different in their expression levels compared with age-matched Myo-Tg mice.

The expression of various cytokine and growth factor genes is shown in Table 1.

DISCUSSION

The most significant finding in this study is that knockdown of the myotrophin gene, using a lentivirus-mediated shRNA delivery approach, caused a significant regression of CH associated with an inhibition of NF-κB activation.

Using shRNA of the myotrophin gene in a lentiviral vector, we studied the mechanism of myotrophin-induced myocyte growth. siRNAs represent an alternative to approaches using antisense oligonucleotides to silence gene expression. Lentiviral delivery of shRNAs transcribed from RNA Pol III promoters situated within the viral backbone and processed into siRNAs has recently been described by many investigators (8, 14, 24, 36). RNAi is mediated by RNA duplexes bound to a protein complex called the RNA-induced silencing complex, which is governed by siRNA to accomplish the specific recognition of homologous mRNA sequences followed by degradation (8, 14, 24, 36). Our study showed that shRNAs could be expressed in a lentiviral long terminal repeat backbone driven by the U6 promoter. Lentiviral gene transfer allows the stable integration of the shRNA expression cassette in neonatal myocytes as well as into the myocardium and lasts for several days without showing any detrimental effects. Our study also showed a partial inhibition of the myotrophin gene, providing
evidence of the knockdown effect. A longer treatment with L-sh-109 may have resulted in even better regression of hypertrophy.

Our data showed five lines of evidence that substantiate these findings. First, using RNAi technology, we established an important role of myotrophin in the CH process in neonatal rat myocytes. In this report, we first validated the efficacy of siRNA made against myotrophin in lentiviral vectors to achieve a better expression system within the cells. The benefit of using lentiviral vectors is that we can achieve a constitutive expression of shRNA using the U6 promoter in the experimental system. Our data showed that of the four constructs of L-sh-Myo, L-sh-109 showed the best silencing/inhibitory effect, both at the transcriptional and translational levels, in the presence or absence of myotrophin (Fig. 1). Using L-sh-109, we determined the role of L-sh-109 in CH gene expression. We observed significant inhibition of ANF and β-MHC gene expression in the presence of myotrophin (Fig. 2). This study further confirms that myotrophin is one causal factor...
for myocardial hypertrophy (myocyte growth). Collectively, our data provide strong evidence that myotrophin might be a target for the treatment/prevention of CH that progresses to HF.

Second, we determined the association between myotrophin and NF-κB in L-sh-Myo (L-sh-55, L-sh-109, L-sh-196, and L-sh-267)-transduced myocytes in the presence versus absence of myotrophin. Our data showed that L-sh-109-transduced cells significantly inhibited myotrophin-induced NF-κB activation (Fig. 3). Our data suggest that L-sh-109 is responsible for the initiation of CH in neonatal rat myocytes.

Third, to examine the efficacy of lentivirus-mediated gene transfer in vivo, L-sh-109 was directly injected into the hearts of Myo-Tg mice. A significant reduction in cardiac mass, associated with an attenuation of ANF and β-MHC genes (marker genes for CH) in L-sh-109-transduced Myo-Tg mice (Fig. 5, C and D) compared with untreated Myo-Tg mice. This is the first report of an in vivo demonstration of the effects of silencing the myotrophin gene in cardiac remodeling.

Fourth, to determine the functional significance of L-sh-109 in CH, we examined the status of the NF-κB activation cascade in L-sh-109-transduced Myo-Tg mice. Our data revealed that NF-κB signaling components (NF-κB activation, IkB-α protein, and p65) were significantly inhibited in L-sh-109-transduced Myo-Tg mice (Fig. 6). Taken together, our data demonstrate the association between myotrophin, NF-κB, and cardiac remodeling. Our data also suggest that silencing the myotrophin gene with si-109 resulted better regression or prevention of CH compared with si-p65.

Fifth, we analyzed the cytokines and growth factor changes by Oligo GE microarray analysis in Myo-Tg and L-sh-109-treated Myo-Tg mice. Our data showed the upregulation of many IL and TNF family genes in Myo-Tg mice, as shown in Table 1. Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are not constitutively expressed in the normal heart (18). The upregulation and production of these cytokines represent an intrinsic or innate stress response against myocardial injury (22). This observation suggests that overexpression of the myotrophin gene stimulates cytokines/growth factors

### Table 1. Expression of various cytokine and growth factor genes

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<th>Gene Name</th>
<th>Gene</th>
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<th>Percent Inhibition Over Myo-Tg</th>
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<td>Interleukin 1 α</td>
<td>II1a</td>
<td>99.21</td>
<td>45.2</td>
<td>54.4</td>
<td>&lt;0.01</td>
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<td>Interleukin 1 β</td>
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<td>99.13</td>
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<td>Interleukin 1 family, member 7</td>
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<td>96.80</td>
<td>47.9</td>
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<tr>
<td>Interleukin 1 receptor antagonist</td>
<td>II1m</td>
<td>106.14</td>
<td>59.45</td>
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<tr>
<td>Interleukin 2</td>
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<td>104.17</td>
<td>34.19</td>
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<td>Interleukin 20</td>
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<td>21.26</td>
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<td>Similar to interleukin 21</td>
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<td>98.7</td>
<td>48.95</td>
<td>50.4</td>
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<td>Interleukin 4</td>
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<td>Interleukin 5</td>
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<td>25.33</td>
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<td>Interleukin 6</td>
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<td>24.62</td>
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<td>Leukemia inhibitory factor</td>
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<td>27.31</td>
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<td>Oncoprotein-induced transcript 1</td>
<td>Oti1</td>
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<td>Oncostatin M</td>
<td>Osm</td>
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<td>Tumor necrosis factor receptor superfamily, member 11b</td>
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<td>41.399</td>
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<td>90.1</td>
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Myo-Tg, myotrophin transgenic.

H634  ABLATION OF MYOTROPHIN GENE ATTENUATES CARDIAC HYPERTROPHY
that promote CH. Cytokines also have the unique ability to self-amplify through a positive feedback loop targeting the transcription factor NF-κB. Activation of NF-κB will, in turn, accelerate the production of various cytokines, including TNF-α and IL-6. Interestingly, L-sh-109-transduced Myo-Tg mice showed significant inhibition of many cytokines/growth factors, notably TNF, IL, and BMP family genes. These cytokines and growth factors are not constitutively expressed in the normal heart but are upregulated in Myo-Tg mice as a result of CH or HF (31). One possible reason is that these cytokines/growth factors were inhibited is the inhibition of NF-κB; all these inflammatory genes are regulated by NF-κB, as a consequence of inhibition of the myotrophin gene by L-sh-109. It has been reported that the inhibition of NF-κB reduces the expression of various inflammatory genes in rat model challenged with lipopolysaccharide (20). Furthermore, it has been shown in other in vitro studies that the inhibition of NF-κB significantly attenuates NF-κB-dependent genes (19, 26, 38, 40).

In summary, the present data demonstrated that silencing of the myotrophin gene using RNAi technology resulted in the attenuation of CH. Our data showed that inhibition of the myotrophin gene resulted in a decrease in cardiac mass, associated with a significant reduction in ANF and β-MHC gene expression in the L-sh-109-Myo-Tg model. The reduction in cardiac mass was further linked with the downregulation of several known cytokines and growth factors in the L-sh-109-transduced Myo-Tg model. Our study also suggests that the lentivirus-mediated gene delivery technique is not toxic to the animal, and the effects appeared to be specific for myotrophin and lasted over several weeks, a long enough period of time to observe and analyze the phenotypic changes.

In conclusion, we demonstrated that, when administered directly into myocardium, U6 promoter-based shRNA constructs targeted against myotrophin reduce cardiac mass significantly by inhibiting the NF-κB signaling cascade in a Myo-Tg mouse model. Myotrophin could therefore be used as a marker for CH and may provide a new target for future therapeutic possibilities in designing drugs for human use. Our data also suggest that RNAi can be used to specifically analyze the role of a specific gene product. We envision an expansion in the application of RNAi in our animal model to improve our understanding of CH and HF. The findings may provide a better understanding of the mechanisms of cardiac remodeling and new insights into the development of novel therapeutic strategies in CH.

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GRANTS

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REFERENCES


