Identification of Nogo as a novel indicator of heart failure

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Bullard TA, Protack TL, Aguilar F, Bagwe S, Massey HT, Blaxall BC. Identification of Nogo as a novel indicator of heart failure. Physiol Genomics 32: 182–189, 2008. First published October 30, 2007; doi:10.1152/physiolgenomics.00200.2007.—Numerous genetically engineered animal models of heart failure (HF) exhibit multiple characteristics of human HF, including aberrant β-adrenergic signaling. Several of these HF models can be rescued by cardiactargeted expression of the Gβγ-inhibitory carboxy-terminus of the β-adrenergic receptor kinase (βARKct). We recently reported microarray analysis of gene expression in multiple animal models of HF and their βARKct rescue, where we identified gene expression patterns distinct and predictive of HF and rescue. We have further investigated the muscle LIM protein knockout model of HF (MLP−/−), which closely parallels human dilated cardiomyopathy disease progression and aberrant β-adrenergic signaling, and their βARKct rescue. A group of known and novel genes was identified and validated by quantitative real-time PCR whose expression levels predicted phenotype in both the larger HF group and in the MLP−/− subset. One of these novel genes is herein identified as Nogo, a protein widely studied in the nervous system, where it plays a role in regeneration. Nogo expression is altered in HF and normalized with rescue, in an isomform-specific manner, using left ventricular tissue harvested from both animal and human subjects. To investigate cell type-specific expression of Nogo in the heart, immunofluorescence and confocal microscopy were utilized. Nogo expression appears to be most clearly associated with cardiac fibroblasts. To our knowledge, this is the first report to demonstrate the relationship between Nogo expression and HF, including cell-type specificity, in both mouse and human HF and phenotypic rescue.

Previously, we reported microarray investigation of the development, progression, and regression of HF using two mouse models of HF. Deletions of the muscle LIM protein (MLP), an actin-associated cytoskeletal protein, and cardiarcstricted overexpression of the calcium binding protein calsequestrin (CSQ) both produce an HF phenotype that replicates various aspects of human dilated cardiomyopathy (2, 7), including aberrant β-adrenergic receptor (β-AR) signaling (16, 33). The MLP−/− mice appear to closely replicate human dilated cardiomyopathy, with a progressive reduction in cardiac function beginning at 4–6 mo, whereas the CSQ mice develop a rapid-onset dilated cardiomyopathy resulting in mortality by 9–14 wk. Cardiac-specific inhibition of the interaction between the G protein dimer Gβγ and the β-AR kinase 1 (βARK1, a.k.a. GRK2) by myocardial-targeted expression of the carboxy terminus of βARK (βARKct) increased survival and normalized β-AR signaling and cardiac function in the MLP−/− and CSQ mice (16, 33). Using these animal models of HF (MLP−/−, CSQ) and “rescue” (MLP−/−/βARKct and CSQ/βARKct), we conducted a robust microarray study (n = 53) to identify several known and novel genes associated with development, progression, and regression of HF (3). In the current study, we further investigated the gene expression data associated specifically with the MLP−/− model of human dilated cardiomyopathy and its βARKct-mediated rescue.

Left ventricular assist devices (LVADs) have proven an effective intervention for end-stage HF, both as a bridge to cardiac transplant as well as destination therapy (35). Furthermore, LVADs have been shown to lead to partial normalization of myocardial structure and function, including increased β-AR responsiveness (30); decreased myocyte volume (40), width (41), and diameter (22); and improved myocyte contractility (10) in a process termed “reverse remodeling.” We were the first to report both global and etiology-specific differential gene expression using paired tissue samples from patients in end-stage HF and following LVAD-mediated reverse remodeling, followed by several other reports (4, 5, 15, 24). These tissue samples provide a powerful model to investigate progression and regression of HF with patients as their own reference, eliminating the confounding factors of human heterogeneity. Among the novel genes we identified in both our prior and current microarray study of murine HF was a gene formerly characterized only as a novel clone, which we now identify as Nogo. Nogo and its three splice variants A, B, and C are members of the reticulon (rtn 4) family of proteins (14) whose functions have been most widely studied in the central nervous system, where they inhibit axonal regeneration. A functional role for Nogo in vascular pathology was originally reported by Acevedo et al. (1), who found that tightly regulated expression levels of the Nogo-B splice variant play an important role in...
cell migration and vascular remodeling in response to vascular injury. Subsequent studies have demonstrated a role for Nogo-B in vascular endothelial cell response to injury (26, 31, 32) and further suggest an important role for locally decreased Nogo-B expression in atherosclerosis (34).

Human nonfailing, pre- and post-LVAD tissue samples provide us the distinct advantage of validating novel genes such as Nogo, identified in our mouse microarray studies, in human cardiac tissue that represents both the progression and regression of myocardial disease. Herein we describe strain-specific microarray analysis of the MLP-/- model of HF and its rescue by Gβγ inhibition with βARKct. Furthermore, we report the discovery of Nogo as one of the novel clones identified in our microarray study of the development, progression, and rescue of HF. Finally, we validate differential expression and cell-specific localization of Nogo in mouse and human HF, elucidating a potential role for differential Nogo expression and localization in mouse and human HF and cardiovascular disease.

METHODS

Microarray Data

Microarray data were prepared according to the “minimum information about a microarray experiment” (MIAME) recommendations and were originally submitted with our prior manuscripts (3, 4). The mouse microarray data can be accessed via the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/ with the GEO accession numbers GSM-10178 through GSM-10283 (3).

Statistical Analyses

Affymetrix probe-set data were first converted to normalized gene expression values using robust multiarray analysis (RMA, available at www.bioconductor.org, based on R 1.7 software, available at www.r-project.org) (18, 38). Following normalization, genes included in statistical analyses were found to be present above background on at least two arrays per group. Resulting gene expression data was then subjected to data visualization [multidimensional scaling (MDS), see below] and two independent statistical analyses: 1) significance analysis of microarrays (SAM; available at http://www.stat.stanford.edu/~tibs/SAM/) with an estimated overall false-discovery rate cutoff of 0.10 (36), and 2) Bayesian regression based on permutation-tested t-statistic priors, followed by leave-one-out cross-validation or singular value decomposition, as we have previously described (3). Data visualization was achieved with MDS, a data structure visualization and analysis tool considered more robust than hierarchical clustering, which, in the simplest of terms, is a mapping of high-dimensional microarray data (~11,000 genes per animal) into low-dimensional space, such that the distances between points mapped in low-dimensional Euclidean space represent, in this case, dissimilarities or distances of the high-dimensional original data (DR). Herein, metric MDS was performed based on Pearson dissimilarity [defined as (1 – r)/2, where r is the Pearson correlation] and mapped into two-dimensional space (initialized with points drawn randomly from a uniform distribution in the range of −1 to 1). Figures are generated by MDS to minimize a measure of badness of fit, called stress, which depends on the point-to-point dissimilarities in the high-dimensional data compared with the distances in low-dimensional space; all images presented herein had stress values from 0.005 to 0.079. Subsequently, MDS data from the dissimilarity matrix were subjected to randomization and a single- or double-exact test for validation of nonrandom segregation. For further details, see Ref. 3.

Genes identified by both statistical methods were considered for further analysis, and we required >25% change in expression for consideration. The list of 20 genes presented in Table 2 represents genes common to both analytical methods that met all criteria. Gene identity of Affymetrix probes (expressed sequence tags) was determined using an annotation database (available at www.netaffx.com) or basic local alignment search tool (BLAST) search (http://www.ncbi.nih.gov/BLAST).

Tissue Sampling

Mouse tissue. LV myocardial tissue was dissected out of hearts freshly harvested from nine mice, weighed, immediately frozen in liquid nitrogen, and then stored at −80°C as described previously (3). Cardiac phenotypes were validated by conscious echocardiography prior to death (3). All animal procedures and tissue harvesting were performed in accordance with National Institutes of Health (NIH) guidelines and were approved by the University of Rochester IACUC.

Human tissue. Tissue was harvested from the LV free wall near the apex of 11 male patients at the time of LVAD placement (HeartMate, Thoratec, Pleasanton, CA) and at the time of myocardial explant. Duration of LVAD support in all patients ranged between 2 and 4 mo. Nonfailing tissue was obtained from the LV free wall (toward the apex) of five male organ donor hearts rejected for transplant for physical incompatibility. LV tissue obtained from surgery was immediately frozen in liquid nitrogen and stored at −80°C. All surgical procedures and tissue harvesting were performed in accordance with NIH guidelines and were approved by the University of Rochester Institutional Review Board. Caution was exercised to utilize only viable, nonischemic cardiac tissue from both pre- and post-LVAD samples for RNA extraction.

RNA Extraction

Total RNA extraction from human left ventricular tissue weighing ~120–150 mg was performed with the RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. We used the Eppendorf Biophotometer (Eppendorf North America, Westbury, NY) to measure RNA concentration for each sample.

Table 1. Primers for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer</th>
<th>F</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogo-A</td>
<td>GGGAAGGCTAGCTAAATAACCTCGAG</td>
<td>ATCTTGCCATCCTTGGACAC</td>
<td>ATCTTGCCATCCTTGGACAC</td>
</tr>
<tr>
<td>Nogo-B</td>
<td>GCTATCTCTGCTGTTTCTTATTGAC</td>
<td>GGCCATCCACAGTCTTCT</td>
<td>GGCCATCCACAGTCTTCT</td>
</tr>
<tr>
<td>Nogo-C</td>
<td>GGAGGGCTGCGGGGCACGTGAG</td>
<td>GGAGGGCGGCAACTCTTCTTACG</td>
<td>GGAGGGCGGCAACTCTTCTTACG</td>
</tr>
<tr>
<td>Nogo Receptor</td>
<td>CTGGAGGATGACCAACCTACG</td>
<td>ATCTTGCCATCCTTGGACAC</td>
<td>ATCTTGCCATCCTTGGACAC</td>
</tr>
<tr>
<td>FHL-1</td>
<td>CTTGGAGGGAAGAAGATCTGAG</td>
<td>ATCTTGCCATCCTTGGACAC</td>
<td>ATCTTGCCATCCTTGGACAC</td>
</tr>
<tr>
<td>ANF</td>
<td>CTGGAGGATGACCAACCTACG</td>
<td>ATCTTGCCATCCTTGGACAC</td>
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<tr>
<td>CTGF</td>
<td>AGACGGGGGGGAAGAAGATCTGAG</td>
<td>ATCTTGCCATCCTTGGACAC</td>
<td>ATCTTGCCATCCTTGGACAC</td>
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<tr>
<td>B-MHC</td>
<td>TGGAAGGACGTCACGCCTTGAT</td>
<td>GGGGAGGAGCCGGAATCGTGAC</td>
<td>GGGGAGGAGCCGGAATCGTGAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ATACTCTCCTTTCCCCAGTGAGCC</td>
<td>ATACTCTCCTTTCCCCAGTGAGCC</td>
<td>ATACTCTCCTTTCCCCAGTGAGCC</td>
</tr>
<tr>
<td>Nogo-A human</td>
<td>CATGCTGCTTATTTTCGTAAGTTAGG</td>
<td>CATGCTGCTTATTTTCGTAAGTTAGG</td>
<td>CATGCTGCTTATTTTCGTAAGTTAGG</td>
</tr>
<tr>
<td>Nogo-B human</td>
<td>CCAATTTGCTGCTTATTTTCGTAAGTTAGG</td>
<td>CCAATTTGCTGCTTATTTTCGTAAGTTAGG</td>
<td>CCAATTTGCTGCTTATTTTCGTAAGTTAGG</td>
</tr>
<tr>
<td>Nogo-C human</td>
<td>GTCAGGCAAGAAGATCTGAG</td>
<td>GTCAGGCAAGAAGATCTGAG</td>
<td>GTCAGGCAAGAAGATCTGAG</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
Reverse Transcription

Two hundred nanograms of total RNA was utilized for reverse transcription. The Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used according to manufacturer’s instructions. The size of RT products and specificity of primers were tested by running samples on 2% agarose (Seachem LE Agarose; Cambrex Bio Science Rockland, Rockland, ME) gels containing ethidium bromide and visualized by ultraviolet light.

Primer Design

Mouse and human primers were purchased from Invitrogen and IDT (Coralville, IA). The sequences were obtained using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were subsequently BLASTed against the National Center for Biotechnology Information database. Human and mouse primer sequences are listed in Table 1.

Real-time PCR

Real-time PCR was performed using the ABI Prism 7900HT Sequence Detection System (ABI, Foster City, CA). All assays were performed in a 384-well plate with each reaction mixture having a final volume of 20 µl. All samples were run in triplicate. Briefly, the reaction mixture comprised 5 µl of RT products diluted 1:100 in DNase-free water, 900 nM forward primer, 900 nM reverse primer, 1× Syber Green Master Mix (ABI) and DNase-free water. The reaction conditions were denaturation step of 95°C for 10 min, 40 cycles of amplification and quantification steps of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s.

Gene expression was analyzed by the $2^{-\Delta Ct}$ method described by Livak and Schmittgen (21). Threshold values were set to 0.2 and the threshold cycle number (Ct) was determined for each gene of interest and GAPDH, and the $\Delta Ct$ was calculated ($Ctgene of interest - CtGAPDH$). To compare differences in gene expression between the treatment groups, $\Delta\Delta Ct$ values were calculated ($\Delta Ct Pre LVAD - \Delta Ct Non Failing$) or ($\Delta Ct Post LVAD - \Delta Ct Non Failing$). In calculating the error for each sample set, the error was estimated by evaluating the $2^{-\Delta\Delta Ct}$ term using $\Delta\Delta Ct$ plus the standard deviation and $\Delta\Delta Ct$ minus the standard deviation and is referred to as the data range. This produces an asymmetrical distribution because the values from an exponential process are converted to a linear range of values. Therefore, presenting the data as a mean and standard deviation or standard error of the mean is impossible using this method (21).

Immunohistochemistry

Mouse hearts and human left ventricular tissue were frozen in liquid nitrogen and stored at −140°C. Samples were embedded in Tissue-Tek O.T.C. compound, and 8 µm sections were made on a cryostat at −20°C, collected on glass slides, and stored at −80°C until use. Once slides were removed from −80°C, they were allowed to come to room temperature, then were fixed in acid alcohol for 5 min, and rinsed three times with PBS. Slides were blocked in 5% BSA and then 5% normal rabbit serum in 1% BSA for 1 h each. The Nogo primary antibody (N-18 sc-11027; Santa Cruz Biotechnology) was diluted 1:100 in antibody diluent (Dako). Slides were incubated in the primary antibody overnight at 4°C. Slides were rinsed in three changes of PBS and incubated for 1 h at 37°C in a rhodamine-conjugated affiniPure rabbit anti-goat secondary antibody diluted 1:200 in antibody diluent. Slides were

Table 2. Effect of diabetes on mouse HDL-induced nitric oxide production

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Ionomycin</th>
<th>Acetylcholine</th>
<th>Serum</th>
<th>LPDS</th>
<th>HDL</th>
<th>LDL/VLDL</th>
<th>Serum + Acetylcholine</th>
<th>LPDS + Acetylcholine</th>
<th>HDL + Acetylcholine</th>
<th>LDL/VLDL + Acetylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Citrate</td>
<td>18.2</td>
<td>256± 7</td>
<td>254± 6</td>
<td>19± 3</td>
<td>263± 9</td>
<td>22± 5</td>
<td>255± 7</td>
<td>251± 8</td>
<td>257± 9</td>
<td>256± 8</td>
<td>257± 9</td>
</tr>
<tr>
<td>Diabetic Streptozotocin</td>
<td>19.4</td>
<td>251± 5</td>
<td>253± 5</td>
<td>18± 5</td>
<td>17± 3</td>
<td>19± 4</td>
<td>18± 5</td>
<td>19± 3</td>
<td>258± 7</td>
<td>20± 4</td>
<td>259± 8</td>
</tr>
<tr>
<td>C57BL/ks</td>
<td>18.4</td>
<td>253± 8</td>
<td>251± 6</td>
<td>20± 4</td>
<td>259± 6</td>
<td>21± 4</td>
<td>256± 5</td>
<td>252± 9</td>
<td>257± 4</td>
<td>255± 9</td>
<td></td>
</tr>
<tr>
<td>L57BL/kslepr/db</td>
<td>20.5</td>
<td>254± 6</td>
<td>255± 7</td>
<td>20± 5</td>
<td>19± 5</td>
<td>21± 4</td>
<td>20± 3</td>
<td>17± 5</td>
<td>261± 9</td>
<td>19± 4</td>
<td>256± 7</td>
</tr>
</tbody>
</table>

fmoles [3H]citrulline/well, n = 7, mean ± SE.
then incubated in 488 Phalloidin (1:200, Molecular Probes) and TOPRO-3 (1:500, Molecular Probes) and coverslipped. Slides were viewed and analyzed with the 40X oil immersion objective of an Olympus IX70 laser scanning confocal microscope and Olympus Flow view software.

RESULTS

To investigate differential gene expression following data normalization and filtering (see METHODS), we first performed data visualization analysis by MDS (3). We replicated our ability to clearly distinguish between normal (wild type), HF (MLP−/−), and rescue (MLP−/−βARKct) mice (Fig. 1). MDS data from the resulting Pearson dissimilarity matrix were subjected to randomization and a single- or double-exact test for validation of nonrandom segregation, demonstrating nonrandom MDS segregation of the microarray data (P < 0.001, not shown). Subsequently, we performed two statistical analyses of the microarray data, including SAM using a false discovery rate cutoff of <0.10 (36), and Bayesian regression with leave-one-out crossvalidation, as we have previously described (3, 4). Several known genes and novel cDNA clones were associated with HF and rescue/reverse remodeling in two models of HF (3). Interestingly, all genes identified as the top 20 significant predictors of cardiac phenotype in the MLP−/− group (Table 2) were common with our prior grouped analysis of both MLP−/− and CSQ animals (3).

![Validation of the novel gene Nogo in left ventricular specimens obtained from patients with end-stage heart failure prior to obtaining the LVAD (PRE); and from the same patients at the time of heart transplant following –2 mo LVAD support (POST) by real-time PCR. Combined heart failure samples. Both Nogo-A and C increased during heart failure, however, only Nogo-A normalized in response to LVAD support. Nogo-B decreased during heart failure and returned to values above the control. Values were separated out according to disease etiology (IDC and ISC, see Table 5). Data was normalized to GAPDH and analyzed using the 2−ΔΔCt method (see METHODS). Values represent the fold change compared with control samples, the data range for each sample set is included in Table 5. All Samples were run in triplicate. NF, nonfailing; PRE, prior to LVAD support; POST, after LVAD support.](image)

**Table 3. Real-time PCR validation of gene expression in MLP−/− mice**

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT (data range)</th>
<th>Heart Failure (data range)</th>
<th>Rescue (data range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>CTGF</td>
<td>1.00 (0.25–3.95)</td>
<td>7.03 (5.126–9.634)</td>
<td>0.66 (0.3–1.48)</td>
</tr>
<tr>
<td>FHL-1</td>
<td>1.00 (0.69–1.45)</td>
<td>2.1 (1.83–2.4)</td>
<td>1.12 (0.793–1.58)</td>
</tr>
<tr>
<td>B-MHC</td>
<td>1.00 (0.09–10.81)</td>
<td>14.7 (5.57–38.78)</td>
<td>2.15 (1.614–2.86)</td>
</tr>
<tr>
<td>ANF</td>
<td>1.00 (0.21–4.88)</td>
<td>8.15 (2.96–22.44)</td>
<td>2.94 (0.77–11.28)</td>
</tr>
</tbody>
</table>

**Table 4. Real-time PCR validation of Nogo expression in MLP−/− mice**

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT (data range)</th>
<th>Heart Failure (data range)</th>
<th>Rescue (data range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Nogo-A</td>
<td>1.00 (0.23–4.40)</td>
<td>1.76 (1.51–4.40)</td>
<td>0.74 (0.64–0.86)</td>
</tr>
<tr>
<td>Nogo-B</td>
<td>1.00 (0.19–5.14)</td>
<td>0.44 (0.27–0.74)</td>
<td>0.62 (0.39–0.98)</td>
</tr>
<tr>
<td>Nogo-C</td>
<td>1.00 (0.06–16.25)</td>
<td>0.2 (0.09–0.46)</td>
<td>1.47 (1.29–1.57)</td>
</tr>
<tr>
<td>NogoReceptor</td>
<td>1.00 (0.33–3.02)</td>
<td>0.06 (0.01–0.47)</td>
<td>0.14 (0.01–1.79)</td>
</tr>
</tbody>
</table>

Validation of Known Genes in HF and Rescue

We previously identified numerous known and novel genes that were predictive for HF and rescue. Of the known genes identified in the current and prior study, four and a half LIM domain-1 (FHL-1) expression has been reported to be upregulated in two mouse models of cardiac hypertrophy and dilated cardiomyopathy, suggesting a role for this molecule in the development of heart disease (8). Additionally, connective tissue growth factor (CTGF) has also been shown to be an indicator of cardiac myocyte hypertrophy and fibrosis (25); therefore, we sought to validate our model by examining the expression of FHL-1 and CTGF as indicators of HF and rescue. Although both sets of genes increased during failure and normalized with rescue, we observed a greater increase of CTGF RNA during failure than FHL-1 (Fig. 2 and Table 3). To further validate the mouse model and our microarray data, we examined changes in atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC). ANF and β-MHC increased during HF and at least partially normalized with rescue (Fig. 2 and Table 3).

Characterization of Nogo Expression in Mouse HF and Rescue

Nogo has been demonstrated to have a role in modulation of vascular, endothelial cell, and skeletal muscle pathologies,
with particular focus on the Nogo isozyme Nogo-B (1, 26, 31, 32, 34). Having identified Nogo as one of the novel genes in our prior and current microarray study of murine HF, and considering the role of Nogo-B in vascular pathology, we investigated whether cardiac expression of specific Nogo isozymes were associated with HF or rescue. Nogo-A expression increased, while Nogo-B and C expression decreased, in the left ventricle of MLP−/− mice. Expression of all three Nogo isozymes were at least partially normalized with rescue (Fig. 3 and Table 4), suggesting an important role for Nogo, perhaps in an isozyme-specific manner, in HF and rescue.

**Characterization of Nogo Expression in Human HF**

Having observed differential Nogo expression in our mouse model of HF and its partial normalization with rescue, we sought to determine whether Nogo expression was altered in human HF. Nogo expression was investigated in using left ventricular tissue harvested at the time LVAD implantation, and subsequently at the time of transplant. Nonfailing donor hearts were utilized as a control comparison. Expression of all three Nogo isoforms was substantially altered during HF; however, only Nogo-A and B normalized following LVAD implantation. Expression of Nogo-A and C increased in HF while Nogo-B expression decreased (Fig. 4 and Table 5). In response to LVAD implantation, Nogo-A and C increased in HF, while Nogo-B expression decreased (Fig. 4 and Table 5). In response to LVAD implantation, Nogo-A and B normalized while Nogo-C remained elevated (Fig. 4 and Table 5). There appears to be Nogo isomorph specificity in the response to HF and LVAD-mediated reverse remodeling.

**Immunohistochemical Localization of Nogo**

To validate Nogo protein expression in normal and pathological tissue samples we performed immunohistochemical staining. Confocal microscopic analysis of samples obtained from the left ventricular walls of human and mouse hearts revealed positive Nogo immunoreactivity, further confirming our RT-PCR data. The positive staining was confined to fibroblasts and was associated with vasculature, while Nogo immunoreactivity was absent in myocytes (Fig. 5). In the fibroblasts, we found positive staining on the cell membrane and uniform staining pattern throughout the cytoplasm with an absence of staining in the nucleus. Isolated neonatal and adult mouse ventricular myocytes showed a lack of staining (data not shown), indicating that cardiac Nogo expression appears to be nonmyocyte.

**DISCUSSION**

Using multiple statistical methods and quantitative real-time PCR, we have identified and validated differential expression of several known and novel genes in mouse and human HF and their phenotypic rescue. MLP-specific reanalysis of our original data identified several genes that were highly predictive of HF, concordant with our prior study (Table 2). Secondary analysis of several of these genes, including CTGF, FHL-1, ANF, and β-MHC, validated their expression was indeed increased in HF and normalized in rescue, consistent with prior reports (8).

Analysis of our MLP−/− mouse microarray data identified a novel cDNA clone associated with HF, which, at the time, was listed as an unknown gene. Further investigation revealed this novel gene was in fact Nogo. Although Nogo expression has been widely investigated in the central nervous system, several other studies have demonstrated that Nogo is expressed in nonneuronal tissues such as skeletal muscle (23) and vascular tissue (1) and has been associated with skeletal muscle denervation (23) as well as amyotrophic lateral sclerosis (ALS) (12, 19) and various aspects of vascular remodeling (1, 26, 31, 32, 34). Northern and Western blot analyses have demonstrated that Nogo is expressed in the adult human heart (17), and immunohistochemical studies suggest Nogo may be present in the embryonic heart (28). As differential Nogo expression has been associated with vascular and skeletal muscle pathology, we sought to examine its cardiac expression in mouse and human models of HF and rescue.

Nogo encodes three isoforms, and we observed substantial changes in the cardiac expression of all three in HF and at least partial normalization in phenotypic rescue from HF. Specifically, we found Nogo-A increased while Nogo-B and Nogo-C decreased in HF [similar to data in ALS (23)]; all three were at least partially normalized in rescue. Nogo-B in particular appears to play an important role in cardiovascular physiology (1, 26, 31, 32, 34). Specifically, enhancing/replacing vascular Nogo-B expression significantly reduces vascular remodeling (1); this group has recently reported identification of a Nogo-B receptor (26). Our data demonstrate normalization of cardiac Nogo expression for all three isoforms is associated with normalization of cardiac function. Importantly, our real-time PCR experiments utilized LV homogenates, which include several cell types in addition to myocytes. Therefore, the cardiac Nogo-B we detected could be due to its expression in various cell types within the heart, e.g., in cardiac fibroblasts or the cardiac vasculature; its decreased expression may be a reflection of cardiac vascular pathology associated with HF.
Nogo and Fibrosis

Nogo staining of cardiac tissue was associated with fibroblasts, but not myocytes. Our results are consistent with those described by Dodd et al. (11) and Magnusson et al. (23). Dodd et al. (11) reported finding Nogo-A, -B, and -C on the surface of 3T3 fibroblasts by immunofluorescence, and Magnusson et al. (23) reported that the connective tissue surrounding muscle fibers and nerves were Nogo-B immunopositive. We found Nogo staining to be present on the cell membrane and uniform throughout the cytoplasm of fibroblasts, while Dodd et al. (11) reported Nogo-A staining to be punctate, nonuniform, and often localized to areas of cell-cell junction in isolated 3T3 cells and immature myoblasts. Discrepancies are likely due to differences between cell culture and intact tissues, antibody specificity, and/or antibody availability in cultured cells compared with tissue sections. Importantly, we used a commercially available antibody that recognizes all three isoforms of Nogo, while Dodd et al. (11) used a Nogo-A specific antibody.

Cardiac fibroblast Nogo expression has many implications. Most importantly, it suggests that Nogo may have a role in cardiac fibrosis and remodeling by regulating fibroblast migration. Several groups have examined the role of Nogo in cellular migration. Nogo has three regions that have been shown to inhibit neurite outgrowth and 3T3 fibroblast spreading (29). The inhibitory regions of Nogo include the NH2-terminal region, termed NiG, which is only found in Nogo-A (6), and the Nogo-66 domain, which is common to all isoforms (13). Nogo-B has been shown to promote the migration of vascular endothelial cells while inhibiting the migration of vascular smooth muscle cells (1, 26, 31, 32, 34). Recent studies have shed light on signaling molecules associated with Nogo. Experiments using NiG and Nogo-66 demonstrated that Nogo-A activates RhoA and suppresses Rac1 (27). Additionally, the use of RhoA or Rho kinase pharmacological inhibitors abolished the inhibitory effects of neurite outgrowth (27). The GTP binding proteins Rho and Rho-kinase have been shown to play a role in mediating cell migration, proliferation, and cardiac remodeling (7), thereby suggesting a mechanism for Nogo mediated migration in fibroblasts.

We report for the first time that Nogo-A, -B, and -C expression is altered during mouse and human HF normalized in phenotypic rescue. Interestingly, results from our human HF patients further characterized HF etiology-specific alterations in gene expression (See Supplemental Table 1).

Further analysis of Nogo protein expression by immunohistochemistry demonstrated that Nogo was expressed in cardiac fibroblasts to a much greater extent than in myocytes, indicating a potential role for Nogo in pathologic (including fibrotic) cardiac remodeling. These results corroborate our microarray findings and strongly suggest that, like in vascular pathology, Nogo may play a critical role in myocardial pathophysiology.

GRANTS

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REFERENCES


11. Dodd DA, Niederoest B, Bloehinger S, Dupuis L, Loeffler JP, Schwab ME. Nogo-A, -B, and -C are found on the cell surface and often localized to areas of cell-cell junction in isolated 3T3 fibroblasts, while Dodd et al. (11) used a Nogo-A specific antibody.


