Am J Physiol Heart Circ Physiol 293: H949–H958, 2007.
First published April 6, 2007; doi:10.1152/ajpheart.01341.2006.

Rescue of tropomyosin-induced familial hypertrophic cardiomyopathy mice by transgenesis

Ganapathy Jagatheesan,[1] Sudarsan Rajan,[1] Natalia Petrashevskaya,[2] Arnold Schwartz,[2] Greg Boivin,[3] Grace M. Arteaga,[4] R. John Solaro,[4] Stephen B. Liggett,[5] and David F. Wieczorek[1]

[1]*Department of Molecular Genetics, Biochemistry, and Microbiology,* [2]*Institute of Molecular Pharmacology and Biophysics, Department of Surgery, and* [3]*Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio;* [4]*Department of Physiology and Biophysics, University of Illinois, Chicago College of Medicine, Chicago, Illinois; and* [5]*Department of Medicine, University of Maryland, Baltimore, Maryland*

Submitted 8 December 2006; accepted in final form 4 April 2007

Jagatheesan G, Rajan S, Petrashevskaya N, Schwartz A, Boivin G, Arteaga GM, Solaro RJ, Liggett SB, Wieczorek DF. Rescue of tropomyosin-induced familial hypertrophic cardiomyopathy mice by transgenesis. *Am J Physiol Heart Circ Physiol* 293: H949–H958, 2007. First published April 6, 2007; doi:10.1152/ajpheart.01341.2006.—Familial hypertrophic cardiomyopathy (FHC) is a disease caused by mutations in contractile proteins of the sarcomere. Our laboratory developed a mouse model of FHC with a mutation in the thin filament protein α-tropomyosin (TM) at amino acid 180 (Glu180Gly). The hearts of these mice exhibit dramatic systolic and diastolic dysfunction, and their myofilaments demonstrate increased calcium sensitivity. The mice also develop severe cardiac hypertrophy, with death ensuing by 6 mo. In an attempt to normalize calcium sensitivity in the cardiomyofilaments of the hypertrophic mice, we generated a chimeric α-/β-TM protein that decreases calcium sensitivity in transgenic mouse cardiac myofilaments. By mating mice from these two models together, we tested the hypothesis that an attenuation of myofilament calcium sensitivity would modulate the severe physiological and pathological consequences of the FHC mutation. These double-transgenic mice "rescue" the hypertrophic phenotype by exhibiting a normal morphology with no pathological abnormalities. Physiological analyses of these rescued mice show improved cardiac function and normal myofilament calcium sensitivity. These results demonstrate that alterations in calcium response by modification of contractile proteins can prevent the pathological and physiological effects of this disease.

hypertrophy; contractile function; genetically altered mice; calcium sensitivity

FAMILIAL HYPERTROPHIC CARDIOMYOPATHY (FHC) is inherited as a Mendelian autosomal dominant trait and is caused by mutations in any 1 of 10 genes, each encoding protein components of the cardiac sarcomere. Mutations in cardiac α- and β-myosin heavy chain (MHC), myosin binding protein C, cardiac troponin (Tn)T, regulatory and essential myosin light chains, titin, α-tropomyosin (TM), α-actin, and cardiac TnI are associated with hypertrophic cardiomyopathy. This genetic diversity is compounded by intragenic heterogeneity, with ~ 200 mutations now identified; most of these are missense mutations with a single amino acid residue substitution (11, 24). Pathologically, FHC is generally characterized by left ventricular hypertrophy in the absence of an increased external load, myofibrillar disarray, and fibrosis and oftentimes leads to increased Ca^{2+} sensitivity of myofilaments.

Eight mutations have been defined in α-TM that lead to FHC. Four of the mutations occur in the TnT binding region (Asp175Asn, Glu180Gly, Glu180Val, Leu185Arg); three mutations lie at the amino end of the TM molecule (Glu62Gln, Ala63Val, Lys70Thr); and one mutation lies in the middle (Val95Ala) (4, 8, 9, 11, 15). To understand the biochemical, morphological, and physiological effects of TM mutations in the heart, we developed two transgenic mouse models of FHC with mutations at amino acids 175 (Asp → Asn) and 180 (Glu → Gly) (14, 21, 22). Both mutant proteins confer increased Ca^{2+} sensitivity to myofilaments and result in decreased myocardial function. However, the α-TM180 mutation causes severe concentric hypertrophy of the heart, and the mice die by 6 mo of age. Of the eight mutations reported for α-TM, at least five confer increased Ca^{2+} sensitivity to the myofilaments, which may be causative for the development of the FHC phenotype (6, 12, 14, 21, 22, 28). We hypothesized that attenuating the increased Ca^{2+} sensitivity of myofilaments by contractile protein modification may prevent the development of FHC and its subsequent lethality. To test this hypothesis, we generated a chimeric α-/β-TM transgene [α-/β-TM (α-TM amino acids 1–257 and β-TM amino acids 258–284)]; previous work showed that the encoded α-/β-TM chimeric protein decreases Ca^{2+} sensitivity of myofilaments without altering cardiac morphology in transgenic Chimera 1 mice (7). To examine whether attenuation of increased myofilament Ca^{2+} sensitivity may curtail the development of cardiac hypertrophy, we generated double-transgenic (DTG) mice by mating the α-TM180 mice with Chimera 1 mice. We obtained the four expected genotypes: nontransgenic (NTG), α-TM180, Chimera 1 (Chi 1) transgenic (TG), and DTG. While α-TM180 and Chimera 1 TG mice exhibit their defined phenotypes (7, 21), DTG mice exhibit a phenotype similar to that of NTG mice. These DTG mice exhibit normal Ca^{2+} sensitivity of cardiac myofilament force development, have no gross morphological alterations, including hypertrophy, and have dramatically improved cardiac function. Our results suggest that modification of the contractile proteins can normalize myofilament Ca^{2+} sensitivity and prevent the pathological and physiological effects of FHC.

Address for reprint requests and other correspondence: D. F. Wieczorek, Dept. of Molecular Genetics, Biochemistry, and Microbiology, Univ. of Cincinnati College of Medicine, Cincinnati, OH 45267-0524 (e-mail: david.wieczorek@uc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

All animal procedures were conducted in conformance with the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society. The Institutional Animal Care and Use Committee of the University of Cincinnati College of Medicine approved the handling and maintenance of animals.

Generation of DTG mice. α-TM180 TG mice (line 33, transgene copy number 18) (21) were crossed with Chimera 1 mice (line 14, transgene copy number 4) (7) to generate DTG mice. The α-TM180 mice utilize an α-MHC cardiac-specific promoter to express a FHC α-TM Glu180Gly cDNA (21) (Fig. 1*A*). The Chimera 1 TG mice use the α-MHC promoter ligated to a cDNA encoding amino acids 1–257 of α-TM and amino acids 258–284 of β-TM (7) (Fig. 1*B*). The DTG mice express both α-TM180 mutant and chimeric α-/β-TM proteins in the same heart. All mice were of the FVB/N genetic background strain. Polymerase chain reaction (PCR) and Southern blot analysis on genomic DNAs were used to identify the four different genotypes (NTG, α-TM180 TG, Chimera 1 TG, and DTG mice) and to determine their respective transgene DNA copy numbers. The accession numbers of the mouse TM sequences are X64831 (α-TM) and M81086 (β-TM).

RNA and protein analysis. Real-time reverse transcription-PCR (RT-PCR) analysis was conducted to determine mRNA levels of the specific TM transcripts. cDNA was synthesized for 50 min at 50°C in a 20-μl reaction containing 1× First-Strand Buffer, 5 μg of total heart RNA, 50 ng of random hexamers, 2 μM dNTPs, 40 U of RNase inhibitor, and 200 U of Superscript III reverse transcriptase (Invitrogen). Specific primers that were used for the 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′; Chimera 1 forward: 5′-TGA AAC TCG GGC TGA GTT TGC-3′, Chimera 1 reverse: 5′-CAG TGG GGA CTC AGA GGG AAG-3′; α-TM180 mutant forward: 5′-AAG CGA CCT GGA ACG TGC AAG-3′, α-TM180 mutant reverse: 5′-AGC CTC CTT CAG CTT GTC-3′; and endogenous wild-type α-TM 5′ untranslated region (UTR) forward: 5′-AAG TAT TGG CTG TCC TAA GGA ATG-3′, endogenous wild-type α-TM 5′ UTR reverse: 5′ GCG TCC ATG GTG GCG GTG GC 3′. Real-time RT-PCR was performed in a 20-μl reaction in 96-well format [0.2 μl cDNA, each primer at 250 nM, 1× DyNAmo HS SYBR Green Master mix (Finnzymes)] with 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 (19).

To analyze and quantify TM composition in the hearts, myofibrillar proteins were prepared from ventricular myocardium as described previously (13) with minor modifications. In brief, the enriched total myofibrillar proteins were extracted with rigor buffer supplemented with 5 mM ATP, pH 7, and 5 mM $CaCl_2$ and were used without further differential centrifugation. Two-dimensional gel electrophoresis was performed on myofibrillar protein preparations according to the method of O'Farrell (16) with minor modifications. In brief, isoelectric focusing was carried out with 25 μg of the protein samples denatured in 300 μl of dehydration buffer [8 M urea, 2 mM tributylphosphine, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% Bio-Lytes (4.7–5.9; Bio-Rad)]. Each sample was used to hydrate a 17-cm ReadyStrip pH 4.7–5.9 for 12 h. Isoelectric focusing was performed in three stages of applied potential difference: 250 V for 15 min, 10,000 V for 1 h, followed by 8,000 V for up to 6 h, until 40,000 Vh were achieved. Focused strips were then soaked in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris·HCl, pH 8.8, 20% glycerol) containing 130 mM DTT for 10 min, followed by equilibration buffer containing 135 mM iodoacetamide for another 10 min. Strips were then applied to 10% acrylamide gels for SDS-PAGE, followed by transblotting onto polyvinylidene difluoride membrane. Western blot analysis using the striated muscle TM-

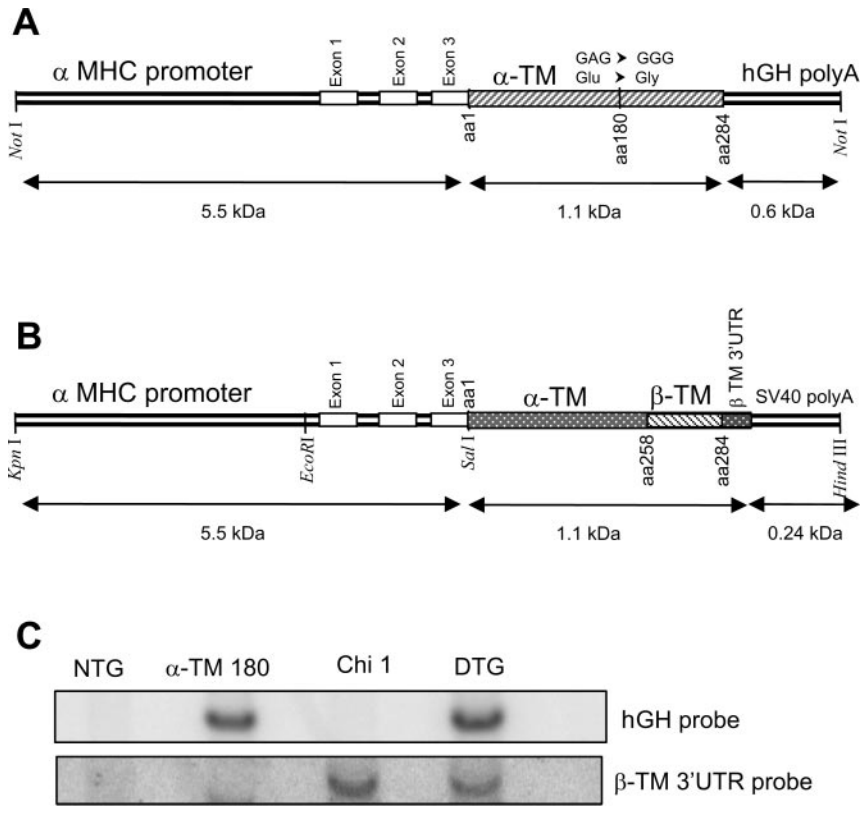


Fig. 1. *A*: familial hypertrophic cardiomyopathy (FHC) α-tropomyosin (TM)180 construct. The α-TM180 construct was made with the α-myosin heavy chain (MHC) cardiac-specific promoter, the α-TM cDNA with an encoded substitution at amino acid 180 (Glu180Gly), and the human growth hormone (hGH) termination and polyadenylation cassette at the 3′ end. *B*: Chimera α-/β-TM 1 construct. The Chimera 1 construct encodes amino acids 1–257 of α-TM and amino acids 258–284 and the 3′ untranslated region (UTR) of β-TM. This TM cDNA was linked to the α-MHC promoter at the 5′ end and to the SV40 polyadenylation/termination cassette at the 3′ end. *C*: genomic Southern blot of the 4 distinct genotypic mice: nontransgenic (NTG), α-TM180, Chimera 1 (Chi 1), and double transgenic (DTG). Mouse tail genomic DNA was digested with *Eco*RI, Southern blotted, and hybridized to ^{32}P-radiolabeled probes from hGH or β-TM 3′ UTR as designated.

specific CH1 antibody was conducted with a 1:5,000 dilution (7). Protein spots that reacted with the TM-specific antibody were quantified by ImageQuant analysis, using three separate blots for each genotype. Quantified values were assessed with a Student's *t*-test, and results are presented as means ± SD.

To quantify the total amount of TM, 5 μg of the myofibrillar fraction from each sample was run on 10% SDS-PAGE gels and transferred to nitrocellulose filters. Filters were first incubated with striated TM-specific CH1 antibody (Sigma), followed by second antibody-horseradish peroxidase conjugate, and the blots were developed with an enhanced chemiluminescence kit (Pierce). The blots were then stripped of their first and second antibodies according to the membrane manufacturer's instructions. Blots containing the myofibrillar fraction were reacted with sarcomeric anti-actin antibody (clone 5C5 from Sigma) and developed as described above. The intensity of the bands was quantified with ImageQuant 5.1 software. Cardiac actin levels were used to normalize TM values in the myofibrillar fractions. Western blot analysis was performed three times for each genotype, and mean ± SD values were calculated.

Isolated anterograde-perfused heart preparation. Control and transgenic mice were anesthetized intraperitoneally with 100 mg/kg Nembutal sodium and 1.5 U of heparin to prevent intracoronary microthrombi (7). Anterograde work-performing perfusion was initiated at a workload of 250 mmHg ml/min as described previously (7). Heart rate (HR), left ventricular pressure (LVP), and 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 (RT½). Starling curves were generated by linear regression with Origin software (version 4.0, Microcal Software). For the regression lines, the average slopes were calculated using only the initial part of the Frank-Starling curve (at cardiac work from 0 to 350 $mmHg \cdot ml \cdot min^{-1}$). Data are presented as means ± SE.

Skinned fiber bundle preparation and force measurements. Force developed by bundles of detergent-extracted fibers dissected from papillary muscle was measured as previously described (14, 29). Isometric tension was plotted as a function of pCa and fitted to the Hill equation by applying nonlinear least-squares regression analysis with Prism software (GraphPad version 2.0). Isometric tensions measured at submaximally activating Ca^{2+} concentrations were expressed as a percentage of the maximum tension. Half-maximally activating pCa values (pCa_{50}) were computed from individual Hill fits of each pCa-tension relation and then averaged. Experimental values are expressed as means ± SE.

RESULTS

Generation of transgenic mice. The cDNA constructs used to generate the α-TM180 and Chimera 1 mice are shown in Fig. 1, *A* and *B*, respectively. FHC α-TM180 mice develop severe cardiac hypertrophy, extensive fibrosis, impaired systolic and diastolic function, and increased myofibrillar sensitivity to Ca^{2+} (21, 22). Chimera 1 mice have delayed rates of cardiac muscle contraction and relaxation, with decreased myofibrillar Ca^{2+} sensitivity, but with no morphological changes in the heart (7). To test our hypothesis that normalization of cardiac myofilament Ca^{2+} sensitivity by modification of contractile proteins will attenuate the hypertrophic phenotype associated with FHC α-TM180 mice, we developed a DTG mouse model by mating α-TM180 mice with Chimera 1 mice. Four different genotypes were identified by PCR and confirmed by Southern blot analysis. When genomic DNA from TG or NTG mice is endonuclease restricted and subjected to Southern blot hybridization with a ^{32}P-radiolabeled human growth hormone (hGH) probe, only the α-TM180 and DTG DNAs show hybridization bands (Fig. 1*C*). Hybridization to a β-TM 3′ UTR probe exhibits a unique transgene-associated band (6.8 kb) only in the chimera α-/β-TM and DTG DNAs. Genomic DNA from NTG mice did not hybridize with the hGH probe, but hybridization to the endogenous 4-kb band occurs when probed with the β-TM 3′ UTR (data not shown). Quantified results demonstrate that the DTG mice have both sets of transgenes with copy numbers identical to the original parental mouse lines: Chimera 1 mice: 4 copies of the Chimera 1 transgene; α-TM180 mice: 18 copies of the α-TM180 transgene; DTG mice: 4 copies of the Chimera 1 transgene plus 18 copies of the α-TM180 transgene.

α-TM180, Chimera 1, and DTG transcript and protein expression. After RNA isolation from hearts of TG and NTG littermates, we measured TM expression with real-time RT-PCR analyses. When normalized to GAPDH mRNA (levels reported in arbitrary units), the total amount of TM mRNA produced in the TG mice is greater than in NTG mice (Fig. 2). The normalized level of endogenous α-TM mRNA is 56.5 ± 2.6 in NTG hearts. In Chimera 1 hearts, the endogenous α-TM level is 34.0 ± 1.2 and 37.2 ± 2.4 for Chimera 1 mRNA. In α-TM180 hearts, the endogenous α-TM level is 44.9 ± 0.1 and 27.5 ± 2.6 for α-TM 180 mRNA. In the DTG hearts, endogenous α-TM levels are 37.2 ± 2.4, Chimera 1 mRNA levels are 32.9 ± 0.1, and α-TM180 mRNA levels are 38.6 ± 0.7. In addition, the results show that the α-TM180 mRNA is only expressed in the DTG and α-TM180 hearts and expression of Chimera 1 mRNA is found only in the DTG and Chimera 1 mice. We hypothesize that the suppression of endogenous α-TM mRNA in the TG mice is due to differences in the 5′ and 3′ UTR sequences; the transgenes incorporate an α-MHC 5′ UTR and either a hGH or SV40 polyA-3′ UTR which may account for the increased transcription, mRNA stability, and/or translation of the transgene transcripts or proteins.

To examine TM protein expression in the control and TG mice, cardiac myofibrillar proteins from all genotypes were subject to two-dimensional gel electrophoresis followed by immunoblotting with a striated muscle-specific TM antibody. The results show that under reduced conditions, heart myofi-

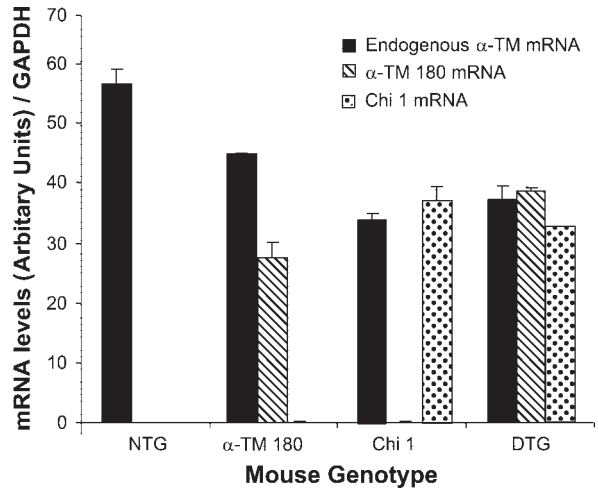


Fig. 2. Quantification of TM mRNA levels in the hearts of NTG and transgenic (TG) mice. Real-time RT-PCR analysis was conducted on NTG and TG cardiac RNA with primers specific for endogenous α-TM, α-TM180, or Chimera 1 TM mRNAs. Values were normalized to GAPDH levels.

brillar protein samples from NTG mice show a single spot, labeled α-TM, which is reactive with the TM antibody (Fig. 3*A*). In agreement with previous studies on TM composition in cardiac musculature, the results show that the α-TM isoform is predominant in the heart. Hearts from α-TM180 mice express both the endogenous α-TM and α-TM180 protein isoforms (42.64 ± 0.22% and 57.36 ± 0.22%, respectively), which is in agreement with previous work (22) (Fig. 3*B*). Also, the results show that the Chimera 1 TG mice express the endogenous α- and chimeric α-/β-TM protein isoforms (48.70 ± 3.25% and 51.30 ± 3.24%, respectively). In DTG hearts, the endogenous α-TM protein isoform level is 33.4 ± 1.89%, the α-/β-TM chimeric protein isoform level is 26.5 ± 0.23%, and the α-TM180 protein isoform is 40.1 ± 2.28% of the total cardiac myofibrillar TM (Fig. 3*B*). Previous studies demonstrated that 35% α-TM180 protein clearly triggers a hypertrophic lethal phenotype (21, 22); thus the DTG mice are not simply rescued because of decreased levels of endogenous wild-type or FHC mutant TM.

A quantitative analysis was conducted using ImageQuant to determine the amount of TM incorporated into the myofilaments of the TG hearts after Western blot analysis. The total amount of incorporated striated muscle TM (generated from both endogenous and transgene sources) was normalized to striated muscle actin; the ratio of myofilament TM to actin for NTG hearts was set at 100%. The data show that there are no significant differences in the level of total TM incorporation into the myofilaments in the TG hearts: 98.5 + 1.6% for α-TM180 mice, 91.2 + 7.0% for Chimera 1 mice, and 97.9 + 7.1% for DTG mice. Additional results show that there are no changes in the total amount of TM or in non-TM cardiac contractile protein expression profiles (including actin) among NTG, α-TM180 mutant, chimera α-/β-TM transgenic, and DTG mice (data not shown).

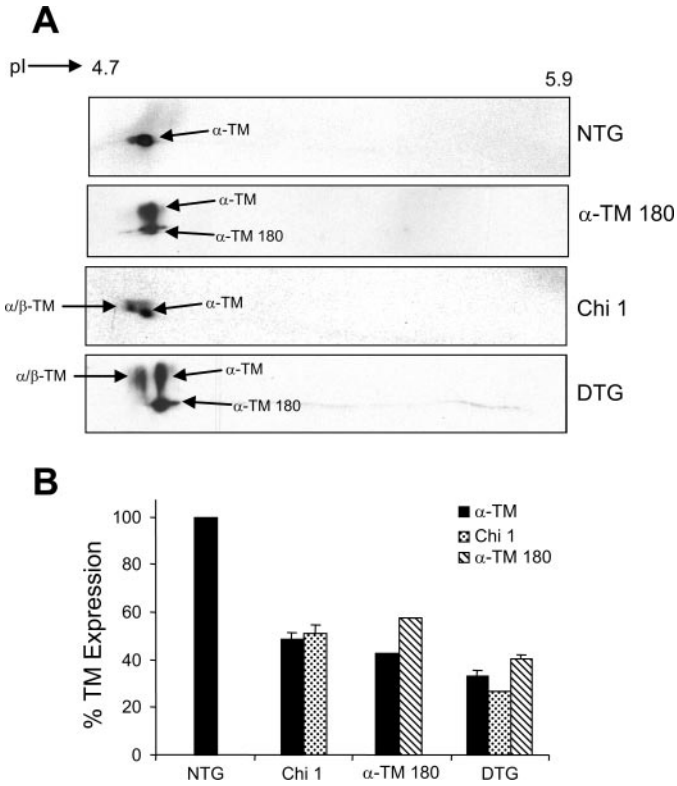
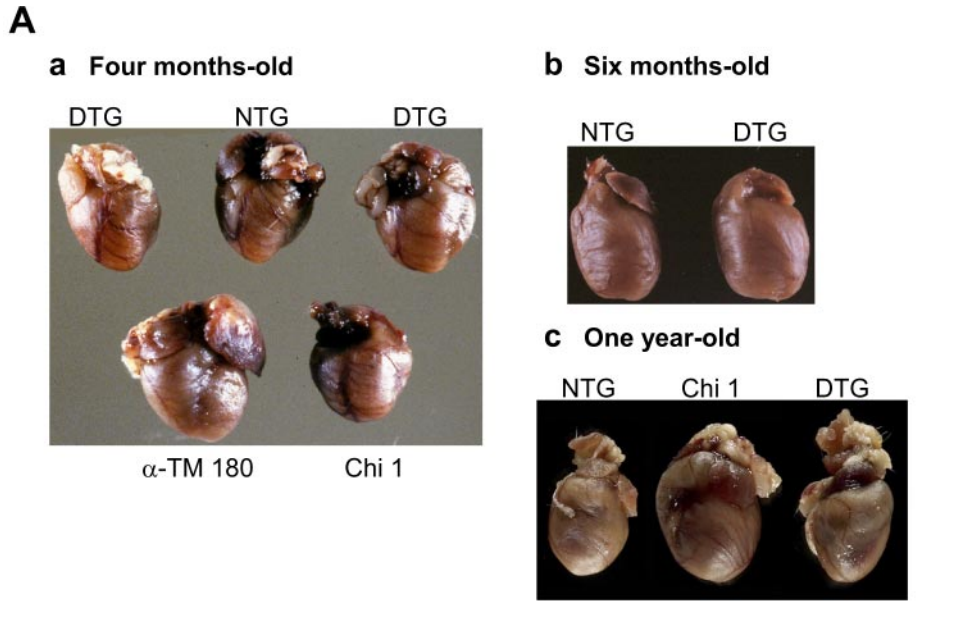


Fig. 3. *A*: 2-dimensional electrophoretic analysis with Western blot analysis of NTG and TG cardiac myofibrillar proteins. Myofibrillar TM subunit composition was analyzed under reduced conditions with 2-dimensional PAGE as described in EXPERIMENTAL PROCEDURES. Isoelectric point (pI) values are shown. SDS-PAGE of 10% was used in the second dimension. Positions of the α-TM, α-TM180, and α-/β-TM Chimera 1 subunits are marked. *B*: quantification of TM protein isoform distribution in NTG and TG hearts. The signal intensity of the TM isoform distribution in the NTG and TG hearts was quantified with ImageQuant version 5.1. The TM level found in NTG hearts was set at 100%.

We conducted PCR analyses on TG mouse tail genomic DNA to negate the possibility that an unexpected mutation or deletion of the α-TM180 and or chimeric α-/β-TM DNA sequences occurred during transgenesis. PCR fragments were generated with oligonucleotides corresponding to the α-MHC and β-TM 3′ UTR sequences as 5′ and 3′ primers for Chimera 1 mice and α-MHC and hGH poly (A) sequences as 5′ and 3′ primers for α-TM180 mice. Nucleotide sequencing of these fragments shows that there are no mutations or deletions in the incorporated Chimera 1 or α-TM180 sequences present in the TG mice.

Histological analysis of DTG mice. Mice from the four different genotypes and ranging in age from 4 to 12 mo were examined for morphological and histological changes. α-TM180 mutant mice do display severe morphological and histopathological alterations (21, 22): at 4 mo, these mice display severe hypertrophy, fibrosis, enlarged left atria, and calcification of left atria. On a gross level, there are no obvious morphological differences in 4- or 6-mo or 1 yr-old NTG, DTG, or Chimera 1 mouse hearts (Fig. 4). Histochemical analyses of the heart sections show no signs of cardiac hypertrophy, fibrosis, thrombi, necrosis, or any other pathological condition in NTG, Chimera 1, and DTG mice. Also, there are no differences in percent heart-to-body weight ratios among these three genotypes (Table 1). These results show that incorporation of chimeric α-/β-TM protein into the DTG myofibers, which also contain the α-TM180 protein, prevents the development of hypertrophy and severe pathological alterations. This attenuation of cardiac hypertrophy and fibrosis lasts at least 1 yr.

Physiological analyses of DTG mice. We pursued several lines of experiments to address cardiac muscle performance in DTG mice. An isolated anterograde-perfused heart preparation was used to carry out a functional analysis of hearts from all four genotypes at 4 mo of age and three genotypes (NTG, Chimera 1, and DTG) at 12 mo of age. Previous work using echocardiography and the isolated work-performing heart method show that at 2.5–3 mo of age the α-TM180 hearts exhibit normal heart rates (22); by 4 mo of age, these hearts are severely stressed (Table 1). At 4 mo, Chimera 1 and α-TM180 hearts produce systolic pressures that are significantly lower than the control hearts, but DTG hearts have a similar level of systolic pressure compared with the NTG controls (Table 1). The maximal rate of pressure development for contraction (+dP/d*t*) is significantly decreased in the Chimera 1 and α-TM180 hearts, but DTG hearts display similar rates as NTG hearts. TPP is faster in control and DTG hearts than in either the Chimera 1 or α-TM180 hearts (Table 1). Maximal −dP/dt is also decreased in all TG groups; however, RT½ is only increased in α-TM180 hearts.



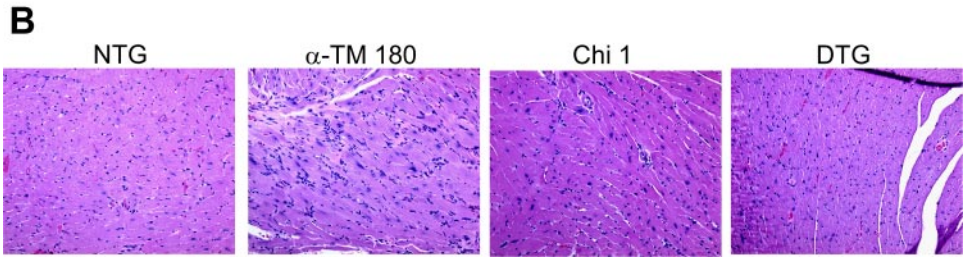


Fig. 4. *A*: NTG, α-TM180, Chimera 1, and DTG hearts at 4 mo (*a*), 6 mo (*b*), and 1 yr (*c*). Note the extreme size of the atrial and ventricular chambers in the α-TM180 hearts and the similarity in size between the NTG and DTG hearts. One-year-old hearts are shown for NTG, Chimera 1, and DTG mice. Note that the increased size of the Chimera 1 mouse heart at 1 yr is not a consistent observation. *B*: pathology of 4-mo-old hearts. Left ventricular wall tissue from the hearts of the 4 distinct genotypic mice is shown. The α-TM180 mutant shows increased numbers of picnotic nuclei, whereas the DTG hearts exhibit minimal pathological effects.

To determine whether the improved phenotype of the DTG mice would persist for greater than 4 mo, we aged the mice and performed the isolated work-performing analysis on 1-yr-old hearts (α-TM180 mice do not survive beyond 6 mo). The results show that there were no differences between the NTG and DTG hearts for the heart weight-to-body weight ratio, intrinsic heart rates, or systolic parameters in 1-yr-old mice (Table 2). However, hearts from DTG and Chimera 1 mice exhibit several altered cardiac parameters, including increased diastolic and end-diastolic pressures and a prolongation of RT½, possibly indicative of impending cardiac disease and failure. Previous studies show that overexpression of wild-type α-TM in transgenic mice does not lead to alterations in morphology or physiological performance of the heart (21, 29).

Response to change in workload. The Frank-Starling relationship reflects the increase in cardiac performance in response to increased intraventricular pressure or volume load. To determine to what extent the DTG mice could be loaded with increasing workload, cardiac minute work was varied from 50 mmHg·ml·min^{-1} to the maximal level of mean aortic pressure (afterload) that can be generated at a given venous return (preload) of 5 ml/min. The hearts from all four groups are sensitive to increases in afterload for +dP/d*t* at 4 mo of age (Fig. 5*A*). However, there is no increased response for −dP/d*t*

Table 1. *Parameters describing cardiac function at 4 mo of age in NTG and TG hearts*

Parameters	NTG (n = 7)	DTG (n = 7)	Chi 1 (n = 6)	α-TM180 (n = 6)
Systolic pressure, mmHg	105±1.3	99.0±3.9	87±5.7[a]	71.1±1.25[c]
Diastolic pressure, mmHg	−13.2±6.8	−16.2±1.1	−12.6±2.9	−0.6±5.9[b]
End-diastolic pressure, mmHg	0.856±1.53	2.14±1.4	2.04±0.22	1.76±1.73
Maximal +dP/d*t*, mmHg/s	4,095±52	3,989±120	3,031±197[a]	2,833±188[c]
Maximal −dP/d*t*, mmHg/s	3,478±156	2,963±73[d]	2,625±226[a]	1,833±93[c]
Heart rate, beats/min	359±16	356±14	404±19[a]	177±13[c]
TPP, ms/mmHg	0.39±0.011	0.41±0.015	0.56±0.04[a]	0.65±0.007[b]
$RT_{1/2}$, ms/mmHg	0.62±0.053	0.64±0.036	0.71±0.057	1.16±0.13[c]
Heart wt/body wt × 100	50.8±2.2	52.3±2.9	51.6±1.8	64.2±4.1[f]
Body wt, g	25.9±3.9	27.6±3.5	32.2±4.5	32.4±1.3[e]

Values are means ± SE; n, no. of mice. TG, transgenic; NTG, nontransgenic; DTG, double transgenic; Chi 1, Chimera 1; TM, tropomyosin; +dP/d*t*, rate of pressure development; −dP/d*t*, rate of relaxation; TPP, time to peak pressure; $RT_{1/2}$, time to half-relaxation. [a]$P < 0.05$, NTG vs. Chi 1; [b]$P < 0.01$; [c]$P < 0.001$, α-TM180 vs. DTG, NTG; [d]$P < 0.01$, DTG vs. NTG; [e]$P < 0.05$, NTG vs. α-TM180; [f]$P < 0.01$, NTG vs. α-TM180.

Table 2. *Parameters describing cardiac function at 1 yr in NTG and TG hearts*

Parameters	NTG (n = 4)	Chi 1 (n = 3)	DTG (n = 3)
Systolic pressure, mmHg	83.7±7.8	73±4.8*	86.3±7.1
Diastolic pressure, mmHg	−4.5±1.8	−5.9±3.5	4.5±0.82†
End-diastolic pressure, mmHg	6.0±1.8	16.2±5.9*	18.3±5.7*
Maximal +dP/dt, mmHg/s	3,437±217	2,505±158*	3,395±198
Maximal −dP/dt, mmHg/s	3,173±224	2,115±225*	2,680±95*
Heart rate, beats/min	303±21	360±10*	329±5
TPP, ms/mmHg	0.47±0.03	0.62±0.03*	0.52±0.04
$RT_{1/2}$, ms/mmHg	0.65±0.06	0.82±0.0†	0.89±0.01†
Heart wt/body wt × 100	48.4±8.3	64.5±8.7	54.3±9.1
Body wt, g	34.8±3.1	29.9±5.3	34.9±8.4

Values are means ± SE; n, no. of mice, *$P < 0.05$, †$P < 0.01$, NTG vs. DTG, Chi 1.

(diastolic state) in the α-TM180 mice at this age, most likely because of the severe pathological condition of the hearts at this time. Also, there are no significant differences in the responses between the NTG and DTG hearts to increased workload at 4 mo. By 1 yr, the plots of cardiac work versus +dP/dt demonstrate an overall reduction for the Chimera 1 mice, with no significant differences between the NTG and DTG mice (Fig. 5*B*). There is, however, a diminished response in the DTG from the NTG mice at 1 yr in the diastolic state (−dP/dt), where the y-intercept is significantly lower for the DTG and Chimera 1 mice than for the NTG mice. The slopes of the regression curves for the DTG at 1 yr are not different, indicating that maximal −dP/dt and +dP/dt were sensitive to length-dependent regulation to an extent similar to that in the NTG hearts.

Response to β-adrenergic stimulation. We examined the effect of isoproterenol in working hearts to evaluate the possible mechanical abnormalities in cardiac function under hemodynamic stress. At 4 mo, DTG hearts display positive inotropic and chronotropic responses to β-adrenoreceptor stimulation, but the α-TM180 hearts fail to respond (Fig. 6). The lusitropic effect (−dP/dt, mmHg/s) is restored to control levels in DTG mice, indicating that depressed relaxation can be overcome by protein kinase A (PKA)-dependent phosphorylation, which increases the rates of Ca^{2+} cycling and reuptake by the sarcoplasmic reticulum. The positive chronotropic effects of isoproterenol were preserved even at 1 yr of age in DTG hearts and are similar among NTG, Chimera 1, and DTG mice (data not shown). Stimulation with β-adrenoagonist also increased +dP/dt and −dP/dt in DTG hearts to values similar to

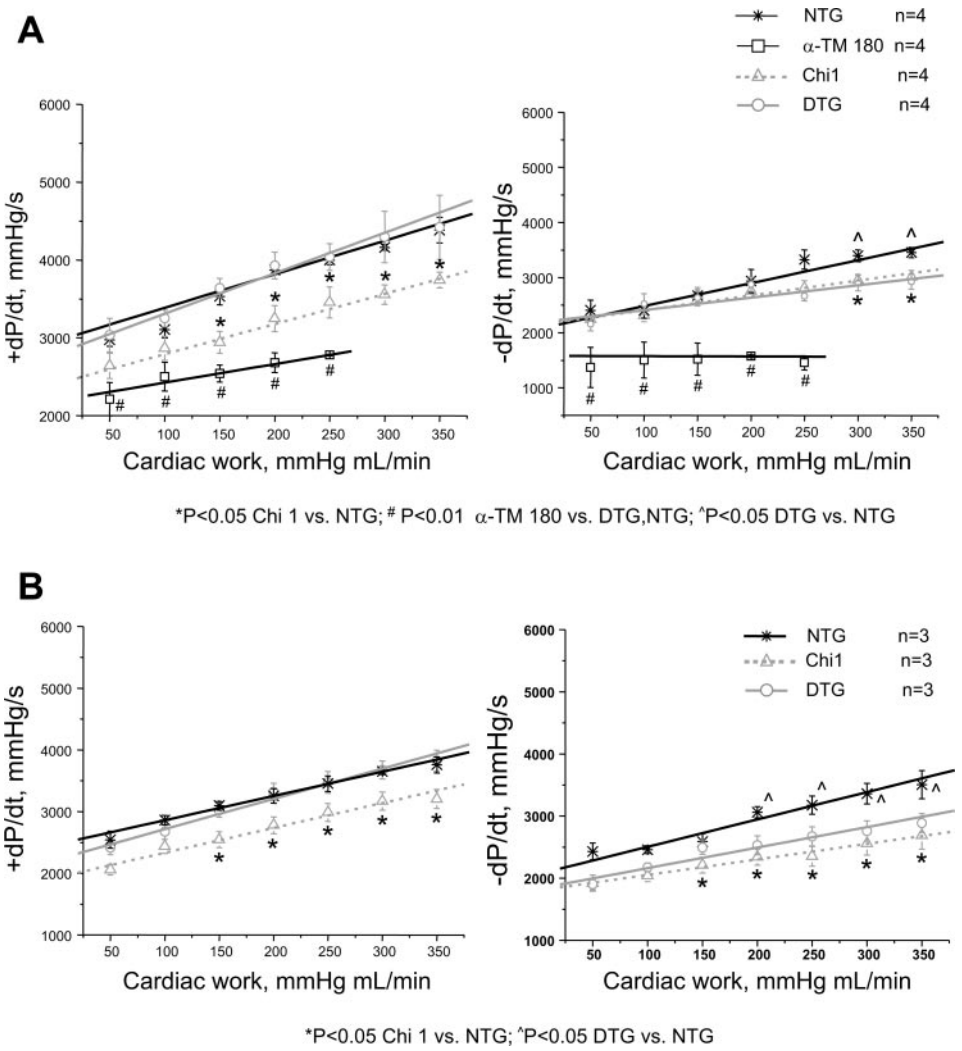


Fig. 5. Response of isolated work-performing hearts to preload over a range of cardiac work from 50 to 350 mmHg/ml min. *A*: 4 mo-old hearts. *B*: 1-yr-old hearts. The baseline recordings are measured under similar conditions: mean aortic pressure (afterload, 50 mmHg), venous return (preload, 5 ml/min), left ventricular minute work 350 mmHg·ml·min^{-1}. Rate of pressure development (+dP/dt) of experimental groups was plotted against gradually increasing afterloads at a constant preload (venous return) of 5 ml/min. The hearts of the experimental groups increase force of contraction of loading.

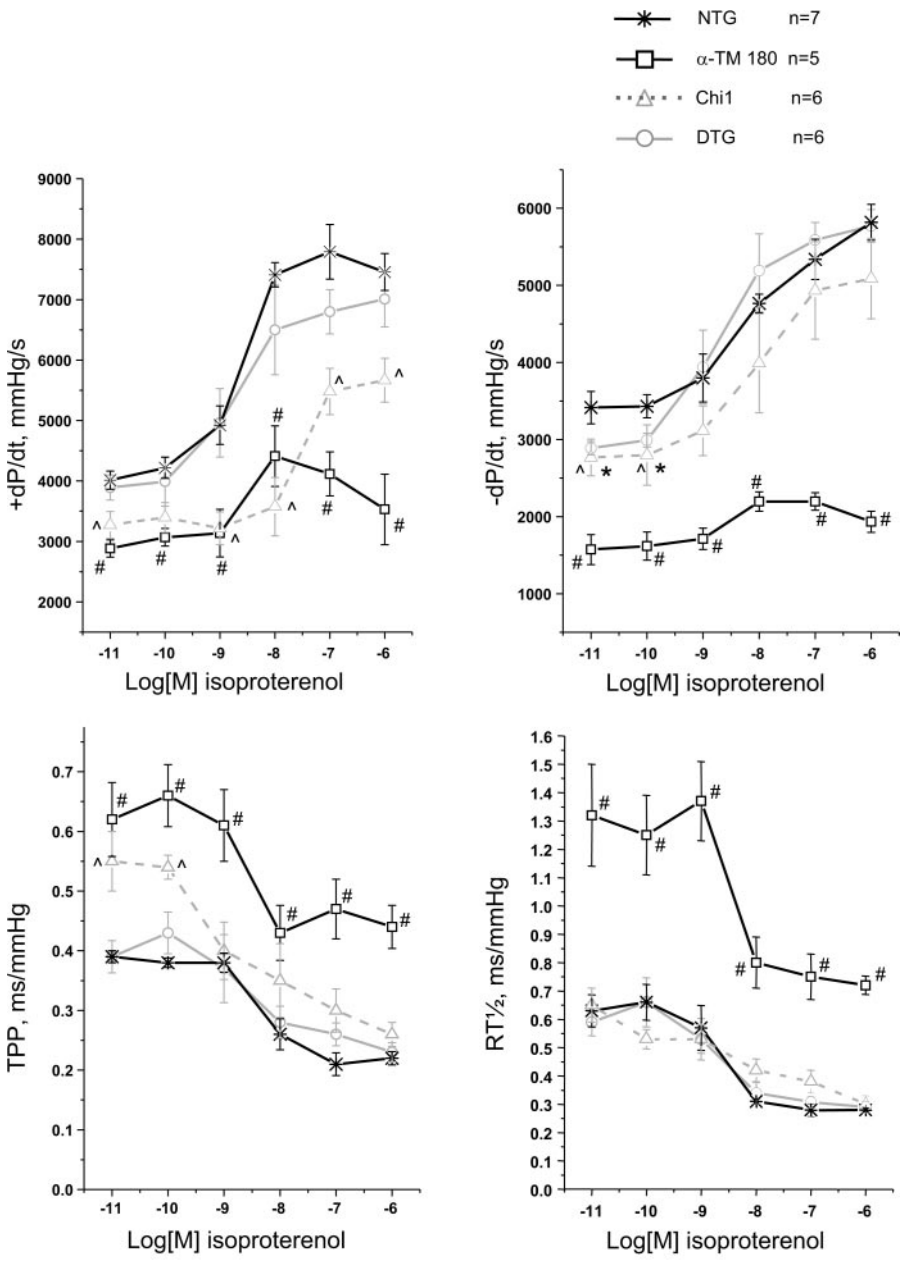


Fig. 6. Isoproterenol dose-response curves in TG and NTG mouse hearts at 4 mo of age. Hearts from NTG, α-TM180, Chimera 1, and DTG mice were subjected to isolated heart analyses with increasing concentrations of isoproterenol (10^{-11}–10^{-6} M).

NTG littermates. Also, DTG hearts show a positive response with respect to TPP and RT½ measurements. The aged DTG hearts reveal preservation of β-adrenergic transduction and subsequent stimulation of Ca^{2+} cycling. The latter translated into enhanced cardiac performance and relaxation. Furthermore, correction of cardiomyocyte Ca^{2+} sensitivity had a beneficial effect not only on baseline systolic cardiac function but also on β-adrenergic responsiveness in DTG mice over long periods of time.

Ca^{2+}-force measurements in skinned fiber bundles. To examine the correlation between physiological results from the whole heart and transgene expression at the sarcomere level, experiments were conducted with detergent-extracted (skinned) fiber bundles. Figure 7 shows data comparing pCa-% maximum force relations obtained from NTG, Chimera 1, α-TM180, and DTG hearts. Parameters describing these relations are summarized in Table 3. Within each specific genotype, the increase in Ca^{2+} sensitivity is sarcomere length dependent. The data indicate that pCa_{50} values (−log of free Ca^{2+} concentration required for half-maximum activation) are similar between NTG and DTG fiber bundles at both sarcomeric lengths (Table 3; Fig. 7). However, there are significant differences between NTG and α-TM180 myofilaments, which exhibit an increased sensitivity to Ca^{2+} at both 1.9- and 2.3-μm lengths, and Chimera 1 myofilaments, which exhibit a decreased sensitivity to Ca^{2+}. These results clearly show that incorporation of Chimera 1 protein into myofilaments containing α-TM180 protein attenuates the effect of the α-TM180 mutation on myofilament Ca^{2+} sensitivity at both sarcomeric lengths.

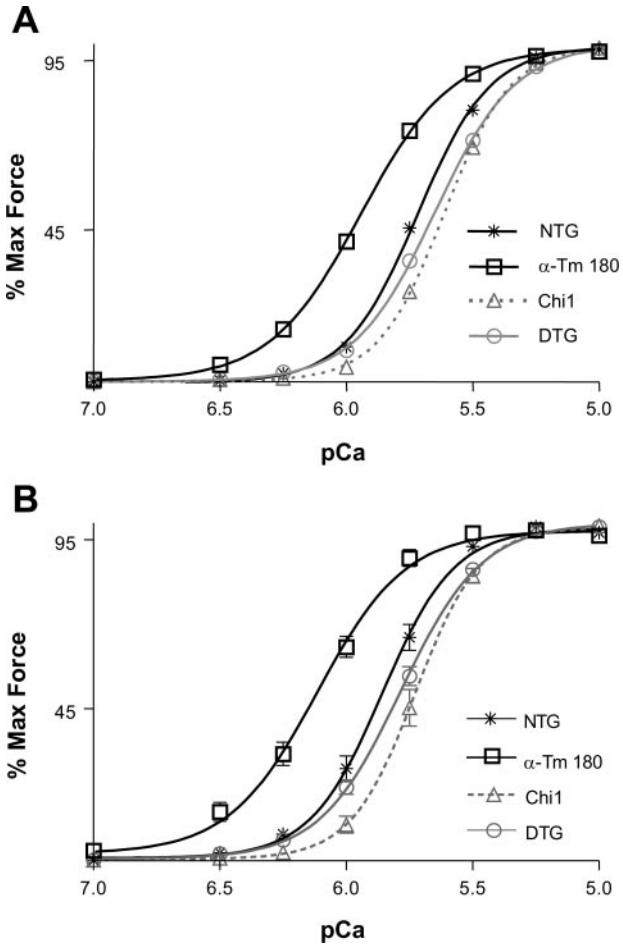


Fig. 7. pCa-force relation of skinned fiber preparations obtained from NTG, Chimera 1, DTG, and α-TM180 hearts at 2 sarcomere lengths (SL): 1.9 μm (*A*) and 2.3 μm (*B*). There was a significant difference in pCa_{50} in skinned fiber bundles from NTG vs. Chimera 1, NTG vs. α-TM180, and α-TM180 vs. DTG. There was no significant difference in half-maximally activating pCa (pCa_{50}) values between DTG and Chimera 1, or DTG and NTG groups. See Table 3 for a summary of parameters describing the pCa-force relations.

We also determined Hill coefficient (n_H) values as a measure of cooperative Ca^{2+} activation of the myofilaments from hearts of the four distinct genotypes. Within each genotype, the n_H values appear constant and independent of sarcomere length (Table 3). Interestingly, there is a decrease in cooperativity in the DTG myofilaments associated with increased sarcomere length. Significant differences in n_H were observed between α-TM180 and Chimera 1 myofilaments at both sarcomeric lengths. There was also a significant difference between Chimera 1 n_H values and those of DTG at 1.9 μm.

DISCUSSION

Numerous studies demonstrate that aberrant Ca^{2+} levels in the cardiomyocyte can lead to severe pathological consequences, including hypertrophy and heart failure (5, 31). Excessive Ca^{2+} levels can also lead to defective excitation-contraction coupling through abnormal ryanodine receptor function, altered sarcoplasmic reticulum function, and aberrant myofibrillar function. This investigation suggests that altering the myofilament response to Ca^{2+} can reverse the effect of mutations in α-TM that impair sarcomeric performance and cause hypertrophy. What is unique in this study is that this reversal occurs through modification of the contractile proteins themselves and not through a Ca^{2+}-handling protein. Thus our data suggest that the response of the cardiac thin filament to Ca^{2+} is a major determinant in signaling the development of cardiac fibrosis and hypertrophy.

Burkart et al. (2) showed that the FHC Glu180Gly mutation in α-TM not only affects the TM-TnT interaction but causes secondary alterations in the signaling of TnC and TnI. These structural alterations most likely account for the increased Ca^{2+} sensitivity in the FHC α-TM180 myofilaments. TnT can sense the changes in the state of TnC and TnI in a signaling cascade that is ultimately responsible for TM movement allowing strong cross-bridge binding. The amino terminus of TnT interacts with the Cys190 region of TM, which is near the FHC mutation Glu180Gly. Thus modulation of this region affects both the activation of the thin filament by Ca^{2+} binding and also cross-bridge binding.

The carboxy region of TM (amino acids 258–284) plays critical roles in thin filament structure and function, including head-to-tail interactions, cooperativity, and interactions with actin and TnT. With the DTG mice, there are substantive amino acid changes in both TnT binding regions (TM amino acids 175–190 and 258–284). We hypothesize that the effect of the FHC Glu180Gly amino acid substitution is offset by the exchange of the α- for β-TM carboxy region. Since the two transgenes result in opposite effects in myofilament Ca^{2+} sensitivity, and the two associated TM proteins are found within myofibers, their joint presence in the sarcomere normalizes Ca^{2+} sensitivity. Our present results support this hypothesis. Surprisingly, these two TM molecules also normalize virtually all of the cardiac functional parameters and the cardiac phenotype. The precise mechanism as to how this dramatic rescue occurs is under investigation. Although we cannot negate the possibility that a slight decrease in the level of the α-TM180 protein in the DTG mice is responsible for the "rescue" phenotype, the data suggest that this is unlikely because previous studies (21, 22) show that TG mice having α-TM180 protein levels equivalent to the levels in DTG mice exhibit severe cardiac hypertrophy, abnormal cardiac function, and death by 6 mo. Thus we hypothesize that the normalization of cardiac performance in the DTG mice involves a signaling process that acts through the Tn complex. Previous studies show that normalization of Ca^{2+} dynamics through Ca^{2+} buffers, increased Ca^{2+} uptake by the sarcoplasmic reticulum,

Table 3. *Parameters describing Ca^{2+}*-dependent activation of tension in skinned fiber bundles from NTG and TG hearts

Sarcomere Length, μm	pCa_{50}	n_H	*n*
NTG 1.9	$5.72 \pm 0.02^{b,d}$	3.39 ± 0.17	13
NTG 2.3	5.86 ± 0.03^{a}	3.41 ± 0.15	
Chi 1 1.9	$5.62 \pm 0.02^{b,e}$	3.84 ± 0.22^{e}	15
Chi 1 2.3	5.73 ± 0.02^{a}	4.05 ± 0.31^{e}	
α-TM180 1.9	$5.94 \pm 0.03^{c,d,e}$	2.58 ± 0.15^{e}	10
α-TM180 2.3	6.12 ± 0.03^{a}	2.60 ± 0.19^{e}	
DTG 1.9	5.65 ± 0.02^{c}	3.02 ± 0.09^{f}	16
DTG 2.3	5.78 ± 0.01^{a}	2.83 ± 0.09	

Values are means ± SE; *n*, no. of mice. pCa_{50}, half-maximally activating pCa; n_H, Hill coefficient. $^{a}P < 0.05$, sarcomere length within same group; $^{b}P < 0.05$, Chi 1 vs. NTG; $^{c}P < 0.001$, DTG vs. α-TM180; $^{d}P < 0.001$, NTG vs. α-TM180; $^{e}P < 0.001$, Chi 1 vs. α-TM180; $^{f}P < 0.05$, Chi 1 vs. DTG.

β-adrenergic blockade, or calcineurin inhibitors can improve hemodynamic function in cardiomyopathic hearts (3, 20, 23, 27, 30). Previous work shows there are Ca^{2+} cycling defects in the FHC α-TM180 mice as exhibited by decreased expression of phospholamban and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)2a (23). Our preliminary studies demonstrate that crossing FHC α-TM180 mice with phospholamban-knockout mice also prevents the development of hypertrophy and cardiac dysfunction (18), and gene transfer of SERCA2a into FHC α-TM180 neonates improves cardiac morphology and in situ hemodynamic performance (17). Thus myofilament response to Ca^{2+} and the associated signaling pathways play an integral role in cardiac performance and disease.

Surprisingly, missense mutations or isoform exchanges between the TnT binding regions of α- and β-TM can lead to either increased or decreased myofibrillar Ca^{2+} sensitivity. Although myofibrillar sensitivity to Ca^{2+} does appear to play a major role in cardiac function, it is not a true indicator of altered diastolic performance. For example, while phosphorylation of cardiac TnI by PKA increases cross-bridge kinetics, it desensitizes the myofilaments to Ca^{2+} and enhances the relaxation rate (10). On the other hand, protein kinase C phosphorylation of cardiac TnT also decreases myofilament Ca^{2+} sensitivity by depressing cross-bridge kinetics (26). Why are there such varied alterations in cardiac performance and myofibrillar Ca^{2+} sensitivity with changes in TM expression, and why is the Chimera 1 TM molecule able to "rescue" the hypertrophic phenotype in the FHC α-TM180 mouse? We assume that cardiac muscle functions as an integrated whole in determining sarcomeric tension and relaxation; whether this occurs through the summation of small individual regions that preferentially incorporate a specific TM protein or is a total unit of random TM protein integration is unknown. An area of future investigation is to determine whether there is preferential TM dimer formation of the endogenous α-TM with either α-TM180 or Chimera 1 proteins, and whether α-TM180 and Chimera 1 protein preferentially dimerize. Regardless, increased Ca^{2+} sensitivity and its effect on myofilament function appear to play a critical role in the development of pathological hypertrophy, and modulation of Ca^{2+} sensitivity through various pathways may prove to be a viable approach for treatment of cardiovascular disease (25).

ACKNOWLEDGMENTS

We thank Jon Neumann for production of the transgenic mice and Maureen Luehrmann for care of the animals.

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-22619 (to D. F. Wieczorek and A. Schwartz), HL-71952 awarded to D. F. Wieczorek, HL-22231 and HL-62426 awarded to R. J. Solaro, and K01-HL-67709 awarded to G. M. Arteaga.

REFERENCES

1. **Bronson DD, Schachat FH.** Heterogeneity of contractile proteins. Differences in tropomyosin in fast, mixed, and slow skeletal muscles of the rabbit. *J Biol Chem* 257: 3937–3944, 1982.
2. **Burkart E, Arteaga G, Sumandea M, Prabhakar R, Wieczorek DF, Solaro RJ.** Altered signaling surrounding the C-lobe of cardiac troponin C in myofilaments containing an α-tropomyosin mutation linked to familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 35: 1285–1293, 2003.
3. **Coutu P, Bennett C, Favre E, Day S, Metzger J.** Parvalbumin corrects slowed relaxation in adult cardiac myocytes expressing hypertrophic cardiomyopathy-linked α-tropomyosin mutations. *Circ Res* 94: 1235–1242, 2004.
4. **Coviello D, Maron B, Spirito P, Watkins H, Vosberg H, Thierfelder L, Schoen F, Seidman J, Seidman C.** Clinical features of hypertrophic cardiomyopathy caused by mutation of a "hot spot" in the alpha-tropomyosin gene. *J Am Coll Cardiol* 29: 635–640, 1997.
5. **Hasenfuss G, Pieske B.** Calcium cycling in congestive heart failure. *J Mol Cell Cardiol* 34: 951–969, 2002.
6. **Hernandez O, Housmans P, Potter J.** Pathophysiology of cardiac muscle contraction and relaxation as a result of alterations in thin filament regulation. *J Appl Physiol* 90: 1125–1136, 2001.
7. **Jagatheesan G, Rajan S, Petrashevskaya N, Schwartz A, Boivin G, Vahebi S, de Tombe P, Solaro RJ, Labitzke E, Hilliard G, Wieczorek DF.** Functional importance of the carboxyl-terminal region of striated muscle tropomyosin. *J Biol Chem* 278: 23204–23211, 2003.
8. **Jongbloed R, Marcelis C, Doevendans P, Schmeitz-Mulkens J, Van Dockum W, Geraedts J, Smeets H.** Variable clinical manifestation of a novel missense mutation in the alpha-tropomyosin (TPM1) gene in familial hypertrophic cardiomyopathy. *J Am Coll Cardiol* 41: 981–986, 2003.
9. **Karibe A, Tobacman L, Strand J, Butters C, Back N, Bachinski L, Arai A, Ortiz A, Roberts R, Homsher E, Fananapazir L.** Hypertrophic cardiomyopathy caused by a novel α-tropomyosin mutation (V95A) is associated with mild cardiac phenotype, abnormal calcium binding to troponin, abnormal myosin cycling, and poor prognosis. *Circulation* 103: 65–71, 2001.
10. **Kentish J, McCloskey D, Layland J, Palmer S, Leiden J, Martin A, Solaro RJ.** Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. *Circ Res* 88: 1059–1065, 2001.
11. **Maron BJ.** Hypertrophic cardiomyopathy: a systematic review. *JAMA* 92: 1336–1347, 2002.
12. **Michele D, Albayya F, Metzger J.** Direct, convergent hypersensitivity of calcium-activated force generation produced by hypertrophic cardiomyopathy mutant α-tropomyosins in adult cardiac myocytes. *Nat Med* 5: 1413–1417, 1999.
13. **Muthuchamy M, Grupp I, Grupp G, O'Toole B, Kier A, Boivin G, Neumann J, Wieczorek DF.** Molecular and physiological effects of overexpessing striated muscle β-tropomyosin in the adult murine heart. *J Biol Chem* 270: 30593–30603, 1995.
14. **Muthuchamy M, Pieples K, Rethinasamy P, Hoit B, Grupp I, Boivin G, Wolska B, Evans C, Solaro RJ, Wieczorek DF.** Mouse model of a familial hypertrophic cardiomyopathy mutation in α-tropomyosin manifests cardiac dysfunction. *Circ Res* 85: 47–56, 1999.
15. **Nakajima-Taniguchi C, Matsui H, Nagata S, Kishimoto T, Yamaguchi-Takihara K.** Novel missense mutation in alpha tropomyosin gene found in Japanese patients with hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 27: 2053–2058, 1995.
16. **O'Farrell P.** High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007–4021, 1975.
17. **Penia J, Goldspink P, Prabhakar R, del Monte F, Hajjar R, Wieczorek DF, Wolska B.** Neonatal gene transfer of SERCA2a improves the response to β-adrenergic stimulation in the α-tropomyosin (Glu180Gly) mouse model of familial hypertrophic cardiomyopathy (Abstract). American Heart Association Scientific Conference on Molecular Mechanisms of Growth, Death, and Regeneration in the Myocardium, Snowbird, UT, 2003.
18. **Penia J, Urboniene D, Goldspink P, Kranias E, Wieczorek DF, Wolska B.** Phospholamban knockout prevents the development of hypertrophy and cardiac dysfunction in a FHC α-tropomyosin (Glu180Gly) mouse model (Abstract). Keystone Symposium: Molecular Biology of Cardiac Diseases and Regeneration, Steamboat Springs, CO, 2005.
19. **Pfaffl MW.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
20. **Plank D, Yatani A, Ritsu H, Witt S, Glascock B, Lalli M, Periasamy M, Fiset C, Benkusky N, Valdivia H, Sussman M.** Calcium dynamics in the failing heart: restoration by β-adrenergic receptor blockade. *Am J Physiol Heart Circ Physiol* 285: H305–H315, 2003.
21. **Prabhakar R, Boivin G, Grupp I, Hoit B, Arteaga G, Solaro RJ, Wieczorek DF.** A familial hypertrophic cardiomyopathy α-tropomyosin mutation causes severe cardiac hypertrophy and death in mice. *J Mol Cell Cardiol* 33: 1815–1828, 2001.

22. **Prabhakar R, Petrashevskaya N, Schwartz A, Aronow B, Boivin G, Molkentin J, Wieczorek DF.** A mouse model of familial hypertrophic cardiomyopathy caused by a α-tropomyosin mutation. *Mol Cell Biochem* 251: 33–42, 2003.
23. **Sato Y, Kiriazis H, Yatani A, Schmidt A, Hahn H, Ferguson D, Sako H, Mitarai S, Honda R, Mesnard-Rouiller M, Frank K, Beyermann B, Wu G, Fujimori K, Dorn G, Kranias E.** Rescue of contractile parameters and myocyte hypertrophy in calsequestrin overexpressing myocardium by phospholamban ablation. *J Biol Chem* 276: 9392–9399, 2001.
24. **Seidman J, Seidman C.** The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell* 104: 557–567, 2001.
25. **Sorsa T, Pollesello P, Solaro RJ.** The contractile apparatus as a target for drugs against heart failure: interaction of levosimendan, a calcium sensitizer, with cardiac troponin C. *Mol Cell Biochem* 266: 87–107, 2004.
26. **Sumandea M, Pyle G, Kobayashi T, de Tombe P, Solaro RJ.** Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T. *J Biol Chem* 278: 35135–35144, 2003.
27. **Sussman M, Lim H, Gude N, Taigen T, Olson E, Robbins J, Colbert M, Gualberto A, Wieczorek DF, Molkentin J.** Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science* 281: 1690–1693, 1998.
28. **Westfall M, Borton A, Albayya F, Metzger J.** Myofilament calcium sensitivity and cardiac disease. *Circ Res* 91: 525–531, 2002.
29. **Wolska B, Keller R, Evans C, Palmiter K, Phillips R, Muthuchamy M, Oehlenschlager J, Wieczorek DF, deTombe P, Solaro RJ.** Correlation between myofilament response to Ca^{2+} and altered dynamics of contraction and relaxation in transgenic cardiac cells that express β-tropomyosin. *Circ Res* 84: 745–751, 1999.
30. **Yang A, Sonin D, Jones L, Barry W, Liang B.** A beneficial role of cardiac $P2X_4$ receptors in heart failure: rescue of the calsequestrin overexpression model of cardiomyopathy. *Am J Physiol Heart Circ Physiol* 287: H1096–H1103, 2004.
31. **Yano M, Ikeda Y, Matsuzaki M.** Altered intracellular Ca^{2+} handling in heart failure. *J Clin Invest* 115: 556–564, 2005.