Severe Heart Failure and Early Mortality in a Double-Mutation Mouse Model of Familial Hypertrophic Cardiomyopathy

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Background—Familial hypertrophic cardiomyopathy (FHC) is characterized by genetic and clinical heterogeneity. Five percent of FHC families have 2 FHC-causing mutations, which results in earlier disease onset, increased cardiac dysfunction, and a higher incidence of sudden death events. These observations suggest a relationship between the number of gene mutations and phenotype severity in FHC.

Methods and Results—We sought to develop, characterize, and investigate the pathogenic mechanisms in a double-mutant murine model of FHC. This model (designated TnI-203/MHC-403) was generated by crossbreeding mice with the Gly203Ser cardiac troponin I (TnI-203) and Arg403Gln α-myosin heavy chain (MHC-403) FHC-causing mutations. The mortality rate in TnI-203/MHC-403 mice was 100% by age 21 days. At age 14 days, TnI-203/MHC-403 mice developed a significantly increased ratio of heart weight to body weight, marked interstitial myocardial fibrosis, and increased expression of atrial natriuretic factor and brain natriuretic peptide compared with nontransgenic, TnI-203, and MHC-403 littermates. By age 16 to 18 days, TnI-203/MHC-403 mice rapidly developed a severe dilated cardiomyopathy and heart failure, with inducibility of ventricular arrhythmias, which led to death by 21 days. Downregulation of mRNA levels of key regulators of Ca²⁺ homeostasis in TnI-203/MHC-403 mice was observed. Increased levels of phosphorylated STAT3 were observed in TnI-203/MHC-403 mice and corresponded with the onset of disease, which suggests a possible cardioprotective response.

Conclusions—TnI-203/MHC-403 double-mutant mice develop a severe cardiac phenotype characterized by heart failure and early death. The presence of 2 disease-causing mutations may predispose individuals to a greater risk of developing severe heart failure than human FHC caused by a single gene mutation. (Circulation. 2008;117:1820-1831.)

Key Words: cardiomyopathy ■ mutation ■ heart failure ■ death

Familial hypertrophic cardiomyopathy (FHC) is a disorder characterized by marked clinical and genetic heterogeneity.¹ Patients with FHC can present with diverse clinical symptoms that range from mild dyspnea and chest pain to recurrent syncope, heart failure, and, most tragically, sudden death.²,³ In ≈10% of FHC patients, the disease can progress to a dilated, burnt-out phase, with left ventricular chamber dilatation, myocardial wall thinning, impaired systolic function, and overt New York Heart Association class III to IV heart failure.⁴,⁵ In the last 15 years, >400 single mutations in at least 14 genes have been shown to cause FHC. These causative genes most commonly encode proteins that are components of the sarcomere, eg, β-myosin heavy chain (β-MHC) and myosin-binding protein C (MyBP-C).¹,⁶ The long-standing paradigm has been that a single mutation in a single gene leads to FHC, hence the reference to FHC as a “monogenic” disorder.

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Most recently, we and others have described families with FHC in whom 2 disease-causing gene defects have been identified.⁷-⁹ These mutations have been found to occur in either the same gene (compound heterozygotes) or in different genes (double heterozygotes) in up to 5% of families with FHC. Importantly, individuals who carry 2 disease-causing
FHC mutations demonstrate more severe disease, including earlier onset of disease, more severe left ventricular hypertrophy and heart failure, and a higher rate of sudden death events (either resuscitated cardiac arrest or sudden death).7–10 These preliminary observations suggest that the number of genes identified in an individual patient with FHC may be an important determinant of phenotype severity and the clinical outcome of disease.

Animal models of FHC have been particularly useful in confirming the notion that the mutations identified in sarcomere genes cause FHC, with typical features of human FHC observed in these animal models.11–15 Furthermore, investigation of these models has shed light on potential underlying pathogenic mechanisms of FHC and identified candidate therapeutic interventions that have demonstrated both regression and prevention of FHC.13,16 Mutations in sarcomeric genes lead to activation of intracellular signaling mechanisms, cardiac remodeling, and changes in contractile function. Signaling through the latent transcription factor signal transducer and activator of transcription (STAT) 3 has been implicated in linking cardiac myocyte remodeling with various extrinsic and intrinsic stimuli.17,18 Specifically, STAT3 activation, characterized by phosphorylation on a specific tyrosine residue (Y705), promotes cardiac myocyte hypertrophy both in cell culture systems and in animal models.19,20 Previous studies in murine models have also indicated a role for STAT3 in cardiac protection, specifically with transgenic cardiac-specific STAT3 overexpression transducing a protective signal against cardiomyopathy after treatment with the antiinflammatory drug doxorubicin in vivo19 and enhancing vascular formation in the heart in vivo to mediate additional cardiac adaptation under conditions of stress.21 Consistent with this role of STAT3 in cardiac protection, the cardiac-specific knockout of STAT3 enhanced susceptibility to cardiac injury after myocardial ischemia, with reduced cardiac function and increased mortality,22 and resulted in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age.23 In human hearts with end-stage dilated cardiomyopathy, altered STAT3 levels and activation have also been observed.24,25 Taken together, the results of these studies suggest that STAT3 may be an important mediator in the development of cardiac hypertrophy and, through its modulation of cardiac cell death, the subsequent progression to heart failure.

The present study sought to develop and characterize a novel double-mutant murine model of FHC and to investigate the possibility that development of the severe symptoms of FHC, specifically end-stage severe heart failure, may be associated with the presence of 2 disease-causing gene mutations. Furthermore, the specific activation of STAT3 was investigated as a potential signaling mechanism underlying the development of heart failure in the double-mutant murine model of FHC.

**Methods**

**Mouse Models**

The Arg403Gln α-myosin heavy chain (MHC-403) knockout/knock-in mouse model has been described previously.11 In brief, a guanine-to-adenine point mutation at codon 403 that results in an amino acid change from arginine to glutamine (Arg403Gln) was introduced into exon 13 of the mouse cardiac α-MHC gene. These mice develop characteristic features of human FHC by 30 weeks of age.11 The Gly203Ser cardiac troponin I (Tnl-203) transgenic mouse model has been described recently.26 In brief, a transgene was constructed that contained the cardiac-specific mouse α-MHC promoter (kindly provided by Dr Jeffrey Robbins, Robbins Lab, Cincinnati, Ohio), mutant human cardiac Tnl cDNA, and the human growth hormone poly-A tail. Tnl-203 mice develop the characteristic phenotypic features of human FHC by 21 weeks of age, whereas wild-type Tnl mice are indistinguishable from nontransgenic (NTG) mice.26 Both FHC mouse models were bred on the same C57BL/6 genetic background and have each been shown to have a normal life span.11,26 The double-mutant mouse model (designated Tnl-203/MHC-403) was generated by mating female Tnl-203 mice with male MHC-403 mice. Both of these mutations have been described in human FHC previously.27,28 The 4 genotypes studied were therefore NTG, Tnl-203, MHC-403, and Tnl-203/MHC-403 mice.

**Mouse Body Weight and Heart Weight, Histopathology, and Fibrosis Assessment**

At ages 14 days and 16 to 18 days, body weight of littermates was measured with a PB303-L Precision Balance (Mettler-Toledo GmbH, Germany). Mice were euthanized and their hearts excised, washed in PBS, blotted dry on tissue paper, and weighed. Heart weight to body weight (HW:BW) ratio was then calculated. Hearts were then fixed in 10% formalin, and histological analysis performed as described previously.16 In brief, hearts were cut and embedded in a paraffin block, sectioned, and stained with Milligan’s trichrome or Von Kossa stain. Magnified digital images were obtained with a Nikon Eclipse E800 fluorescence microscope (Nikon Instech, Japan). Lungs and livers were also excised, blotted dry on tissue paper, and weighed to determine the ratios of lung weight to body weight and liver weight to body weight. Lungs and livers were fixed in 10% formalin and subsequently embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin. The proportion of fibrotic tissue in each heart section was quantified with KS 400 version 3.0 software for Windows (Carl Zeiss Vision GmbH), and calculated by dividing the fibrotic tissue area by the total area.

**TUNEL Assay and Immunohistochemistry**

TUNEL (terminal dUTP nick end-labeling) assay was performed with a TMR red in situ cell-death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. Briefly, frozen sections were fixed in 4% freshly prepared paraformaldehyde in PBS for 20 minutes and incubated in permeabilization solution (0.1% Triton, 0.1% sodium citrate), followed by incubation with TUNEL reaction mixture at 37°C for 60 minutes in a dark, humidified chamber. Sections were then washed, counterstained with DAPI (0.5 μg/mL in PBS), and mounted with VECTASHIELD medium (Vector Laboratories, Inc, Burlingame, Calif). Six to 10 images per heart (3 to 4 hearts per genotype group) were acquired on a Nikon Eclipse E800 fluorescence microscope (Nikon Instech, Kanagawa, Japan). DAPI images were divided into 6 equal grids for analysis with the “cell counter” plug-in in ImageJ 1.36b (National Institutes of Health, Bethesda, Md). TMR red-positive cells were counted individually. Results were expressed as the percentage of apoptotic cells among the total cell population.

Frozen tissue sections for structural protein immunofluorescent staining were fixed in acetone for 5 minutes followed by air-drying to remove residual acetone. Sections were then blocked with 10% fetal calf serum in PBS for a further 20 minutes, stained with anti-α-actinin (1:100 in 1% BSA in PBS), and detected with Cy3-conjugated anti-mouse secondary antibodies (1:400 in 1% BSA in PBS). FITC-conjugated phallolidin (1:400 in 0.1% BSA in PBS) staining of actin thin filaments was performed at the same time as the secondary antibody incubation. Finally, nuclear staining was performed with Hoechst 33258 (2 μg/mL in 5 mM L-Tris, pH 7.4, 1 mM L-EDTA). Fluorescent images were collected on a Leica Microsystems TCS SP5 laser scanning confocal microscope (Wetzlar, Germany).
Transmission Electron Microscopy
Excised hearts were washed in PBS, cut in the transverse plane into 2 parts, and partially fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. Consequently, 1×1×2-mm tissue cubes were cut from the left ventricle. Tissue samples were fixed in fresh 2.5% glutaraldehyde overnight, rinsed, postfixed in 1% osmium tetroxide for 1 hour, dehydrated in ascending grades of ethanol, then infiltrated and embedded in Spurr’s resin. Semithin sections (350 nm) were stained with toluidine blue to screen for regions with longitudinal myofibrils by light microscopy. Ultrathin sections (70 to 90 nm) were mounted on 100-mesh copper grids and stained with uranyl acetate followed by lead citrate; images were acquired by a Zeiss EM 902A (Oberkochen, West Germany) operating at 80 kV.

Mouse Echocardiography
Two-dimensional and M-mode studies were performed as described previously.16 In brief, transthoracic echocardiography was performed with a 7- to 15-MHz linear-array probe and an HDI 5000 ultrasonograph (Acuson) as described previously.16,26 2D images of the left ventricle in short-axis and long-axis views were obtained. Left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) chamber dimensions and wall thickness were obtained from M-mode tracings based on measurements averaged from 3 separate cardiac cycles. Left ventricular fractional shortening (FS) was calculated with the formula: FS=(LVEDD−LVESD)/LVEDD. Heart rate was recorded throughout the study.

Mouse Electrocardiography
ECG studies were performed in unanesthetized mice (minimum heart rate 450 bpm) as described previously with PowerLab/8sp with Chart 5.4.2 software (ADInstruments, Colorado Springs, Colo).26 Bipolar ECG from leads I, II, and III were obtained by implanting 27-gauge needle electrodes subcutaneously into 3 limbs. The ECG channel was amplified, filtered between 0.3 and 200 Hz, and sampled at a rate of 4000 samples per second. The signal-averaged ECG was calculated with Chart 5 extension for Macintosh SAECG (version 1.1; ADInstruments) by averaging 50 to 300 consecutive R-R intervals. Measurements of all ECG parameters were made, including P-wave duration, PR, QRS, QT, and corrected QT intervals and P and QRS wave heights (using ECG lead II). In a subgroup of mice, ECG was performed, followed by intraperitoneal injection of adrenalin (1 to 20 mg/kg) in an attempt to induce cardiac arrhythmias. After injection, mice were monitored by ECG recordings for 15 minutes.

RNA Extraction, Quantification, and Reverse-Transcription Polymerase Chain Reaction Analysis
RNA extraction was performed as described previously.26 In brief, hearts were excised, washed in PBS, blotted dry on tissue paper, and snap-frozen in dry ice before being stored at −80°C. The ventricles of frozen mouse hearts were added to Trizol and homogenized with a Polytron pt-MR2100 homogenizer (Kinematica AG, Lucerne, Switzerland). After chloroform-based extraction, the quality of extracted RNA was determined by gel electrophoresis and quantified with a fluorometric assay with SYBR Green II dye. A minimum of 9 mouse heart samples (5 males, 4 females) for each of the 4 genotype groups were analyzed in each experiment.

Extracted RNA samples were used as the template for cDNA prepared via a reverse-transcription polymerase chain reaction that was performed in duplicate for all RNA samples. Quantitative real-time reverse-transcription polymerase chain reaction was then used to determine the mRNA expression of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), the cardiac L-type Ca2+ channel (LTCC), cardiac ryanodine receptor (RYr2), sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA2a), phospholamban (PLB), Na+/Ca2+ exchanger, and connective tissue growth factor. GAPDH, β-microglobulin (β2m), hypoxanthine guanine phosphoribosyltransferase (HPRT), peptidyl-propyl isomerase A (PPIA), and β-actin (ACTB) were used as normalizing genes. Quantitative real-time reverse-transcription polymerase chain reaction was performed in an Mx3000P Real-Time PCR System (Stratagene, La Jolla, Calif). Results were analyzed with MxPro QPCR software version 2.0 (Stratagene).

Protein Extraction and Immunoblotting
Protein extracts were prepared by lysing heart tissue in solubilizing lysis buffer (5 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 2% sulfobetaine 3-10, 40 mmol/L Tris, 65 mmol/L DTT, and 2 mmol/L TBP), and insoluble material was cleared by centrifugation (14 000 g, 1 hour, 10°C). Protein concentration was then determined by Bradford protein assay. Protein extracts were resolved by SDS-PAGE and transferred to PVDF membranes. Immunoblotting with antibodies specific for phosphorylated STAT1, STAT3, or STAT5 (Cell Signaling Technology, Danvers, Mass), activated mitogen-activated protein kinases, extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38, according to the manufacturer’s instructions. Immunoreactive protein bands were detected by enhanced chemiluminescence with Supersignal (Pierce, Rockford, Ill). Membranes were then stripped and rebotted for total STAT1, STAT3, or STAT5, activated mitogen-activated protein kinases, extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 (Santa Cruz Biotechnology, Santa Cruz, Calif). Protein bands were quantified with Image Gauge (Fujiﬁlm, Tokyo, Japan) version 4.23 software.

Statistical Analysis
Data are expressed as mean±SEM. All statistical analyses were performed with SPSS version 13.0 for Macintosh (SPSS Inc, Chicago, Ill). Comparisons between the 4 genotype groups (NTG, TnI-203, TnI-403, and TnI-203/MHC-403) were made with 1-way or 2-way ANOVA, followed by Bonferroni post hoc tests where appropriate. A probability value <0.05 was considered significant for ANOVA. Bonferroni post hoc tests had an adjusted significance cutoff probability value of 0.008. Kaplan–Meier curves for survival analysis were compared by the log-rank test. All data analyses were performed by a person blinded to the genotype of the mice being studied.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Survival Analysis
TnI-203/MHC-403 mice demonstrated 100% mortality by 21 days of age, with survival declining rapidly from 16 days of age (Kaplan–Meier survival analysis; Figure 1; n=144 mice). The mean life span of double-mutant males was found to be 16.8 days, which was significantly shorter than the mean life span of 18.3 days in double-mutant females (log-rank P=0.018). One NTG mouse and 2 MHC-403 mice died before day 11 of malnutrition, unrelated to cardiac disease.
Figure 2. Histopathological analysis. A, Whole hearts at age 14 days, with corresponding light microscopy of low-magnification (scale bar=500 μm) coronal heart sections (below) and high-magnification (scale bar=50 μm) myocardial sections stained with Milligan’s trichrome. Myocytes stained red, collagenous tissue stained blue. B, Top, Planimetric quantification of fibrotic tissue in ventricular myocardium (*P<0.0001 vs NTG, P<0.0001 vs MHC-403). Bottom, relative connective tissue growth factor (CTGF) mRNA expression (*P<0.0001 vs NTG, P<0.0001 vs Tnl-203, P<0.0001 vs MHC-403). C, Lung sections of 18-day-old mice stained with hematoxylin and eosin at low magnification (scale bar=500 μm) and high magnification (scale bar=100 μm). There was normal alveolar structure of NTG and single-mutant mice compared with pulmonary congestion and edema in double-mutant mice. D, Liver sections of 18-day-old mice stained with hematoxylin and eosin (scale bar=100 μm). Mononuclear infiltrates (arrows) and vacuoles (asterisks) indicate development of steatohepatitis in double-mutant mice. E, Von Kossa staining of left ventricular myocardium in Tnl-203/MHC-403 mice (scale bar=50 μm). Brown staining (arrow) represents areas of necrosis. No necrosis was evident in the other 3 genotype groups.
Genotype studies of mice at age 10 days showed an approximately equal proportion of all 4 genotypes (NTG = 22%, TnI-203 = 25%, MHC-403 = 26%, and TnI-203/MHC-403 = 27%; n = 279 mice; P = 0.757), which suggests there was no in utero mortality of the TnI-203/MHC-403 mice.

MHC-403 mice were also crossed with wild-type cTnI transgenic mice (TnI-WT/MHC-403) to determine whether the TnI transgene (independent of the mutation at position 203) was contributing to the 100% mortality in TnI-203/MHC-403 mice. In summary, the TnI-WT/MHC-403 mice had normal survival, had no cardiac phenotype at age 21 days, and were indistinguishable from NTG mice (data not shown). By age 30 weeks, the TnI-WT/MHC-403 mice developed a mild FHC phenotype, characterized by moderate cardiac hypertrophy, associated with mild myofiber disarray and interstitial fibrosis, and were indistinguishable from MHC-403 mice described previously.11,16

Cardiac Pathology and Histology
Gross examination of TnI-203/MHC-403 mouse hearts at age 14 days showed marked 4-chamber cardiac enlargement, with prominent bialtrial dilatation (Figure 2A). Premorbid left atrial thrombus was observed in a subgroup of mouse hearts. HW:BW varied significantly between the 4 genotype groups, with increased HW:BW in TnI-203/MHC-403 mice. This increase was statistically significant for TnI-203/MHC-403 mice compared with single-mutant mice, both at age 14 days and at 16 to 18 days of age (Table 1). The ratio of lung weight to body weight was also increased in TnI-203/MHC-403 mice compared with single-mutant and NTG mice (Table 1). Body weight alone did not differ significantly between genotype groups (P = 0.772). Histopathological analysis of both transverse and longitudinal sections of hearts from NTG, TnI-203, and MHC-403 mice showed normal cardiac histology with no visible myocyte hypertrophy, myofiber disarray, or interstitial fibrosis (Figure 2A). TnI-203/MHC-403 mice showed abnormal myofiber alignment and myocyte hypertrophy and a large proportion of blue-staining collagenous tissue, indicative of interstitial fibrosis (Figure 2A).

The proportion of fibrotic tissue in the ventricular myocardium varied significantly between the 4 genotype groups (n = 4 to 5 per group; Figure 2B). The increased fibrosis in TnI-203/MHC-403 mice was statistically significant compared with NTG and MHC-403 mice. The proportion of fibrotic tissue did not vary significantly between NTG and TnI-203, NTG and MHC-403, or TnI-203 and MHC-403 mice. Expression levels of connective tissue growth factor mRNA were also markedly increased in TnI-203/MHC-403 mice compared with the other 3 genotype groups, consistent with a fibrotic response (Figure 2B). The severe heart failure observed was supported by significant congestion and inflammation in both lung and liver tissues from TnI-203/MHC-403 mice (Figure 2C and 2D). Areas of necrosis were present in left ventricular myocardial sections from near-death TnI-203/MHC-403 mice aged 17 days (Figure 2E) but not in the other 3 genotype groups. No statistically significant increase in TUNEL-positive cells in TnI-203/MHC-403 mice compared with NTG or single-mutant mice was observed (mean apoptotic index: NTG = 0.28 ± 0.05, TnI-203 = 0.36 ± 0.09, MHC-403 = 0.24 ± 0.07, and TnI-203/MHC-403 = 0.69 ± 0.17; ANOVA P = 0.043; TnI-203/MHC-403 not significantly different from the other groups).

Myocyte Structure
Changes in myocyte structure were observed in TnI-203/MHC-403 mice (Figure 3A and 3B). Significantly altered alignment of myofibrils was seen in TnI-203/MHC-403 mice, and early changes were also noted in MHC-403 mice (Figure 3A). Images obtained by electron microscopy displayed several ultrastructural abnormalities in TnI-203/MHC-403 mice hearts, including myocyte atrophy and fragmentation, altered distribution of mitochondria between myofibrils, myofibril disarray and discontinuity, degeneration and loss of myocyte structure, collagen accumulation, greater infiltration of other cells, and enlarged extracellular spaces (Figure 3B). Early pathological changes in cardiac ultrastructure of MHC-403 mice, such as misalignment of Z-disks and clusters of mitochondria, were also evident (Figure 3B).

Hypertrophy-Related Gene Expression
Mean relative expression of ANF and BNP mRNA in NTG, TnI-203, MHC-403, and TnI-203/MHC-403 mice is shown in Figure 4. Both ANF and BNP gene expression were found to vary significantly with genotype group. Relative ANF and

### Table 1. Body Weight, HW:BW, and LW:BW in 14- and 16 to 18-Day-Old Mice

<table>
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<tr>
<th></th>
<th>NTG</th>
<th>TnI-203</th>
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<td><strong>14 days old</strong></td>
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<tr>
<td>Body weight, g</td>
<td>6.5 ± 0.2 (39)</td>
<td>6.8 ± 0.2 (34)</td>
<td>6.9 ± 0.2 (29)</td>
<td>6.6 ± 0.1 (40)</td>
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<td>HW:BW, mg/g</td>
<td>6.2 ± 0.1 (39)</td>
<td>6.0 ± 0.1 (34)</td>
<td>5.9 ± 0.1 (29)</td>
<td>6.9 ± 0.1* (40)</td>
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<td>LW:BW, mg/g</td>
<td>13.5 ± 0.3 (24)</td>
<td>14.1 ± 0.3 (16)</td>
<td>13.5 ± 0.3 (17)</td>
<td>14.9 ± 0.4 (16)</td>
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<td><strong>16 to 18 days old</strong></td>
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<td>Body weight, g</td>
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<td>7.0 ± 0.2 (21)</td>
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<td>HW:BW, mg/g</td>
<td>6.6 ± 0.2 (15)</td>
<td>6.2 ± 0.1 (19)</td>
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<td>7.9 ± 0.1* (20)</td>
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<td>LW:BW, mg/g</td>
<td>12.9 ± 0.3 (16)</td>
<td>13.5 ± 0.3 (21)</td>
<td>12.9 ± 0.2 (32)</td>
<td>16.1 ± 0.4* (21)</td>
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Values are mean ± SEM (No. of mice in each group).

*P<0.0001 vs NTG, TnI-203, and MHC-403.
†ANOVA P=0.02.
Figure 3. Analysis of myofilament structure. A, Fluorescent images of frozen cardiac sections of mice aged 14 days stained with anti-α-actinin (red), phalloidin (green), and Hoechst 33258 (blue) showing compromised alignment of myofibrils in MHC-403 and Tnl-203/MHC-403 mice. Scale bar=20 μm. B, Transmission electron micrographs of mice (scale bar=5 μm). Misalignment of Z-disks (arrows) and clusters of mitochondria (asterisks) are evident for MHC-403 and Tnl-203/MHC-403 mice.
myopathy, with significant enlargement of left ventricular TnI-203/MHC-403 mice developed a severe dilated cardiomyopathy. Echocardiographic parameters are summarized in Table 2.

In Vivo Cardiac Function and ECG Analysis

BNP mRNA expression was significantly greater in TnI-203/MHC-403 mice than in either NTG or MHC-403 mice, and ANF expression was greater in TnI-203/MHC-403 mice than in TnI-203 mice (Figure 3A and 3B). A small upregulation of ANF and BNP gene expression was seen in the TnI-203 genotype group compared with the NTG and MHC-403 groups. This increase was statistically significant for ANF gene expression but not for BNP gene expression. No significant variation was observed between the NTG and MHC-403 groups. This increase was statistically significant for ANF expression (*P<0.0001 vs NTG, †P<0.0001 vs TnI-203, ‡P<0.0001 vs MHC-403). B, Relative expression of BNP mRNA (*P<0.0001 vs NTG, †P<0.0001 vs MHC-403, ‡P<0.0001 vs TnI-203). In both ANF and BNP studies, no significant variation was seen in the TnI-203/MHC-403 mice (Figure 3A and 3B). A small upregulation of ANF expression was greater in TnI-203/MHC-403 mice than in either NTG or MHC-403 mice, and BNP mRNA expression was significantly greater in TnI-203/MHC-403 mice than in either NTG or MHC-403 mice, and ANF expression was greater in TnI-203/MHC-403 mice than in TnI-203 mice (Figure 3A and 3B).

Echocardiographic parameters are summarized in Table 2. TnI-203/MHC-403 mice developed a severe dilated cardiomyopathy, with significant enlargement of left ventricular cardiac chambers (increased left ventricular end-diastolic and end-systolic diameter) and a reduction in fractional shortening. This reduction in cardiac function at age 16 to 18 days was highly variable owing to the different rates of progression of disease in individual mice, with fractional shortening in some mice reduced to below 20%.

ECG parameters are summarized in Table 3. Compared with age- and gender-matched NTG, TnI-203, and MHC-403 mice, TnI-203/MHC-403 mice at age 14 days showed significant prolongation of the PR interval, consistent with first-degree atrioventricular block associated with the development of cardiomyopathy (Table 3; Figure 5A). TnI-203/MHC-403 mice had a prolonged R-R interval and increased P-wave height, consistent with atrial dilatation. The corrected QT interval was also increased compared with NTG, TnI-203, and MHC-403 mice (Table 3; Figure 5A). Ventricular arrhythmias were induced after adrenaline administration in some TnI-203/MHC-403 mice (Figure 5B) but not in mice from the other 3 genotype groups.

Intracellular Ca\(^{2+}\) Cycling Gene Expression

The expression of genes encoding LTCC, RyR2, SERCA2a, PLB, and Na\(^{+}/Ca^{2+}\) exchanger was assessed in duplicate cDNA samples from a minimum of 9 mice per genotype group (5 males, 4 females). There was a significant reduction in LTCC, RyR2, SERCA2a, and PLB mRNA expression levels in TnI-203/MHC-403 mice at age 14 days (Figure 6). No significant gender differences were noted in expression levels of LTCC, RyR2, SERCA2a, and PLB. Na\(^{+}/Ca^{2+}\) exchanger gene expression did not differ significantly between the TnI-203/MHC-403 and NTG or single-mutant groups (data not shown).

STAT3 Phosphorylation/Activation in TnI-203/MHC-403 Mice

Immunoblotting for the activated mitogen-activated protein kinases, extracellular signal-regulated kinase, c-Jun NH\(_2\)-terminal kinase, and p38, did not reveal detectable differences between TnI-203/MHC-403 mice and single mutants or NTG control groups (data not shown). In contrast, active STAT3, indicated by tyrosine phosphorylation, was elevated substantially in hearts from TnI-203/MHC-403 mice aged 14 days compared with the TnI-203 and MHC-403 single-mutant

<table>
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<th>NTG (n=4)</th>
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<th>MHC-403 (n=12)</th>
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<td>455±11</td>
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<td>LVESD, mm</td>
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<td>0.51±0.13</td>
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<td>FS, %</td>
<td>79±2</td>
<td>75±3</td>
<td>71±5</td>
<td>52±5§</td>
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HR indicates heart rate; LWTT, maximal left ventricular wall thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; and FS, fractional shortening.

Values are mean±SEM, n=No. of mice in each group.

*ANOVA P=0.02; †P=0.09 vs NTG, ‡P=0.06 vs TnI-203, §P=0.02 vs MHC-403.

<table>
<thead>
<tr>
<th></th>
<th>NTG (n=4)</th>
<th>TnI-203 (n=6)</th>
<th>MHC-403 (n=12)</th>
<th>TnI-203/MHC-403 (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>481±31</td>
<td>455±11</td>
<td>475±13</td>
<td>413±13*</td>
</tr>
<tr>
<td>LWTT, mm</td>
<td>0.63±0.01</td>
<td>0.58±0.02</td>
<td>0.55±0.02</td>
<td>0.52±0.02</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>1.68±0.05</td>
<td>1.68±0.07</td>
<td>1.69±0.08</td>
<td>2.12±0.09†</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>0.36±0.02</td>
<td>0.42±0.05</td>
<td>0.51±0.13</td>
<td>1.05±0.15‡</td>
</tr>
<tr>
<td>FS, %</td>
<td>79±2</td>
<td>75±3</td>
<td>71±5</td>
<td>52±5§</td>
</tr>
</tbody>
</table>

HR indicates heart rate; LWTT, maximal left ventricular wall thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; and FS, fractional shortening.

Values are mean±SEM, n=No. of mice in each group.

*ANOVA P=0.02; †P=0.09 vs NTG, ‡P=0.06 vs TnI-203, §P=0.02 vs MHC-403.
mice or NTG controls (Figure 7A). Elevated STAT3 phosphorylation was specific to the heart and was not detected in brain, kidney, liver, testis, skeletal muscle, or lung tissue (data not shown). STAT3 phosphorylation appeared in TnI-203/MHC-403 mice at 10 days of age but was not found in hearts from mice aged 5 days (Figure 7A). Tyrosine phosphorylation levels of STAT1 and STAT5 isoforms did not vary between the 4 groups at age 14 days (Figure 7B). Quantitation of phospho-STAT isoform bands in hearts from mice aged 14 days confirmed significantly higher STAT3 phosphorylation (≈3-fold increase) in the TnI-203/MHC-403 group than in the single-mutant or NTG groups, whereas STAT1 and STAT5 phosphorylation were not different (Figure 7C).

**Discussion**

We describe a novel double-mutant mouse model of FHC in which affected mice develop a severe cardiac phenotype, with rapid onset of dilated cardiomyopathy, significant cardiac fibrosis, conduction system abnormalities, severe heart failure, and death by age 21 days. These findings suggest a possible mechanism for the development of severe FHC, whereby the presence of 2 FHC-causing gene mutations results in a rapid progression to a dilated phenotype without a prolonged hypertrophic phase (Figure 8). These findings may explain why some patients with FHC develop rapidly progressive disease that results in heart failure and the need for cardiac transplantation.

The presence of 2 causative mutations has recently been described in up to 5% of FHC families.7–9 Descriptions of large cohorts of FHC patients who have undergone genetic testing have revealed individuals who have compound MyBP-C or MyBP-C/troponin T (TnT), MyBP-C/TnI, MyBP-C/tropomyosin, β-MHC/TnT mutations, and homozygous MyBP-C, β-MHC, and TnT mutations.7–10 Collectively, FHC individuals with 2 mutations have been identified to have earlier onset of disease, more severe hypertrophy, and a greater incidence of sudden death events than FHC individuals with single-gene mutations.7–10 Identification of FHC individuals with 2 gene mutations is therefore likely to be important as part of the risk stratification process in clinical management.

The mechanism of phenotype development in double-heterozygote individuals remains unknown. The double-mutant model presented here supports the notion that the presence of 2 gene defects, in this case in the cardiac MHC and TnI genes, significantly accelerates both the onset and progression of FHC disease. Furthermore, compared with the TnI-203 and MHC-403 single-mutant mouse models in which survival is normal, double-mutant mice develop severe disease and premature death. Importantly, these mice do not appear to progress through a prolonged hypertrophic phase but progress directly to a dilated cardiac phenotype, with heart failure and premature death. This finding is consistent with data from homozygous MHC-403 mice, which also develop a severe neonatal dilated cardiomyopathy with mortality within 7 days of birth.29 Furthermore, FHC individuals have been described in which homozygosity of the causative gene defect, eg, in the cardiac TnT gene, can result in severe

**Table 3. ECG Analysis in 14-Day-Old Mice (Heart Rate >450 bpm)**

<table>
<thead>
<tr>
<th></th>
<th>NTG (n=16)</th>
<th>TnI-203 (n=12)</th>
<th>MHC-403 (n=13)</th>
<th>TnI-203/MHC-403 (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>584±12</td>
<td>588±10</td>
<td>595±12</td>
<td>494±8*</td>
</tr>
<tr>
<td>RR, ms</td>
<td>104±2</td>
<td>103±2</td>
<td>101±2</td>
<td>122±2*</td>
</tr>
<tr>
<td>PR, ms</td>
<td>32.4±0.9</td>
<td>35.2±0.4</td>
<td>33.3±0.8</td>
<td>41.5±1.1*</td>
</tr>
<tr>
<td>QRS, ms</td>
<td>9.2±0.2</td>
<td>9.1±0.2</td>
<td>9.0±0.3</td>
<td>10.8±0.3*</td>
</tr>
<tr>
<td>QT, ms</td>
<td>22.5±1.2</td>
<td>24.5±1.4</td>
<td>24.1±1.2</td>
<td>37.0±1.6*</td>
</tr>
<tr>
<td>QTc, ms</td>
<td>22.1±1.2</td>
<td>24.1±1.2</td>
<td>24.0±1.3</td>
<td>33.4±1.4*</td>
</tr>
<tr>
<td>QRS-H, mV</td>
<td>1.658±0.062</td>
<td>1.458±0.111</td>
<td>1.277±0.049</td>
<td>1.579±0.067†</td>
</tr>
<tr>
<td>P-H, mV</td>
<td>0.210±0.007</td>
<td>0.196±0.015</td>
<td>0.202±0.015</td>
<td>0.251±0.015‡</td>
</tr>
</tbody>
</table>

n=No. of mice in each group.

*P<0.0001 vs NTG, TnI-203, and MHC-403.
†ANOVA P=0.004; P=0.03 vs MHC-403.
‡ANOVA P=0.02; P=0.13 vs NTG, P=0.03 vs TnI-203, P=0.06 vs MHC-403.
biventricular hypertrophy associated with premature sudden death. An extrapolation of these findings is that the presence of a double-gene mutation in FHC is an important risk factor for development of end-stage FHC (Figure 8). The presence of 2 gene defects in FHC is likely to result in a higher proportion of patients developing more severe disease and a dilated cardiac phenotype associated with left ventricular chamber dilatation, wall thinning, and systolic dysfunction than with single-gene mutation FHC individuals, of whom only ~10% develop this severe phenotype (Figure 8). The final cause of death in these end-stage dilated FHC patients is progressive heart failure. In the TnI-203/MHC-403 mouse model presented here, the majority of mice developed progressive heart failure, had evidence of myocyte necrosis, and were notably deteriorating clinically for at least 12 hours before death. However, the prolonged QT interval observed in these mice potentially predisposes them to develop ventricular tachyarrhythmias that could result in sudden death. Significantly increased areas of interstitial fibrosis in TnI-203/MHC-403 mice provide a substrate for malignant arrhythmias, which further supports the evidence of inducibility of

Figure 6. RNA analysis of Ca\textsuperscript{2+} cycling molecules. A, Relative expression of LTCC mRNA (*P<0.0001 vs NTG, †P<0.0001 vs Tnl-203). B, Relative expression of RyR2 mRNA (P=0.006 vs NTG, †P<0.0001 vs Tnl-203). C, Relative expression of SERCA2a mRNA (P=0.005 vs NTG, †P<0.0001 vs NTG, †P<0.0001 vs Tnl-203, P<0.0001 vs MHC-403). D, Relative expression of PLB mRNA (*P=0.001 vs NTG, †P<0.0001 vs NTG, P=0.0001 vs Tnl-203).
ventricular arrhythmias in the double-mutant mice. Collectively, these data suggest the death of these double-mutant mice is due to ventricular arrhythmias in the setting of severe progressive heart failure.

An important consideration is whether the phenotype observed in the TnI-203/MHC-403 model is specific for the 2 particular genes and the mutations they harbor, or whether these findings are applicable to other mutations and other sarcomere genes known to cause FHC. In humans, double- and compound-heterozygote mutations in the β-MHC gene have been described; however, a mutation in the cardiac TnI gene has only been described in 1 individual in the setting of multiple mutations. This individual had a truncated MyBP-C Arg943X and TnI Ser166Phe mutation, and no clinical information was reported. Given the critical role in sarcomere function of both of these encoded proteins, with β-MHC being the key component of the thick filament and TnI a key inhibitory “switch” molecule essential for activation of cardiac contraction, concurrent mutations in both of these molecules are likely to cause significant disruption to the cardiac contractile apparatus. This is supported by the severe cardiac phenotype observed in the TnI-203/MHC-403 model and other single-mutant animal models of FHC caused by either cardiac MHC or TnI gene mutations. However, the findings of the present study may not apply to all situations in which double or compound mutations occur in humans. Certain combinations of mutations in specific genes may result in varying clinical phenotypes. Although in some cases, a second mutation may lead to more severe disease, it is feasible that in other cases, a second mutation may not be as detrimental or, indeed, may have protective effects.

Disruption of normal β-MHC and TnI function likely explains the alteration in key molecules involved in intracellular Ca²⁺ regulation observed in this model. Specifically, downregulation of RyR2, SERCA2a, LTCC, and PLB expression in TnI-203/MHC-403 mice is consistent with previous studies of both the single-mutant TnI-203 and MHC-403 mice, which show Ca²⁺ dysregulation to be an important early pathogenic event in FHC. The changes in key Ca²⁺ handling genes at age 14 days, before the severe clinical decline in TnI-203/MHC-403 mice, suggests Ca²⁺ dysregulation may be an important pathogenic event in FHC caused by double mutations.

Alternatively, the presence of a mutation in the cardiac TnI gene alone may be the critical “second hit” factor in accelerating the cardiac phenotype, particularly in light of the rarity of human FHC double-heterozygote individuals in whom 1 of the mutations is in the TnI gene. The role of mutations in the cardiac TnI gene in human disease has been of great interest recently. A number of single-gene mutations have been identified not only in patients with FHC but also in individuals with restrictive cardiomyopathy and a recessive form of dilated cardiomyopathy. The identification of mutations in the cTnI gene in families with these diverse cardiomyopathies likely reflects the critical role of cTnI in normal cardiac biology. Although these mutations have been found throughout the cTnI gene, the common clinical end points of these cardiomyopathies, in particular chronic heart failure, suggests cTnI may be an important common link in the pathogenesis of human heart failure. Most recently, studies in isolated myocytes and whole animals have indicated that modulation of the cTnI gene, by the introduction of a single histidine residue present in the fetal cardiac TnI isoform into the adult cardiac TnI isoform, can improve cardiac function and protect against pathological stimuli in the setting of hypoxia, ischemia, and heart failure. Such myofilament-based inotropic therapy may be an important platform for the development of new therapies in human FHC.

The link between the contractile deficits caused by genetic mutations and the development of FHC is poorly understood. In the present study, we have reported a very specific activation of the STAT3 isoform of the latent transcription factor family, which correlated with the development of FHC in the double-mutant mice. The absence of STAT1 and STAT5 activation or significant STAT3 activation in noncardiac tissues suggests STAT3 is not activated merely as an inflammatory consequence to disease progression. It is unclear precisely how mutations in the sarcomere could lead to STAT3 activation. The observed early activation of STAT3 may play a key role in signaling mechanisms downstream of the TnI-203/MHC-403 mutations to modulate cardiac remod-
eling. STAT3 is activated by multiple prohypertrophic stimuli such as growth factors, cytokines, the renin-angiotensin axis, and mechanical stretch.\textsuperscript{17,18} Thus, paracrine factors in the double mutants are likely intermediates that lead to STAT3 activation.

Although not defined in the present study, the effect of STAT3 activation would be predicted to be cardioprotective, because cardiac-specific STAT3 knockout mice have previously been shown to experience enhanced sensitivity to cardiac stresses, and the overexpression of STAT3 in the heart appeared protective against cardiomyopathy after doxorubicin treatment in vivo.\textsuperscript{19} The present model is not only the first to show STAT3 activation in an animal model of FHC, but it also shows that the level and/or timing of STAT3 activation under these conditions is not sufficient to prevent heart failure. Previously, STAT3 activation had been described in human dilated cardiomyopathy, but those studies did not delineate the underlying causes of disease in individuals.\textsuperscript{25} Significantly, there has been the subsequent observation of decreased total and phosphorylated STAT3 in human heart failure samples.\textsuperscript{24} Whether the latter observations reflect differences in the underlying causes of cardiomyopathy in the various human patient cohorts or other differences such as the time course for the development of the heart failure events requires further consideration with more detailed evaluation of human heart STAT3 signaling pathways.

Conclusions

The identification of multiple mutations in FHC is an important consideration in explaining both the clinical and genetic heterogeneity characteristically seen in patients with FHC. TnI-203/MHC-403 double-mutant mice develop a severe cardiac phenotype characterized by dilated cardiomyopathy, severe heart failure, and early death. Activation of STAT3 may represent an important underlying mechanism regulating this process. The presence of 2 disease-causing mutations may predispose individuals to the development of a severe dilated phenotype and heart failure compared with individuals with single-gene mutation. The present double-mutant model and generation of other similar models will be important molecular tools to facilitate an improved understanding of the pathogenesis of FHC, particularly in the development of end-stage heart failure and premature death.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

The well-accepted paradigm in autosomal-dominant monogenic medical diseases is that 1 gene mutation in a single gene is the direct cause of disease. This is seen in a number of medical genetic disorders spanning all subspecialties, including neurologic disorders, familial cancer syndromes, and thalassemia. Until recently, cardiac genetic disorders have been thought to involve only single-gene defects; however, several groups have recently reported families with familial hypertrophic cardiomyopathy in which 2 disease-causing gene defects have been identified. These clinically affected patients with 2 mutations develop more severe disease, including earlier age of onset, more severe cardiac hypertrophy, and a greater number of sudden cardiac death events. The present study combined 2 separate animal models of single-mutation familial hypertrophic cardiomyopathy to produce a unique double-mutation model of familial hypertrophic cardiomyopathy, characterized by rapid development of a dilated cardiomyopathy, severe heart failure, and premature death. These mice also demonstrated alterations in both calcium handling and STAT3 signaling. This model provides evidence that the number of disease-causing mutations identified in a patient may explain, at least in part, the clinical heterogeneity observed in human familial hypertrophic cardiomyopathy, particularly in the small subgroup of familial hypertrophic cardiomyopathy patients who develop a burn-out end-stage heart failure phenotype. Elucidation of how multiple mutations lead to diverse clinical outcomes, using appropriate animal models as presented in this study, will likely affect a range of clinical considerations, including genetic diagnosis and improved risk stratification, and therefore will provide the opportunity to initiate earlier treatment and prevention strategies.
Severe Heart Failure and Early Mortality in a Double-Mutation Mouse Model of Familial Hypertrophic Cardiomyopathy
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