Dilated Cardiomyopathy Mutant Tropomyosin Mice Develop Cardiac Dysfunction With Significantly Decreased Fractional Shortening and Myofilament Calcium Sensitivity


Abstract—Mutations in striated muscle α-tropomyosin (α-TM), an essential thin filament protein, cause both dilated cardiomyopathy (DCM) and familial hypertrophic cardiomyopathy. Two distinct point mutations within α-tropomyosin are associated with the development of DCM in humans: Glu40Lys and Glu54Lys. To investigate the functional consequences of α-TM mutations associated with DCM, we generated transgenic mice that express mutant α-TM (Glu54Lys) in the adult heart. Results showed that an increase in transgenic protein expression led to a reciprocal decrease in endogenous α-TM levels, with total myofilament TM protein levels remaining unaltered. Histological and morphological analyses revealed development of DCM with progression to heart failure and frequently death by 6 months. Echocardiographic analyses confirmed the dilated phenotype of the heart with a significant decrease in the left ventricular fractional shortening. Work-performing heart analyses showed significantly impaired systolic, and diastolic functions and the force measurements of cardiac myofibers revealed that the myofilaments had significantly decreased Ca2+ sensitivity and tension generation. Real-time RT-PCR quantification demonstrated an increased expression of β-myosin heavy chain, brain natriuretic peptide, and skeletal actin and a decreased expression of the Ca2+-ATPase and ryanodine receptor. Furthermore, our study also indicates that the α-TM54 mutation decreases tropomyosin flexibility, which may influence actin binding and myofilament Ca2+ sensitivity. The pathological and physiological phenotypes exhibited by these mice are consistent with those seen in human DCM and heart failure. As such, this is the first mouse model in which a mutation in a sarcomeric thin filament protein, specifically TM, leads to DCM. (Circ Res. 2007;101:205-214.)

Key Words: mouse model □ transgenic □ myocardial contractility □ thin filament

Tropomyosin (TM) is an α helical coiled-coil fibrous protein that binds actin filaments providing structural stability and modulation of filament function. In striated muscle, TM along with the troponin complex regulates Ca2+-mediated actin–myosin crossbridges. Numerous mutations in many of the contractile proteins of the cardiac sarcomere have been associated with dilated and hypertrophic cardiomyopathy, where the myocardial performance is compromised. In humans, 2 dilated cardiomyopathy (DCM)-associated mutations (Glu54Lys and Glu40Lys) have been identified in α-tropomyosin (α-TM) (or TPM1), in contrast to the 8 distinct mutations in the same gene that are associated with familial hypertrophic cardiomyopathy (FHC). The DCM mutations in α-TM are located in a region (amino acids 40 to 100) where half of the reported human FHC mutations occur (Glu62Gln, Ala63Val, Lys70Thr, Val95Ala); this region does not interact with troponin (Tn)T.

Protein-modeling studies on the TM filaments harboring Glu54Lys and Glu40Lys substitutions show that both of them create a strong local increase in the positive charge in an otherwise negatively charged region of the molecule. The 2-Å crystal structure of TM indicates that Glu54 is linked to Lys49 and Glu40 is linked to Arg35. The substitution of glutamic acid for lysine would abolish this interaction and thus destabilize localized TM structure. Furthermore, this mutation occurs in the fifth (or e) position of a highly conserved heptad motif of repeating 7 amino acid units (a-b-c-d-e-f-g). Structural and molecular modeling studies suggest that the fifth and seventh position (e and g) amino acid side chains, which are typically of opposite charge, can

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contribute to the stability of the coiled-coil through formation of a salt bridge.1 Disruption of one salt bridging interaction through charge reversal of an amino acid might cause a local change in TM conformation or lability. Whether the primary effect of a single amino acid substitution is to alter protein stability or surface electrostatic charge characteristics, the integrity of the thin filament is likely to be compromised, and hence a defective force transmission has been attributed to be the cause of DCM.1 Two independent studies of the DCM-causing TM mutations involving an in vitro approach have demonstrated a decreased calcium sensitivity of myofilaments.4,5

In our previous studies using a transgenic (TG) mouse model approach, we extensively analyzed the functional consequences of FHC causing α-TM mutations.2,6–8 In the current study, we adopted a similar transgenic approach to address the effect of the DCM Glu54Lys α-TM mutation on cardiac development and function. We established 5 distinct TG lines that use a cardiac-specific α-MHC promoter to express the α-TM54 mutant protein in the heart. Western blotting and quantitative analyses demonstrated that, in all TG lines, an increase in transgenic protein led to reciprocal decreases in endogenous α-TM levels, and the total myofilament TM protein level remained unchanged.

Echocardiographic analyses of these mice showed that they exhibited a significant DCM phenotype with a marked decrease in the left ventricular (LV) fractional shortening. Histopathological and morphological analyses also revealed a slow progression from DCM to heart failure, with lethality frequently occurring within 6 months. Work-performing heart analyses showed significantly impaired systolic and diastolic functions concomitant with increased time to peak pressure and half-time to relaxation. Interestingly, with high concentrations of β-adrenergic stimulation (isoproterenol), a sudden steep increase in the performance is observed, restoring normal cardiac function. The force measurements of skinned myofiber preparations exhibit a significantly decreased Ca²⁺ sensitivity and tension generation with no alteration in sarcomere length dependence of activation. This mouse model provides an opportunity to delineate the pathophysiological mechanisms of the sarcomeric DCM mutation and to explore the relationship between TM mutations inducing DCM and FHC signal transduction pathways.

Materials and Methods
An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.
Generation of DCM Mutant α-TM54

Transgenic Mice

Cardiac-restricted DCM mutant α-TM54 transgenic mice were generated using vectors containing the mouse α-MHC promoter and α-TM cDNA sequences, as described in the online data supplement. Experimental procedures describing the histologic, morphological, and molecular characterizations of those TG mice are also described in the online data supplement. The Institutional Animal Care and Use Committee approved the handling and maintenance of animals.

Cardiac Function

The cardiac performance of the DCM mutant α-TM54 TG mice was assessed by physiological studies, including echocardiography, work-performing heart model, and skinned fiber preparations, all of which are described in detail in the online data supplement. In these physiological experiments, the high expression mice refer to line 30. Initial studies found no functional differences in the results from the moderate expression mice (lines 67, 71, and 95); for this reason, the subsequent extensive studies concentrated on line 67.

Real-Time RT-PCR Analysis, Bacterial Recombinant Protein Expression, and Circular Dichroism Measurements

For details regarding the methods used, see the expanded Materials and Methods section in the online data supplement.

Results

Generation of DCM Mutant α-TM54 TG Mice

The transgene construct used to generate the α-TM54 TG mice is shown in Figure 1A. Because there is 100% amino acid identity between the striated muscle α-TM proteins of the mouse and humans, the designed DCM mutation is reflective of the change found in human DCM. Work from our laboratory shows that overexpression of wild-type α-TM cDNA (WT-TG) using a similar construct in the heart does not lead to pathological changes nor functional alterations in cardiac or myofiber performance.7,9 Five different transgenic lines were established and genomic Southern blot analysis demonstrated that there were no quantitative changes in their corresponding transgene copy numbers (Figure 1C). Also, the ratio of the mutant α-TM54 to endogenous α-TM protein (Figure 1E) correlates with their corresponding transcript levels with the exception of TG line 30, which has a very high transgene copy number and mRNA expression but a low incorporation of mutant protein in the myofilaments. Further analysis of the cytosolic fraction of the TG mouse hearts revealed that only in TG line 30 was there a significant accumulation of α-TM54 protein found in the cytoplasm (Figure 1D). (Transgenic expression of chimeric TMs or mutant transgenes does not lead to cytoplasmic accumulation of the exogenous protein.)10) The significant accumulation of α-TM54 protein in the cytoplasm in TG line 30 was further confirmed by immunohistochemical analyses of TG heart sections using a TM-specific antibody (Figure 2A). Additional quantitative analysis shows that the amount of total striated muscle α-TM (generated from both endogenous and transgene sources) in total cell lysate remains unchanged when normalized to α-tubulin in the NTG, WT-TG, and α-TM54 TG hearts with the exception of α-TM54 TG line 30 (Figure IA in the online data supplement). The total amount of myofilament-incorporated striated muscle TM remains unchanged when normalized to striated muscle actin levels in the NTG, WT-TG, and α-TM54 TG hearts including TG line 30 (supplemental Figure IB).

Cardiac α-TM54 Mutant Transcript and Protein Expression in TG Mice

Message levels of control and transgene transcripts were assayed by Northern blot hybridization using a radiolabeled α-TM 5′ untranslated region probe and an α-MHC 1- to 2-exon probe, respectively, that were normalized to GAPDH expression. The expression levels of the endogenous α-TM transcript remain constant in all of the α-TM54 TG mouse lines, whereas the transgene expression levels correlate with their corresponding transgene copy numbers (Figure 1C). There is a slight decrease in endogenous α-TM mRNA levels in the WT-TG hearts.

To quantify mutant α-TM54 protein expression, myofibrillar proteins from the hearts of NTG and TG mice were subjected to 1D isoelectric focusing, followed by immunoblotting with a strained muscle-specific TM antibody. On a very narrow pH gradient (pH 4.2 to 4.9), the mutant α-TM54 protein focused at a higher pI when compared with the endogenous α-TM, which correlates with the theoretical pI values of endogenous α-TM protein (4.60) and mutant α-TM54 protein (4.65). Results show there are varying degrees of expression of the mutant TM protein in the hearts of the different lines of α-TM54 TG mice, which is coupled with a concomitant downregulation of endogenous α-TM (Figure 1D). The total amount of myofilament-incorporated striated muscle α-TM remain unchanged when normalized to α-tubulin in the NTG, WT-TG, and α-TM54 TG hearts with the exception of α-TM54 TG line 30 (Figure IA in the online data supplement).}

Cardiac Morphology of DCM Mutant α-TM54

TG Mice

Cardiac structure was characterized in the transgenic mice at various time intervals from 1 to 8 months after birth. Results show that the high-copy TG mouse hearts develop a severe DCM phenotype by 2 weeks, and the dilation is seen in both the ventricles (Figure 2B, panels vi and vii). Morphological analyses of the ventricular wall show moderate myocyte hypertrophy with severe diffuse hyalinization of the myocyte.
The cytoplasmic changes are characterized by loss of striation and a homogenous, ground-glass appearance. By 1 month, the high-copy TG animals had a significant increase in the heart weight-to-body weight ratio (Figure 3A) with most mice dying within 1.5 months (Figure 3B). In contrast, the moderate-copy mice show a tendency of developing DCM after two months of age with hearts showing mild myocyte hypertrophy and disorganization at the base of left ventricle and very mild interstitial fibrosis. By five months, they develop significant dilation of both ventricles (Figure 2B, panels ii and iv) with mild disorganization of the myocytes at the base of the ventricles. A moderate diffuse peribronchiolar neutrophil cuffing was seen in the lungs. Interestingly, the body weight of these TG mice show an increase of 50% (NTG, 24.83 ± 1.8 g, n=8 and TG L67, 36.1 ± 1.8 g, n=8); this increase in weight could be attributable to peripheral edema which was observed in these animals. These findings present a clinical feature associated with heart failure, but surprisingly the lung weight is normal and there was no ascites formation. In spite of the increase in body weight, they still show an increase in heart weight to body weight ratio (Figure 3A). The mice from all 3 moderate-expression lines start dying by 4 to 6 months of age (Figure 3B), and the survival data show that by 8 months, 38% of mice died in line 67, 18% in line 71, and 15% in line 95. The mice that survived beyond 8 months also showed an increased heart weight to body weight ratio and develop a DCM phenotype.

Cardiac Function of DCM Mutant α-TM54 TG Mice

To assess whether any functional changes in cardiac performance occur in the DCM mutant α-TM54 TG mice, we conducted several physiological studies by use of echocardiography, the work-performing heart model, and skinned fiber preparations. An in vivo physiologic assessment of cardiac function was conducted on 5-month-old moderate-copy mice, 1-month-old high-copy mice, and control littermate mice by Doppler echocardiographic analyses. In both moderate- and high-copy TG mice, the hearts demonstrated increased LV diastolic and systolic diameter as well as significant reduction in the LV fractional shortening (Figure 4A, Table 1, and supplemental Table I). The cardiac output was also significantly reduced, but the heart rate was not affected. In total, the echocardiographic results demonstrate the development of a DCM phenotype that can lead to heart failure.

Work-Performing Heart Model

The work-performing heart model was used to obtain an ex vivo assessment of cardiac performance. These measurements were conducted in moderate-copy TG (3 month-old) and high-copy TG (1-month-old) mouse hearts. As seen in Table 2 and supplemental Table II, the rates of relaxation and contraction were significantly reduced concomitant with increased time to peak pressure and half-time to relaxation. End-diastolic and diastolic pressures were significantly increased, whereas the systolic pressure was significantly
decreased documenting their systolic and diastolic dysfunction. Previous work has demonstrated that transgenic mice that overexpress wild-type \( \alpha \)-TM show no significant alterations in cardiac function.\(^7,9\)

We also determined responses to isoproterenol to ascertain if the observed systolic and diastolic dysfunction was associated with impaired \( \beta \)-adrenergic responses. The reduced inotropic and lusitropic performance by hearts was assessed during stimulation with isoproterenol, a \( \beta \)-adrenergic agonist that augments muscle contraction and relaxation by cAMP/protein kinase A–dependent kinase. Interestingly, in the moderate-copy TG mouse hearts, whereas a blunted response was observed at lower concentrations of isoproterenol (10\(^{-11}\) to 10\(^{-8}\) mol/L), a sudden steep increase in the performance was observed at higher concentrations of isoproterenol (>10\(^{-8}\) mol/L), restoring normal cardiac function (Figure 4B). In contrast, in the high-copy TG mouse hearts, there was a blunting of \( \beta \)-adrenergic stimulation at all concentrations of isoproterenol (data not shown).

**Ca\(^{2+}\)–Force Measurements in Skinned Fiber Bundles**

To examine the correlation between physiological results from the whole-heart and mutant \( \alpha \)-TM54 expression at the sarcomere level, a series of experiments was conducted using detergent-extracted (skinned) fiber bundles. These experiments were conducted to compare the relation between Ca\(^{2+}\) and tension developed by myofilaments obtained from control versus mutant \( \alpha \)-TM54 left ventricular fiber bundles of 5 month-old moderate-copy TG mice and 1 month-old high-copy TG mice. In the first set of experiments, we compared the pCa–tension relations for fiber bundles obtained from NTG (n=8) and TG (moderate-copy numbers; n=10) hearts at a sarcomeric length of 2.3 \( \mu \)m. As illustrated in Table 3 and supplemental Figure II, there is a significant reduction in maximum tension and pCa50 (\(-\log\) of free [Ca\(^{2+}\)]) in TG mice fiber bundles compared with NTG controls, with no significant differences in the Hill n values. Similar sets of
Figure 4. A, Representative M-mode echocardiographic images of the left ventricle in NTG and moderate-copy α-TM54 TG mice. The moderate-copy TG mouse heart shows a LV internal diastolic dimension of 5.37 mm vs NTG, 3.7 mm; LV internal systolic dimension of 4.4 mm (TG) vs 2.0 mm (NTG); LV fractional shortening of 18% (TG) vs 46% (NTG); heart rate of 472 (TG) vs 442 (NTG) beats per minute. B, Isoproterenol dose-response curves in moderate-copy α-TM54 TG and NTG mouse hearts at 3 months of age. Hearts from NTG and TG mice were subjected to isolated heart analyses with increasing concentrations of isoproterenol (10^{-11} to 10^{-7} mol/L). Note that at a high concentration of isoproterenol (>10^{-6} mol/L), the α-TM54 cardiac function parameters recover to near normal levels.

** P<0.01 between NTG vs. TG; *** P<0.001 between NTG vs. TG
TABLE 1. Cardiac Function of NTG and Moderate Copy α-TM54 TG Mice at Five Months of Age, As Assessed by M-Mode Echocardiography

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NTG (n=7)</th>
<th>TG (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDD, M-mode, mm</td>
<td>3.65</td>
<td>4.42**</td>
</tr>
<tr>
<td>LVSD, M-mode, mm</td>
<td>1.91</td>
<td>3.42***</td>
</tr>
<tr>
<td>LV fractional shortening, M-mode, %</td>
<td>47.83</td>
<td>22.9***</td>
</tr>
<tr>
<td>IVSD, M-mode, mm</td>
<td>0.8</td>
<td>1.04***</td>
</tr>
<tr>
<td>LVFWD, M-mode, mm</td>
<td>0.71</td>
<td>0.86*</td>
</tr>
<tr>
<td>Vcf, M-mode, Doppler (circumference×sec)</td>
<td>9.83</td>
<td>4.26***</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>416</td>
<td>417</td>
</tr>
<tr>
<td>LV isovolumic relaxation time (Doppler), msec</td>
<td>16.14</td>
<td>27.33***</td>
</tr>
</tbody>
</table>

LVDD indicates LV internal diastolic dimension; LVSD, LV internal systolic dimension; IVSD, interventricular septum in diastole; LVFWD, LV posterior wall in diastole; Vcf, LV circumferential fiber shortening. *P<0.05, **P<0.01, ***P<0.001 NTG vs TG.

TABLE 2. Cardiovascular and Contractile Parameters of NTG and Moderate Copy α-TM54 TG Mouse Hearts in the Isolated Work-Performing Heart Preparations at Three Months of Age

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NTG (n=7)</th>
<th>TG (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>149.8±5.8</td>
<td>74.5±5.3***</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>−7.4±2.6</td>
<td>13.5±2.9***</td>
</tr>
<tr>
<td>End diastolic pressure, mm Hg</td>
<td>7.9±2.1</td>
<td>18.4±2.1**</td>
</tr>
<tr>
<td>+dP/dt, mm Hg/sec</td>
<td>3846±204</td>
<td>1522±320***</td>
</tr>
<tr>
<td>−dP/dt, mm Hg/sec</td>
<td>3511±186</td>
<td>1516±363***</td>
</tr>
<tr>
<td>RT1/2, ms/mm Hg</td>
<td>0.44±0.06</td>
<td>0.62±0.08*</td>
</tr>
<tr>
<td>TPP, ms/mm Hg</td>
<td>0.53±0.05</td>
<td>0.76±0.07***</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>311±8.9</td>
<td>245±28*</td>
</tr>
</tbody>
</table>

+dP/dt indicates maximal rate of pressure development; −dP/dt, maximal rate of pressure decline; RT1/2, half time to relaxation; TPP, time to peak pressure. *P<0.05, **P<0.01, ***P<0.001 NTG vs TG.

TABLE 3. Parameters Describing Ca2+-Dependent Activation of Tension in Skinned Fiber Bundles From NTG and α-TM54 TG Hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>pCa50</th>
<th>nH</th>
<th>Force (mN/mm²)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG (5 months old)</td>
<td>5.75±0.02</td>
<td>3.38±0.40</td>
<td>37.50±4.04</td>
<td>8</td>
</tr>
<tr>
<td>Moderate copy TG</td>
<td>5.67±0.01</td>
<td>3.77±0.21</td>
<td>25.41±1.67*</td>
<td>10</td>
</tr>
<tr>
<td>NTG (1 month old)</td>
<td>5.93±0.03</td>
<td>3.74±0.23</td>
<td>46.09±7.22</td>
<td>8</td>
</tr>
<tr>
<td>High copy TG, 1.9</td>
<td>5.82±0.03</td>
<td>3.78±0.15</td>
<td>22.35±1.48*</td>
<td>8</td>
</tr>
</tbody>
</table>

nH indicates Hill coefficient. *P<0.05, NTG vs TG.

opothy namely, β-MHC, brain natriuretic peptide, and skel-
etal actin (Figure 5A). To determine whether genes that
 regulate myocyte Ca2+ cycling were altered in the TG
 myocardium, we also measured mRNA levels of sarcoplasmic reticulum Ca2+-ATPase, ryamodine receptor, calseque-
strin, L-type Ca2+ channel, and phospholamban. We observed
significant downregulation only in the levels of sarcoplasmic reticulum Ca2+-ATPase and ryamodine receptor transcripts.
All of these RNA levels were normalized to GAPDH values.

Effect of Glu54Lys Mutation on Tropomyosin Thermal Stability
Tropomyosin structure is weakened by FHC mutations,
namely, Glu180Gly, Asp175Asn, Lys70Thr, and Ala63Val.12
Therefore, to investigate the effect of Glu54Lys mutation on
TM structure, circular dichroism was used and thermal
stability measurements were made by following the ellipticity
(θ) of TM at 222 nm as a function of temperature. At
intermediate temperatures, the mutation significantly altered
θ 222, a measure of θ-helical content. Results showed that
the thermal denaturation curve of the mutant protein shifted
forward the right when compared with the wild-type curve
(Figure 5B), implying an increased stability conferred by
the mutation. This change in amino acid causes a decrease in
flexibility, which can influence actin binding13 as well as
myofilament Ca2+ sensitivity.12

Discussion
Two missense mutations (Glu54Lys and Glu40Lys) that alter
the highly conserved residues of α-TM have been linked to
DCM. The phenotypic severity associated with the sarco-
meric mutations in human DCM patients and the altered
sarcomeric function associated with the mutations is not well
established. The family pedigree associated with the DCM
α-TM54 mutation appears to be quite severe; these individ-
uals all died at relatively early ages (26, 27, and 49 years).1
Cardiac phenotypic measurements of the proband are in
agreement with those findings from both moderate and
high-copy TM mice, namely increased LV internal diastolic
dimension and LV internal systolic dimension, and decreased
fractional shortening percentage (Table 1 and supplemental
Table I). Because TM protein measurements were not con-
ducted on the proband, the relative ratio of wild type: mutant
TM is unknown. Nevertheless, the α-TM54 TG mice appear
to be a good model system for studying DCM.
The results of this study show that expression of α-TM encoding an amino acid change of glutamic acid (negative side chain) to lysine (positive side chain) at codon 54 induces DCM. Physiological alterations include impairment in both cardiac contractile and relaxation functions, with hearts exhibiting a significantly reduced LV fractional shortening and a decrease in myofilament Ca\(^{2+}\) sensitivity. This is the first demonstration that exogenous expression of a sarcomeric thin filament protein encoding a known human DCM mutation in the mouse heart results in pathological and physiological defects associated with DCM and thus provides an excellent opportunity to understand the disease pathology.

There has been a recent report of a DCM mouse model for a sarcomeric thick filament mutation in β-MHC that also exhibits the cardiomyopathic phenotype. Other reported murine models of sarcomeric DCM have not yet been shown to be related to human DCM, including cardiac overexpression of tropomodulin and homozygous expression in mice of a mutant form of the sarcomeric myosin-binding protein C.

The altered cardiac structure and function of the DCM α-TM54 mutation are thought to be a consequence of impairment in actin-binding capability by TM coupled with a decreased Ca\(^{2+}\) sensitivity of the myofilaments. This hypothesis is reinforced by the results from measurements with skinned fiber preparations of the α-TM54 TG mouse hearts. As summarized in Table 3, at 5 months of age, compared with matched controls, moderate-copy TG myofilaments demonstrate significant desensitization to Ca\(^{2+}\) as well as a depression in maximum tension. Higher-copy TG myofilaments, at 1 month of age, also demonstrate a more severely depressed maximum tension and reduced Ca\(^{2+}\) sensitivity when compared with age-matched controls. Moreover, the reduced myofilament sensitivity to Ca\(^{2+}\) and depressed tension correlates with our finding of depressed cardiac function as determined by echocardiography and/or by studies in isolated working hearts. Studies using the in vitro motility assay also agree that alterations in the TM function and decreased myofilament Ca\(^{2+}\) sensitivity are associated with the Glu54Lys amino acid substitution.

The altered expression of the Ca\(^{2+}\) handling proteins as observed by real-time RT-PCR quantification may contribute to a depressed Ca\(^{2+}\) transient, leading to impaired excitation--
contraction coupling. The rescue of cardiac function, after high doses of isoproterenol, from the impaired adrenergic responsiveness in the work performing hearts of moderate-copy TG mice at 3 months of age indicates that they are in the early stages of heart failure. Furthermore, the result also suggests that apart from the altered expression of the Ca\(^{2+}\)-handling proteins, there could also be an altered phosphorylation status of the Ca\(^{2+}\)-handling proteins such as phospholamban, which is a key determinant of β-adrenergic stimulation in the heart.\(^6\) The blunted response of the high-copy TG mice would indicate that their hearts are in too severe of a pathological state to respond to isoproterenol.

Despite a low incorporation of the mutant protein in the high-copy TG myofilaments, we see a severe phenotype culminating in early death. This could be attributed to an overly high transgene copy number in these mice and/or the excessive cytosolic presence of the mutant protein, which may interfere with cytoskeletal structures leading to a defective force transmission. The precise reason for significant cytosolic accumulation of mutant α-TM54 protein with decreased incorporation in the myofilaments is unclear but is an interesting area for future investigation.Interestingly, the muscle LIM protein associated with actin cytoskeleton at the Z-disc has been correlated to DCM in muscle LIM protein–null mice,\(^19\) and it has been proposed that defects in the cytoskeleton are primarily responsible for many human forms of DCM.\(^20,21\) Another possible mechanism of cardiac disease is the aggregate/amyloid formation by unfolded or misfolded proteins which has been reported in heart failure patients and also in desmin-related cardiomyopathic mice overexpressing mutant α-B-crystallin.\(^22\) This pathogenic process may be the cause of an early phenotype and death in the high-copy α-TM54 TG mice. Although we could not detect the cytosolic accumulation of the transgenic protein in the moderate expression lines, DCM still developed. This indicates that the α-TM54 mutation could be the primary cause for the development of DCM.

Circular dichroism titrations at 222 nm showed the temperature stability of the α-TM helix with a Glu54Lys amino acid substitution is greater than wild-type α-TM. In contrast, all FHC-associated tropomyosin mutations (Glu180Gly, Asp175Asn, Lys70Thr, and Ala63Val) are reported to decrease the temperature stability of the α helical coiled-coil. Most FHC thin filament mutations lead to a variety of functional abnormalities in the sarcomere that include increased myofilament Ca\(^{2+}\) sensitivity.\(^5,6,8,12\) However, the DCM α-TM54 mutation is shown to decrease the myofilament Ca\(^{2+}\) sensitivity. These findings help to correlate FHC mutations with increased Ca\(^{2+}\) sensitivity and a destabilized α-TM helix, and DCM mutations with decreased myofilament Ca\(^{2+}\) sensitivity and a stabilized α-TM helix. It is interesting to consider whether these 2 properties are consistent with other TM mutations and whether they are linked to the disease causing pathways of FHC and DCM. Our data indicate that α-TM54 mutation in mice results in significantly decreased myofilament calcium sensitivity and significantly impaired systolic and diastolic functions. This observation, together with the absence of length dependent activation of tension development and the thermal stability data of the mutant α-TM54 protein favoring the defective actin binding capability, supports the notion that defects in force transmission rather than force generation may cause the observed DCM phenotype.\(^1\)

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**Disclosures**

None.

**References**


In an article by Rajan et al. (Circ Res. 2007;101:205–214), Figure 2 was printed incorrectly. The correct figure appears below, as the authors intended. The corrected article is now available at http://circres.ahajournals.org. The Publisher regrets this error.
Materials and Methods

Generation of DCM Mutant α-TM54 Transgenic Mice

The mouse α-TM striated muscle specific cDNA (1.1 kb) (accession number: X64831) was cloned into the pBluescript vector. The single nucleotide change (GAA>AAA) corresponding to an amino acid substitution at codon 54 (Glu54Lys) was carried out using the QuikChange site-directed mutagenesis kit [Stratagene]. The sequence was verified by automated DNA sequencing at the University of Cincinnati and Children’s Hospital DNA core facility and compared with published sequence. The α-TM54 mutant cDNA was cloned into a vector,1 which contains the cardiac-specific α-MHC promoter and the human growth hormone (HGH) poly(A) signal sequence. The transgene construct was purified to generate transgenic mice as described.2 Transgenic mice were generated at the University of Cincinnati using the FVB/N strain. Founder mice were identified by PCR and five lines of TG mice with varied copy numbers of the transgene were confirmed by Southern blot analysis. The mutation in the transgenic lines was verified by nucleotide sequencing of TG mouse genomic DNA.

Genotyping

DNA samples were extracted from tail clips of 10 day-old mice and PCR was employed for genotyping the α-TM54 transgene: The following primers were used: α-MHC forward (5’- GCC CAC ACC AGA AAT GAC AGA -3’); α-TM reverse (5’- TCC AGT TCA TCT TCA GTG CCC-3’); GAPDH forward (5’- AGC GAG CTC AGG ACA TTC TGG -3’) and GAPDH reverse (5’- CTC CTA ACC ACG CTC CTA GCA -3’). GAPDH amplification was used as an internal control.
**Histopathological Analyses**

Mouse hearts at different ages (2 month-old, 3 month-old, 6 month-old) and both the sexes were analyzed. Heart weight-to-body weight ratios were measured to determine if cardiac hypertrophy had occurred. For histological analyses, hearts were fixed in 10% neutral buffered formalin for 48 hrs. The hearts were dehydrated through a gradient of alcohols and xylene, followed by embedding in paraffin. Step-serial sections (5 µm) were taken from the hearts and stained with hematoxylin/eosin or Masson’s trichrome. An expert who was blinded to genotype evaluated the presence of necrosis, fibrosis, myocyte disarray and calcification. Immunohistochemical analyses were performed in paraffin-embedded tissue sections by indirect immunostaining using the CH1 antibody diluted at 1:250 and incubating at 37°C for 1 hr.

**Northern Analyses**

Total RNA (10 µg) from transgenic and control ventricles was purified, separated by electrophoresis, and transferred to nylon membrane. Hybridization was conducted with $^{32}$P-radiolabeled cDNA fragments from the $\alpha$-MHC 1-2 exons to assess transgene expression, and $\alpha$-TM 5’UTR to determine endogenous $\alpha$-TM message levels. After exposure, blots were stripped and re-probed with a radiolabeled murine GAPDH cDNA fragment for normalization of RNA levels.

**Myofibrillar Protein Analyses**

Myofibrillar proteins were prepared from ventricular myocardium as described. One-dimensional isoelectric focusing (IEF) using slab gel electrophoresis was performed on myofibrillar protein preparations as described with some modifications. In brief, 15 cm IEF slab gels were used containing 5% Duracryl [Genomic Solutions], 9.1 mol/L urea, 2% (v/v) Triton X-100, and 0.05x of Pharmalyte 4.2-4.9 [GE Healthcare], with the catalysts ammonium persulfate, and N,N,N,N-tetramethylethylenediamine (TEMED) added separately. Total myofibrillar protein was dephosphorylated and loaded in a buffer
containing 9.2 mol/L urea, 0.02x of Pharmalyte 4.2-4.9, and 2% Triton X-100 with 0.025% bromophenol blue. The cathode buffer consisted of 0.040 mol/L lysine, and the anode buffer consisted of 0.007 mol/L phosphoric acid. The running conditions for a 150 mm slab gel were 1 W for 16 hours and 2 W for 1 hour. The transfer took place in 0.7% acetic acid (pH 3), at 200 mA for 12-14 hours at 4°C by placing the nitrocellulose membrane towards the anode. Western blot analysis using the striated muscle TM specific CH1 antibody [SIGMA] was conducted using a 1:5000 dilution. The intensity of the bands was quantified by using ImageQuant 5.1 software and the results are presented in ± S.D.

**Echocardiographic Measurements**

Transthoracic 2D-targeted M-mode and pulsed Doppler echocardiography (ECHO) were performed with a 15-MHz linear array transducer [Acuson Sequoia C256 system]. The transducer was placed on a layer of acoustic coupling gel that was applied to the left hemithorax; adequate contact was maintained while avoiding excessive pressure on the chest. Mice were imaged in a shallow left lateral decubitus position. M-mode images of the left ventricle were obtained from the parasternal short axis view at the level of the papillary muscles. Interventricular septal and LV posterior wall thicknesses and LV internal dimensions at the end of diastole and systole were measured by the American Society of Echocardiography leading-edge method on the M-mode tracings. Fractional shortening of the left ventricle, a measure of LV systolic function, was calculated from digital images as: LV fractional shortening (FS) (%) = (LVIDd–LVISd)/LVIDd x 100, where LVIDd is the internal diastolic dimension of the LV, and LVISd is the internal systolic dimension of the LV. The mean velocity of circumferential fiber shortening (Vcf) was calculated as Vcf = FS/ET, where ET is the ejection time through the aortic valve.

Diastolic transmitral inflow recordings were acquired from apical four-chamber views using 7 MHz pulsed Doppler ECHO. The probe was positioned substernally at the xyphoid applying minimal pressure. The Doppler range gate depth was set at 4 mm to obtain optimal signals from the LV inflow and outflow tracts. The sample volume was positioned along the long axis in the middle of the mitral ring at the tips of
the opened cusps of the mitral valve. Three parameters of the LV diastolic function were evaluated: (1) 
E/A ratio–ratio of the maximal velocity of E (early LV filling) and A (atrial contraction) waves; (2) E- 
wave deceleration time (DT)–the time from the peak of the E wave to the intersection of the deceleration 
slope of the E wave with the baseline; and (3) LV isovolumic relaxation time (IVRT), which was 
measured from the aortic valve closure to the mitral valve opening.6, 8 The M-mode and Doppler tracings 
were conducted with a paper speed of 200 mm/sec.

**Isolated Anterograde Perfused Heart Preparation**

Control and transgenic (moderate copy, L67 and high copy, L30) mice were anesthetized intraperitoneally 
with 100 mg/kg sodium nembutal and 1.5U heparin to prevent intracoronary micro thrombi. Anterograde 
work-performing perfusion was initiated at a workload of 250 mmHg mL/min as described.4 Heart rate 
(HR), left ventricular pressure (LVP), and the mean coronary perfusion pressure were continuously 
monitored. The pressure curve was used to calculate the rate of pressure development (+dP/dt) and 
decline (-dP/dt), time to peak pressure (TPP) and time to half relaxation (RT1/2) using the software 
“Origin” [Ver. 4.0, Microcal Software, Inc].

**Skinned Fiber Bundle Preparation and Force Measurements**

Tension developed by bundles of detergent-extracted fibers dissected from papillary muscle was 
measured at two sarcomere lengths as previously described.9, 10 Isometric tension was plotted as a 
function of pCa and fitted to the Hill equation by applying non-linear least squares regression analysis 
using Prism software [GraphPad Ver. 2.0]. Half-maximally activating pCa values (pCa50) were computed 
from individual Hill fits of each pCa-tension relation and then averaged.
**Real-time RT-PCR Analyses**

Ventricular tissue RNA from 5 month-old mice hearts was isolated using TRIZOL Reagent [Invitrogen], followed by RNA cleanup using RNeasy Mini Kit [QIAGEN]. The first strand cDNA was synthesized for 50 min at 50°C in a 20-µl reaction containing 1x First-Strand Buffer, 5 µg total RNA, 50 ng of random hexamers, 2 µmol/L dNTPs, 40 units RNase inhibitor, and 200 units Superscript III reverse transcriptase (RT) [Invitrogen]. Real-time PCR was performed in a 20-µl reaction, 96-well format (0.2 µl cDNA; 250 nmol/L of forward and reverse primer; 1x DyNAmo HS SYBR Green Master mix [Finnzymes]) using an Opticon 2 real time PCR machine [MJ Research]. Three samples were measured in each experimental group in triplicate, with a minimum of two independent experiments. The relative amount of target mRNA normalized to GAPDH was calculated according to the method described by Pfaffl. Specific primers that were used for the real-time PCR amplification included:

- **GAPDH** Forward: 5’- TGA CCA CAG TCC ATG CCA TC -3’;
  GAPDH Reverse: 5’- GAC GGA CAC ATT GGG GGT AG -3’;

- **β-MHC** Forward: 5’ - TTC ATC CGA ATC CAT TTT GGG G -3’
  β-MHC Reverse: 5’- GCA TAA TCG TAG GGG TTG TTG G -3’

- **BNP** Forward: 5’ - GAG GTC ACT CCT ATC CTC TGG -3’
  BNP Reverse: 5’- GCC ATT TCC TCC GAC TTT TCT C -3’

- **Skeletal Actin** Forward: 5’- GTGAGATTGTGCGCGACATC -3’
  Skeletal Actin Reverse: 5’- GGCAACGGAAACGCTCATT -3’

- **SERCA2a** Forward: 5’ - CAT TTG CAT TGC AGT CTG GAT -3’
  SERCA2a Reverse: 5’- CTT TGC CAT CCT ACG AGT TCC -3’

- **Calsequestrin** Forward: 5’- AGA GCC TAT GAC CAT CCC AGA -3’
  Calsequestrin Reverse: 5’- AAT GTG GAT TCC ATC CAG GTC -3’

- **RyR2** Forward: 5’ - TCA AAC CAC GAA CAC ATT GAG G -3’
  RyR2 Reverse: 5’ - AGG CGG TAA AAC ATG ATG TCA G -3’

- **L-Type Ca^{2+} Channel** Forward: 5’ - ATGAAACACGAGGATGACGTT -3’
  L-Type Ca^{2+} Channel Reverse: 5’ - ACTGACCGTAGAGATGGTGTC -3’

- **Phospholamban** Forward: 5’ - AAGTGCAATAACCTCACTCG -3’
  Phospholamban Reverse: 5’ - GATCAGCAGCAGACAT -3’
**Bacterial Recombinant Protein Expression**

Both wild type and mutant $\alpha$-TM54 cDNA constructs were designed to include an N-terminal Ala-Ser dipeptide, added to functionally compensate for lack of acetylation of bacterially expressed tropomyosin. The recombinant tropomyosin was expressed and purified using the Champion pET SUMO Expression System [Invitrogen]. In brief, the cDNA constructs were cloned into pET SUMO vector and transformed into chemically competent Mach1-T1R $E. coli$ according to the manufacturer's specifications. The coding sequences of the expression plasmids were confirmed by automated DNA sequencing. The plasmid DNA construct was then transformed into BL21 (DE3) One Shot $E. coli$ and induced by IPTG. The recombinant tropomyosin was then purified using the ProBond resin precharged with Ni$^{2+}$ ions. The N-terminal peptide containing the 6-His tag and SUMO fusion protein was removed employing SUMO Protease.

**Circular Dichroism Measurements**

Thermal stability measurements were made by following the ellipticity ($\theta$) of TM at 222 nm as a function of temperature, beginning at 5°C in 0.5 mol/L NaCl, 10-mmol/L sodium phosphate pH 7.5, 1 mmol/L EDTA, and 0.5 mmol/L DTT using an Aviv model 215 spectropolarimeter. Data were obtained at 2°C intervals with a protein concentration of 3 $\mu$mol/L. The apparent melting temperature and the thermodynamic parameters for TM unfolding were calculated based on the assumption that the unfolding could be fit by up to three independent helix-coil transitions with dissociation accompanying the helix-coil transition at the highest temperature, as previously described.
Supplemental Figure 1A. Western blot analysis of total TM protein expression in NTG, WT-TG and mutant TG hearts. Whole cell homogenate (5 µg) was run on 10% SDS-PAGE gel. Blots were probed with the striated TM-specific antibody and α-tubulin monoclonal antibody. The signal intensity of the bands was quantified with ImageQuant version 5.1. The TM level found in NTG hearts was set at 100%. α-Tubulin was used as loading control.

Supplemental Figure 1B. Western blot analysis of myofilament incorporated TM protein in NTG, WT-TG and mutant TG hearts. Myofibrilar protein preparations (5 µg) were run on 10% SDS-PAGE gel. Blots were probed with the striated TM-specific antibody and α-actin (sarcomeric) monoclonal antibody. The 10% SDS PAGE was unable to separate the wild type and the mutant TM proteins in the TG mice and hence the TM band observed includes both the endogenous and transgenic protein. The signal intensity of the bands was quantified with ImageQuant version 5.1. The TM level found in NTG hearts was set at 100%. Actin was used as loading control.
Supplemental Figure 2. pCa-force relation of skinned fiber preparations obtained from NTG and DCM α-TM54 mice. i) NTG vs. moderate copy TG mice and ii) NTG vs. high copy TG mice. There were significant decreases in tension development and pCa50 in skinned fiber bundles in both moderate and high copy TG mice when compared to matched controls. Note there is no length dependent activation of tension development in both moderate copy and high copy TG mice.

i. Moderate copy TG

![Graph showing the pCa-force relation for NTG and TG mice with moderate copy.]

* P<0.05 between NTG 2.3 and TG 2.3; ** P<0.05 between NTG 1.9 and TG 1.9;

ii. High copy TG

![Graph showing the pCa-force relation for NTG and TG mice with high copy.]

### P<0.001 between NTG 2.3 and TG 2.3
## Supplemental Table 1: Cardiac Function of NTG and high copy TG mice at one month of age, assessed by M-mode Echocardiography

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NTG, n=5</th>
<th>TG, L30 n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd, Left ventricular (LV) internal diastolic dimension (M-mode), mm</td>
<td>3.14</td>
<td>4.23*</td>
</tr>
<tr>
<td>LVISd, LV internal systolic dimension (M-mode), mm</td>
<td>1.55</td>
<td>3.52*</td>
</tr>
<tr>
<td>FS%, LV fractional shortening (M-mode)</td>
<td>50.88</td>
<td>17.47***</td>
</tr>
<tr>
<td>IVSD, Interventricular septum in diastole (M-mode), mm</td>
<td>0.64</td>
<td>0.79*</td>
</tr>
<tr>
<td>LVPWD, LV posterior wall in diastole (M-mode), mm</td>
<td>0.58</td>
<td>0.55</td>
</tr>
<tr>
<td>Vcf, LV circumferential fiber shortening (M-mode, Doppler) (circ.sec)</td>
<td>11.09</td>
<td>3.77***</td>
</tr>
<tr>
<td>CO, Cardiac output, (Doppler) ml/min</td>
<td>12.29</td>
<td>9.37</td>
</tr>
<tr>
<td>HR, Heart Rate, bpm</td>
<td>502.4</td>
<td>470.3</td>
</tr>
<tr>
<td>LV Isovolumic relaxation time (Doppler), msec</td>
<td>15.6</td>
<td>21.33</td>
</tr>
</tbody>
</table>

n, no. of mice, *P<0.05, ** P<0.01 *** P<0.001 NTG vs.TG
## Supplemental Table 2: Cardiovascular and contractile parameters of NTG and high copy TG mouse hearts in the isolated work-performing heart preparations at one month of age

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NTG, n=7</th>
<th>TG, L30 n=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic pressure, mmHg</td>
<td>149.8 ± 7.6</td>
<td>95.5 ± 8.9***</td>
</tr>
<tr>
<td>Diastolic pressure, mmHg</td>
<td>-8.4 ± 3.6</td>
<td>7.1 ± 3.1***</td>
</tr>
<tr>
<td>End diastolic pressure, mmHg</td>
<td>7.7 ± 2.8</td>
<td>17.6 ± 3.3**</td>
</tr>
<tr>
<td>Maximal rate of pressure development (+dP/dt) mmHg/s</td>
<td>3947 ± 252</td>
<td>2129 ± 227***</td>
</tr>
<tr>
<td>Maximal rate of pressure decline (-dP/dt) mmHg/s</td>
<td>3635 ± 233</td>
<td>1832 ± 379**</td>
</tr>
<tr>
<td>Half time to relaxation, (RT½), ms/mmHg</td>
<td>0.44 ± 0.013</td>
<td>0.58 ± 0.04**</td>
</tr>
<tr>
<td>Time to peak pressure (TPP), ms/mmHg</td>
<td>0.51 ± 0.06</td>
<td>0.74 ± 0.1*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>314 ± 11</td>
<td>245 ± 19**</td>
</tr>
</tbody>
</table>

n, no. of mice, *P<0.05, ** P<0.01 *** P<0.001 NTG vs.TG
References


