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Co-expression of skeletal and cardiac troponin T decreases mouse cardiac function

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[1]*Department of Physiology and Biophysics, Case Western Reserve University, and* [2]*Department of Cardiovascular Medicine, Cleveland Clinic Foundation, Cleveland, Ohio;* [3]*Department of Biomedical Sciences, Florida Atlantic University, Boca Raton; and* [4]*Section of Molecular Cardiology, Evanston Northwestern Healthcare and Northwestern University Feinberg School of Medicine, Evanston, Illinois*

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Huang Q.-Q, Feng HZ, Liu J, Du J, Stull LB, Moravec CS, Huang X, Jin J.-P. Co-expression of skeletal and cardiac troponin T decreases mouse cardiac function. *Am J Physiol Cell Physiol* 294: C213–C222, 2008. First published October 24, 2007; doi:10.1152/ajpcell.00146.2007.—In contrast to skeletal muscles that simultaneously express multiple troponin T (TnT) isoforms, normal adult human cardiac muscle contains a single isoform of cardiac TnT. To understand the significance of myocardial TnT homogeneity, we examined the effect of TnT heterogeneity on heart function. Transgenic mouse hearts overexpressing a fast skeletal muscle TnT together with the endogenous cardiac TnT was investigated in vivo and ex vivo as an experimental system of concurrent presence of two classes of TnT in the adult cardiac muscle.This model of myocardial TnT heterogeneity produced pathogenic phenotypes: echocardiograph imaging detected age-progressive reductions of cardiac function; in vivo left ventricular pressure analysis showed decreased myocardial contractility; ex vivo analysis of isolated working heart preparations confirmed an intrinsic decrease of cardiac function in the absence of neurohumoral influence. The transgenic mice also showed chronic myocardial hypertrophy and degeneration. The dominantly negative effects of introducing a fast TnT into the cardiac thin filaments to produce two classes of Ca^{2+} regulatory units in the adult myocardium suggest that TnT heterogeneity decreases contractile function by disrupting the synchronized action during ventricular contraction that is normally activated as an electrophysiological syncytium.

myocardial heterogeneity; transgenic mouse; echocardiography; working heart preparation

CONTRACTION OF STRIATED (cardiac and skeletal) muscle is regulated by Ca^{2+} via the actin thin filament-associated troponin-tropomyosin system (14, 27, 39). Troponin T (TnT) is the tropomyosin-binding subunit of the troponin complex (33). Through interactions with troponin C (TnC), troponin I (TnI), tropomyosin (Tm), and actin in the thin filament, TnT plays a coordinator function in the Ca^{2+}-regulatory system of muscle. Three homologous TnT genes have evolved in vertebrates encoding the muscle type-specific cardiac, slow skeletal muscle, and fast skeletal muscle TnT isoforms (6, 8, 17, 22). Developmentally regulated alternative RNA splicing generates further protein diversities (18, 20, 41). Although the various TnT isoforms are largely conserved in function, they convey quantitative differences in the Ca^{2+} activation of contraction (3, 13, 15, 34, 38).

A major difference between skeletal and cardiac muscles is their TnT isoform contents. Mammalian skeletal muscle fibers express both fast and slow TnT isoforms and multiple alternative splicing variants (7). In contrast, cardiac muscle only expresses cardiac TnT (4). It is known that the ventricular myocardium functions as an electrophysiological syncytium to accomplish synchronized contraction that is critical to the rhythmic pumping function of the vertebrate heart (9). Consistent with a uniformed contractile action, normal adult cardiac muscle contains only adult cardiac TnT corresponding to a homogenous thin filament regulation after the alternative splicing-generated isoform switch during perinatal heart development (23). Similarly, single isoforms of TnI, TnC, and Tm are present in the adult cardiac muscle thin filament (14). These observations led to a hypothesis that the presence of one class of TnT and, therefore, homogenous Ca^{2+} regulatory machinery is critical to myocardial function that requires synchronized contraction.

To test this hypothesis, our strategy is to introduce a noncardiac TnT isoform into the cardiac muscle to generate constant TnT heterogeneity. In the present study, we examined the function of transgenic mouse hearts that overexpress a fast skeletal muscle TnT together with the endogenous cardiac TnT (16). The transgenic mouse cardiac muscle contains two classes of TnT with diverged structure and function, rendering an integrated experimental model of cardiac thin filament heterogeneity. Echocardiograph imaging detected age-progressive reductions of cardiac function; in vivo left ventricular (LV) pressure (LVP) analysis showed decreased myocardial contractility; ex vivo analysis of isolated working heart preparations confirmed an intrinsic decrease of cardiac function in the absence of neurohumoral influence. The transgenic mice also showed evidence of chronic myocardial hypertrophy and degeneration. The dominantly negative effects of incorporating a normal fast TnT into cardiac muscle suggest that chronic thin filament heterogeneity due to the presence of more than one class of TnT in adult myocardium would decrease contractile function by desynchronizing the Ca^{2+} regulatory units in the ventricular muscle that normally functions as an electrophysiological syncytium.

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MATERIALS AND METHODS

Transgenic mice continuously overexpressing a fast skeletal muscle TnT in the adult cardiac muscle. We constructed transgenic mice expressing a cDNA encoding chicken fast skeletal muscle TnT (16) under the control of a cloned mouse cardiac α-myosin heavy chain (MHC) gene promoter (36, generously provided by Dr. Jeffrey Robbins, University of Cincinnati). Mice of both sexes from two independent founder lines (lines 5 and 18) were investigated in the present study to avoid potential line-to-line variations. There was no detectable phenotypic difference between the two transgenic mouse lines. Therefore, the data from the two lines of mice were pooled for functional characterization. All animal procedures were approved by the Institutional Animal Care and Use Committees and were conducted in accordance with the "Guiding Principles in the Care and Use of Animals," as approved by the Council of the American Physiological Society.

Genomic DNA prepared from mouse tail tissue samples by protease K digestion and phenol-$CHCl_3$ extraction was examined by Southern blot analysis to identify the homozygous transgenic mice with doubled transgene copies versus that in hemizygotes for use in the functional characterization. As described previously (16), 20 μg each of the purified genomic DNA was digested by restriction endonuclease *Pvu*II and run on 1.0% agarose gel. The resolved DNA bands were depurinated and transferred to a Zeta-Probe nylon membrane (Bio-Rad Lab) by capillary action. The blotted membrane was prehybridized with mechanically sheared salmon sperm DNA and hybridized at 55°C with a chicken fast skeletal muscle TnT cDNA probe (42) labeled with ^{32}P by random primed DNA polymerase reaction. The membrane was then washed with several changes of 40 mM sodium phosphate buffer containing 1% SDS and 1 mM EDTA at 60°C to remove nonspecific background radioactivity. The chicken fast TnT transgene integrated into the transgenic mouse genome was detected by autoradiography. Normalized by an endogenous band from the mouse genomic DNA hybridized with the chicken fast TnT cDNA probe, the intensity of the transgene-specific bands was quantified to identify the significantly higher transgene dosage in the homozygous transgenic mice compared with the hemizygous controls. A representative result is shown in Fig. 1.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. All of the transgenic mouse hearts used in the functional studies were examined by Western blot analysis using a monoclonal antibody (mAb) 6B8 that specifically recognizes chicken fast TnT (42), alone or mixed together with an anti-cardiac TnT mAb CT3 (21), to confirm the coexpression of the exogenous fast skeletal muscle TnT and the endogenous cardiac TnT in the cardiac muscle. Whole cardiac muscle or Triton X-100-washed cardiac myofibrils (16) were homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS to extract the myofilament proteins. The samples were resolved by Laemmli SDS-PAGE, and the protein bands were transferred to a nitrocellulose membrane using a Bio-Rad Lab semidry electrotransfer apparatus. The nitrocellulose membrane was blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (150 mM NaCl, 50 mM Tris·HCl, pH 7.5) and incubated with the anti-fast and cardiac TnT mAbs diluted in Tris-buffered saline containing 0.1% BSA. The subsequent washes, incubation with alkaline phosphatase-labeled anti-mouse IgG second antibodies (Sigma), and 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium substrate reaction were carried out as described previously (16).

On Western blot analyses using the same antibodies, we have previously quantified the TnT isoform contents in the transgenic mouse cardiac muscle to demonstrate that the chicken fast skeletal muscle TnT expressed in homozygous transgenic mouse hearts effectively incorporated into the cardiac myofilaments to make ~48% of the total myofibril TnT (30), providing an suitable experimental system to investigate the physiological phenotypes of myocardial TnT heterogeneity.

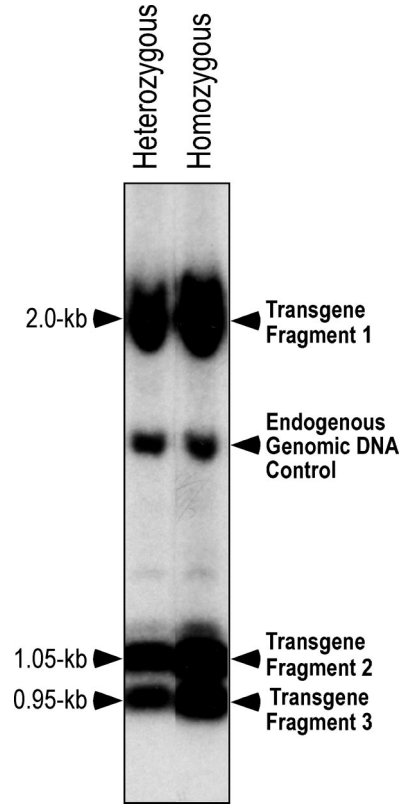

Fig. 1. Southern blot identification of homozygosis of the transgene allele encoding fast skeletal muscle troponin T (TnT) in transgenic mice. With the use of ^{32}P-labeled chicken fast TnT cDNA probe, the autoradiograph shows that the genomic DNA containing homozygous transgene alleles had approximately twofold signal intensity compared with the hemizygous control. The equal intensity of an endogenous mouse fast TnT fragment in the two samples recognized by homologous hybridization with the chicken fast TnT cDNA probe served as an endogenous control for the comparable loading of the genomic DNA.

Two-dimensional gel electrophoresis. Frozen human ventricular muscle samples obtained from explanted failing hearts of anonymous heart transplant patients and anonymous nonfailing donor hearts were homogenized in SDS-PAGE sample buffer as above for Western blot analysis. This investigation was determined to be exempted research by the Cleveland Clinic Foundation Institutional Review Board. The total protein extracted from failing and nonfailing human ventricular muscle was analyzed by two-dimensional gel electrophoresis as described previously (4). The first dimension was isoelectric focusing (IEF) in Bio-Rad Lab mini tube gels containing pH 3.5–10 Ampholine (Amersham BioSciences). After electrophoresis at 350 V for 16 h and 700 V for 1 h, the IEF gel was equilibrated in SDS-PAGE sample buffer for 10 min and loaded on a 14% Laemmli slab mini gel with an acrylamide-to-bisacrylamide ratio of 180:1 for the second dimension SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250 or transferred onto nitrocellulose membrane for Western blot analysis using the anti-cardiac TnT mAb CT3 as above.

Immunofluorescence microscopy. The preparation of mouse cardiac muscle frozen sections and myofibrils for immunofluorescence microscopy was carried out as previously described (16, 24). The cardiac muscle thin sections and myofibrils were incubated with the anti-chicken fast skeletal muscle TnT mAb 6B8 or anti-cardiac TnT mAb CT3 at room temperature for 2 h. After being washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20, the samples were incubated with TRITC-conjugated anti-mouse IgG second antibody (Sigma) and washed again before being mounted on slides for exam-

ination under a Zeiss Axiovert 100H phase contrast-epifluorescence microscope.

In vivo ultrasonic imaging. Cardiac imaging analysis and functional measurements were carried out on transgenic and wild-type mice at 12 or 18 mo of age by using a Vevo 770 high-resolution in vivo imaging system (VisualSonics, Toronto, ON, Canada). As described previously (10), the mice were anesthetized with isoflurane at a concentration of 5% and then maintained at 1.5% isoflurane by a facemask during the whole procedure. Body temperature was monitored with a rectal thermometer. Hair on the precordial region was removed with a Nair lotion hair remover (Church & Dwight Canada, Mississauga, ON, Canada), and the region was covered with prewarmed ultrasound transmission gel (Aquasonic, Parker Laboratory, Fairfield, NJ). The long-axis imaging was taken to mainly visualize left ventricle (LV), right ventricle (RV), ascending aorta, and right ventricular outflow tract (RVOT) by placing the ultrasound scan head on the left parasternal position. The short-axis imaging was taken to view the LV and RV movement during diastole and systole stages by placing the scan head horizontally on the heart area. The examination of each mouse was completed in ~30 min. The data and images were saved and analyzed by the Advanced Cardiovascular Package Software for the quantification of cardiac function (VS-11560, VisualSonics). After the baseline functions were measured, a bolus of dobutamine was given intraperitoneally at 7.5 mg/kg body wt, and cardiac function was measured after a stable period of increased heart rate was observed, indicating the reach of a plateau of β-adrenergic stimulation. Three to five consecutive cardiac cycles were averaged for each measurement and 7–10 mice were examined in each group.

Doppler echocardiography analysis. Mitral pulse Doppler echocardiography was used to evaluate the systolic and diastolic function of the mouse LV. On 12- or 18-mo-old mice anesthetized as above, echocardiographic images were acquired with the use of a high-resolution (40 MHz) transducer with a digital ultrasonic system. Pulse Doppler images were collected with the apical four-chamber view to record the mitral Doppler flow spectra. The Doppler sample volume was placed at the center of the orifice and at the tip level of the valves for the highest velocities. However, for the measurement of the LV systolic and diastolic time intervals, the Doppler sample volume was moved slightly toward the LV outflow tract to intersect with both the mitral inflow and the LV outflow in the same recording. Data analysis was performed offline with the use of a customized version of Vevo 770 Analytic Software. Three to five consecutive cardiac cycles were averaged for each measurement, and 7–10 mice were examined in each group.

In vivo measurement of mouse LV pressure. To further examine the cardiac function of the transgenic mice, left ventricular pressure (LVP) and the first derivative of LVP development ($\pm dP/dt$) were measured in vivo. Eight to 10-mo-old transgenic and control mice were anesthetized with 50 mg/kg body wt ketamine and 100 mg/kg body wt inactin (35). An area corresponding to the midline of the neck and the area above the femoral vein was shaved. Mice were secured to a homeothermic blanket-warming system (Harvard, Natick, MA) to maintain body temperature at 37°C throughout the protocol. During the experimental protocol, mice were assessed for changes in heart rate, respiration rate, and LVP. The mice were monitored by response to foot pinch, and supplemental doses of inactin (25 mg/kg body wt) were given when necessary.

The trachea was exposed by a midline incision from the base of the throat to just above the clavicle. The mice were intubated with a piece of polyethylene-90 tubing. After the tube was secured in place by using a 6-0 silk suture, 100% oxygen was gently blown across the opening. This procedure has been shown previously to maintain blood gases in the normal range (35). The right carotid artery was then dissected out of the surrounding tissue. Care was taken to prevent damage to the vagal nerve. The carotid artery was cannulated with a 1.4-Fr Millar transducer tipped catheter (Millar Instruments, Houston, TX) and carefully advanced into the heart as previously described (28, 35). Once the transducer was placed in the LV chamber as monitored by a change in pressure recording, it was securely tied into place. The Millar catheter was connected to a Gould recorder (Gould Instruments, Cleveland, OH) for collection of data. The mice were allowed to stabilize for 30 min before the beginning of the protocol. During this time, the right femoral vein was cannulated using a polyethylene tubing (200 μm diameter) for the infusion of experimental drugs.

When the mice had stabilized (no change in LVP for 15 min), 30 s of data were collected for the determination of baseline parameters of cardiac function. To assess the effects of β-adrenergic stimulation on the transgenic mouse heart function, an infusion of dobutamine was given via the venous catheter for 5 min (32 $\mu g \cdot kg$ body $wt^{-1} \cdot min^{-1}$). When the response had stabilized, 30 more seconds of data were collected.

Functional measurement of isolated working mouse hearts. Cardiac function of wild-type and transgenic mice were measured at 15–18 mo of age using the Langendorff-Neely isolated working heart preparations. As described previously (2), mice were heparinized and anesthetized with pentobarbital sodium (100 mg/kg body wt ip). The thoracic cavity was opened by a transverse incision to isolate the heart. Retrograde perfusion was established in ~1 min after removal of the heart through aortic cannulation with a modified 6-mm long 18-gauge syringe needle. After we established the left atrial perfusion through pulmonary vein cannulation with another modified 18-gauge needle, the heart was switched to working mode. The hearts were perfused with Krebs-Henseleit bicarbonate buffer aerated with 95% O_2-5% CO_2 at 37°C (pH = 7.4). The buffer contents were as follows (in mM): 118 NaCl, 4.7 KCl, 2.25 $CaCl_2$, 2.25 $MgSO_4$, 1.2 KH_2PO_4, 0.32 EGTA, 25 $NaHCO_3$, and 15 D-glucose. After being calibrated by submerging its tip into saline (37°C) for 30 min and applying a static fluid pressure of 100 mmHg, a 1.4-Fr pressure transducer-tipped catheter (model SPR671; Millar Instruments) was inserted into the LV chamber via a path at the apex made by using a 27-gauge needle. Intraventricular placement of the catheter was confirmed by the systolic and diastolic pressure values recorded.

Function of the isolated working hearts was measured at constant preload of 10 mmHg and afterload of 55 mmHg on intrinsic heart rate at 37°C with no artificial pacing applied. Aortic flow and coronary effluent (as pulmonary arterial flow) were continuously monitored by drop counters. Heart rate and LVP were determined from the pressure catheter signals. The aortic pressure changes were measured by using an MLT844 pressure transducer (Capto, Horten, Norway) attached to the aortic cannula. The aortic pressure signals were amplified with a ML 110 Bridge Amplifier (AD Instruments, Colorado Springs, CO). The analog signals were sampled at 1,000 Hz by a Powerlab/16 SP digital data archiving system (AD Instruments) and stored on computer disk for subsequent analysis. Stroke volume (in μl/g heart tissue) and cardiac output (in $ml \cdot min^{-1} \cdot g$ heart $tissue^{-1}$) were calculated as the sum of aortic flow and coronary effluent, normalized to heart tissue mass and heart rate. LV developed pressure was calculated by LVP_{max} − LVEDP, where LVEDP is LV end-diastolic presure. Stroke work (in $\mu l \cdot mmHg \cdot g$ heart $tissue^{-1}$) was calculated as stroke volume × mean aortic pressure (diastolic pressure + one-third of pulse pressure). The time to peak pressure (TP) and the time for 75% total relaxation (RT_{75}) were measured from the pressure traces. As described by Gauthier et al. (12), pressure work (in $J \cdot min^{-1} \cdot g$ heart $tissue^{-1}$) was calculated as cardiac output (in $ml \cdot min^{-1} \cdot g^{-1}$) × mean aortic pressure (mmHg) × 1.33×10^{-4} $J \cdot min^{-1} \cdot g^{-1}$. Kinetic work was calculated as cardiac output (in $ml \cdot min^{-1} \cdot g$ heart $tissue^{-1}$) × perfusate specific gravity (g/cm^3)/980 $cm/s^2 \times V^2 \times 9.8 \times 10^{-3}$ $J \cdot g^{-1} \cdot m^{-1} \cdot min^{-1} \cdot g^{-1}$.

Histological examination. After mice were euthanized by cervical dislocation, hearts from the transgenic and wild-type mice were rapidly excised, rinsed in PBS, and relaxed in PBS containing 50 mM KCl. The hearts were fixed in 3.7% formaldehyde in PBS containing 50 mM KCl and embedded in paraffin after standard dehydration

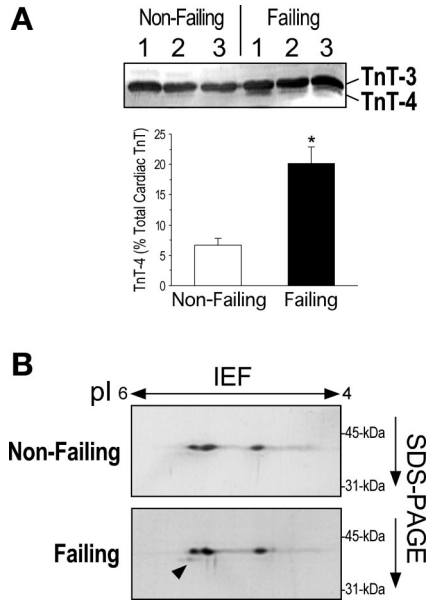

Fig. 2. Expression of an additional cardiac TnT splicing variant in failing human hearts. *A*: one-dimensional Western blot analysis using the anti-cardiac TnT monoclonal antibody (mAb) CT3 and densitometry analysis detected an increased expression of the low-molecular weight TnT-4 in the failing but not nonfailing human ventricular muscle. Data are means ± SD $*P < 0.05$. *B*: two-dimensional gel electrophoresis and Western blot analysis demonstrated the different size and charge of TnT-4 (indicated by the arrow) compared with that of the predominant TnT-3. IEF, isoelectric focusing.

procedures. Thin sections were cut and processed with hematoxylin-eosin staining at service facilities for light microscopic examination.

Data analysis. Densitometry analysis of Western blots was performed on images scanned at 600 dpi using the NIH Image 1.61 software. All functional analyses of the transgenic and wild-type mice were performed in blinded settings. Quantitative data were documented as means ± SD or SE. The statistical significance of differences between the mean values was analyzed by Student's *t*-test or one-way ANOVA.

RESULTS

Heterogeneity of cardiac TnT in failing human hearts. The normal adult human ventricular muscle primarily expresses a single cardiac TnT isoform (TnT-3). The expression of a lower molecular weight cardiac TnT isoform (TnT-4) resulting from NH_2-terminal alternative splicing was reported in hypertrophic and failing hearts (1, 29). Consistently, the Western blots and densitometry analysis in Fig. 2*A* detected the expression of TnT-4 in three samples of failing adult human hearts but not the nonfailing human ventricular muscle controls. The results of the two-dimensional gel electrophoresis Western blotting in Fig. 2*B* further demonstrated the upregulated expression of cardiac TnT-4 in the failing human cardiac muscle with lower molecular weight and a shift of the isoelectric point to the basic side. Cardiac TnT-3 and TnT-4 have shown a detectable functional difference (13). The coexpression of exon 4-excluded cardiac TnT-4 with TnT-3 in human failing heart supports a pathological correlation with myocardial TnT heterogeneity.

Incorporation of both cardiac and fast skeletal muscle TnTs in the transgenic mouse cardiac myofilaments. Driven by the α-MHC promoter, the chicken fast skeletal muscle TnT was successfully expressed in the adult cardiac muscle of transgenic mice and incorporated into the myofibrils (Fig. 3), similar to that previously observed in hemizygous lines (16). The total level of TnT in the transgenic mouse cardiac muscle did not show a detectable change, and the level of endogenous cardiac TnT was lowered in the transgenic hearts, reflecting a competitive incorporation of fast TnT in the myofilament and degradation of unincorporated TnT in the myocytes (43). In contrast to the nearly complete replacement of the endogenous protein seen in transgenic mouse hearts overexpressing β-tropomyosin (31) or slow skeletal muscle TnI (11), both were controlled by the α-myosin heavy chain (α-MHC) promoter used in our study, the expression of endogenous cardiac TnT remained at significant levels in the transgenic mouse heart overexpressing chicken fast skeletal muscle TnT. This outcome produced an experimental model of TnT heterogeneity by constituting an adult cardiac muscle that contains two significantly different classes of TnT in the thin filament regulatory system.

The incorporation of chicken fast skeletal muscle TnT into the transgenic mouse cardiac sarcomere was further demonstrated by immunofluorescence microscopy using the specific anti-chicken fast TnT mAb 6B8 (Fig. 4). The sarcomeres of the transgenic mouse cardiac myofibril were normal in appearance. The 6B8 mAb fluorescence staining detected a uniform expression of chicken fast TnT in the transgenic mouse cardiomyocytes (Fig. 4*A*) and demonstrated a normal I-band localization of the fast TnT in the cardiac sarcomeres (Fig. 4*B*). The 6B8 mAb staining pattern on the transgenic mouse cardiac myofibril was similar to that of the anti-cardiac TnT mAb CT3 (Fig. 4*B*), demonstrating a native incorporation of the fast TnT into the transgenic mouse cardiac muscle thin filaments.

Chronic development of myocardial hypertrophy in the transgenic mice. The heart weight-to-body weight ratios of the two age groups of the transgenic and wild-type mice are shown in Table 1. Although the relative heart weight was not significantly different between the younger transgenic and wild-type mice, the results showed an increased cardiac muscle mass in the older transgenic mice compared with the age-matched wild-type controls, indicating an age-progressive development of myocardial hypertrophy.

Decreased cardiac function in transgenic mice overexpressing fast TnT detected in vivo under noninvasive conditions. Cardiac function of the transgenic mice was evaluated noninvasively in vivo using ultrasonic imaging and Doppler echocardiography. Figure 5 shows representative recordings. The

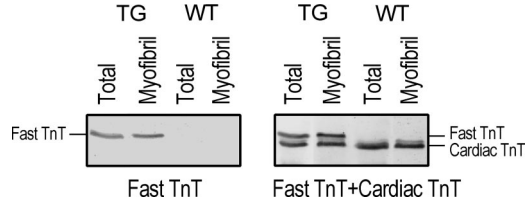

Fig. 3. Co-expression of cardiac and fast skeletal muscle TnTs in transgenic (TG) mouse heart. Total protein extracts from ventricular muscle and isolated cardiac myofibrils of TG and wild-type mouse (WT) hearts were examined by SDS-PAGE and Western blott analysis using a mAb against chicken breast muscle TnT (6B8) alone (*left*) or mixed with an anti-cardiac TnT mAb (CT3) (*right*). The results showed that the transgene-encoded fast TnT (fTnT) and the endogenous cardiac TnT (cTnT) are both expressed at significant levels in the TG mouse heart and incorporated proportionally into the cardiac myofilaments.

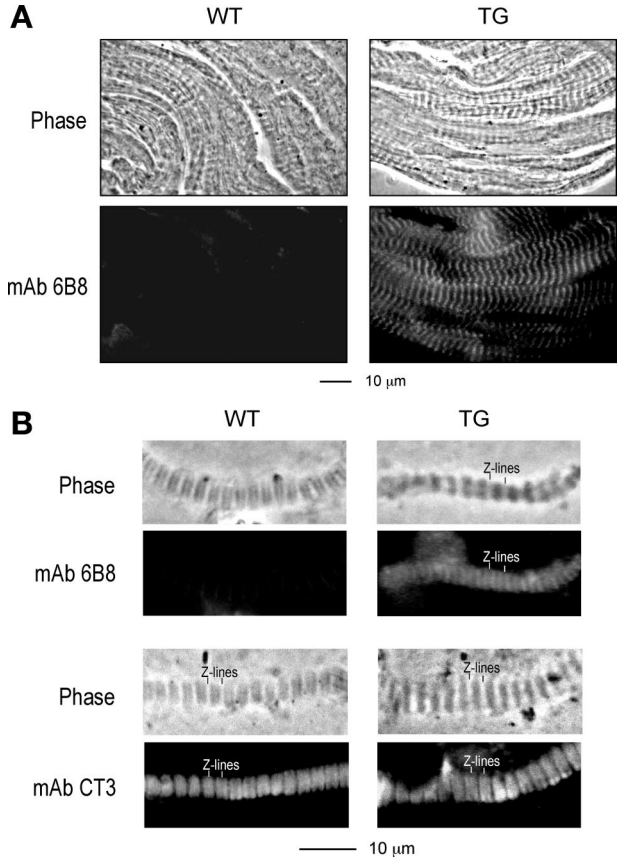

Fig. 4. Incorporation of chicken fast skeletal muscle TnT in transgenic mouse cardiac myofilaments. Phase-contrast and immunofluorescence microscopy was carried out on ventricular sections and myofibrils from the TG and WT mice. *A*: fluorescence staining of anti-chicken fast skeletal muscle TnT-specific mAb 6B8 on thin sections demonstrates a uniform expression of the exogenous fast TnT in the TG mouse cardiac muscle. *B*: mAb 6B8 staining on isolated myofibrils showed an I-band localization of the fast TnT in the TG mouse cardiac sarcomeres similar to the staining pattern of the anti-cardiac TnT mAb CT3.

results of functional analysis are summarized in Table 2. The long-axis and short-axis B-mode data showed no significant difference in the thickness of LV posterior wall between the 12-mo-old transgenic mice and age-matched controls. No difference was detected between the 12- and 18-mo-old transgenic groups. The LV end-diastolic dimension (EDD) did not show significant change. However, ejection fraction (EF) and fractional shortening (FS) were significantly decreased in the transgenic mice compared with wild-type controls, indicating a reduced LV systolic function. Furthermore, the end-systolic dimension (ESD) was increased significantly in the 18-mo-old transgenic mice compared with the 12-mo-old transgenic and control groups. β-Adrenergic stimulation improved EF in all three groups, but the levels in the transgenic mice remained significantly lower than that in the wild-type controls (Table 2).

Doppler echocardiography showed that the wild-type and transgenic mice both had a higher early ventricular filling wave (E wave) and a lower late filling wave (A wave) caused by atrial contraction. The diastolic filling time (DT) did not change significantly in the transgenic mice. However, isovolumic contraction time (IVCT) and isovolumic relaxation time (IVRT) were increased significantly in the transgenic mice compared with that in wild-type controls.

Decreased cardiac function in the transgenic mice measured in vivo by LV catheterization. The functional effect of overexpressing fast TnT in transgenic mouse cardiac muscle on myocardial function was evaluated in vivo by directly measuring LVP development in lightly anesthetized mice 8–10 mo of age. The results are summarized in Table 3. The anesthetization of mice by ketamine-inactin provided a heart rate of 400–550 beats/min. The baseline function data showed that the in vivo heart rate and the rates of LV contraction and relaxation ($+dP/dt$ and $-dP/dt$, respectively) of the transgenic mice were not significantly different from that of the wild-type mice. However, the maximum LVP was significantly decreased in the transgenic mice when compared with the control (Table 3), further indicating decreased systolic function. Whereas dobutamine treatment showed positive β-adrenergic effects on both wild-type and transgenic mouse cardiac function, the transgenic mouse hearts had a significantly lower response in the $+dP/dt$ (Table 3).

Decreased cardiac function measured ex vivo in working heart preparations. Although in vivo measurement of cardiac function best reflects the physiological conditions, neurohumoral regulation and vascular compensation may minimize the effects of changes in cardiac function. Therefore, isolated working heart preparations were analyzed to further characterize the effects of myocardial overexpression of fast TnT on cardiac function excluding the influences from systemic regulation and compensation. The experiments were performed in 15- to 18-mo-old mice, and intrinsic heart rates of over 300 beats/min were obtained. The heart rates were lower than that obtained from 5- to 6-mo-old mice under identical conditions (2) and may reflect an aging-related effect. Nonetheless, the heart rates were stable and near the in vivo range, validating the heart preparations.

Cardiac function was measured for the time segments illustrated in Fig. 6. The results in Table 4 showed that the stroke volume and cardiac output of the transgenic hearts were significantly lower than that of the wild-type hearts. The trans-

Table 1. *Age-progressive cardiac hypertrophy*

	8–10 Mo Old		15–18 Mo Old	
Parameters	WT (n = 11)	TG (n = 14)	WT (n = 8)	TG (n = 5)
Body weight, g	31.37±1.98	33.03±1.99	41.93±1.88*	39.1±1.96†
Heart weight, mg	161.45±8.70	177.14±8.95	204.75±13.18*	240.6±21.62*
HW/BW, mg/g	5.35±0.47	5.65±0.54	4.90±0.25	6.11±0.28‡

Data are means ± SE. WT, wild type; TG, transgenic; HW, heart weight; BW, body weight. Results showed an age progressive increase in heart mass relative to the body weight, indicating chronic myocardial hypertrophy. *$P < 0.01$ vs. 8- to 10-mo-old controls; †$P < 0.05$ vs. 8- to 10-mo-old controls; ‡$P < 0.01$ vs. the age-matched (15–18 mo old) WT controls.

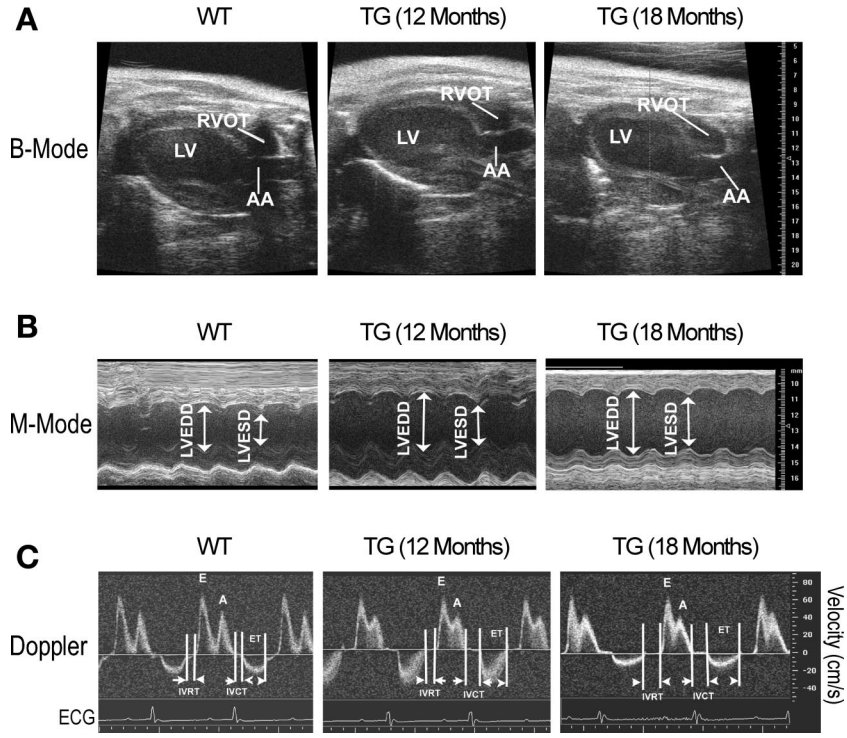

Fig. 5. Echocardiographic analysis of mouse cardiac function in vivo. *A*: two-dimensional B-mode imaging. Representative ultrasound images from left parasternal long-axis view on WT and two age groups of TG mice at the end-diastolic stage showed the structures from the left ventricle (LV) to the ascending aorta (AA) and the right ventricular outflow tract (RVOT). *B*: anatomic M-mode imaging: Representative recordings demonstrate the measurements of LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD). Results showed increased LVEDD and LVESD in the TG versus WT control, indicating decreased cardiac function. *C*: Doppler flow spectral analysis. Representative Doppler flow spectra were obtained from the mitral orifice. The results showed that the WT and TG mice both had a higher early ventricular filling wave (E wave) and a lower late filling wave (from atrial contraction) (A wave). Isovolumic contraction time (IVCT), ejection time (ET), and isovolumic relaxation time (IVRT) were measured to evaluate the change in cardiac function. Electrocardiograph (ECG) was recorded simultaneously to outline the cardiac cycles.

genic mouse hearts also showed decreased LV developed pressure compared with that of the wild-type control. Consistently, stroke work and pressure work were lower in the transgenic mouse hearts than that in the wild-type hearts. $+dP/dt$ and $-dP/dt$ were both lower in the transgenic than that in the wild-type hearts, indicating slower rates of contraction and relaxation, which resulted in decreased kinetic work (Table 4).

Myocardial degeneration in the transgenic mouse hearts. Histological examination of the transgenic mouse cardiac muscle showed that whereas the 3-mo-old transgenic mouse did not show significant morphological changes, the 15-mo-old transgenic hearts showed a sign of LV dilation (Fig. 7*A*). Whereas histological changes were not detected in the young and most old transgenic mouse heart samples (Fig. 7*A*), fibrosis was reproducibly observed in old transgenic mice (over 12 mo of age) compared with similarly aged wild-type controls (Fig. 7*B*). The results indicate myocardial degeneration as a consequence of the chronic presence of two classes of TnT in the cardiac muscle. Under nonstress conditions, the young transgenic mice were apparently normal in daily life activities and had similar body weight to the wild-type controls. The 15- to 18-mo-old groups showed that the transgenic mice had higher body weight than that of the similarly aged controls (Table 1). A possible contribution from cardiac insufficiency-related inactivity is worth investigating, whereas a greater appetite could not be excluded.

DISCUSSION

To investigate the effect of TnT heterogeneity on cardiac function, we characterized transgenic mouse hearts overex-

Table 2. *Echocardiographic measurements of cardiac function*

Parameters Analyzed	Wild Type (12 mo) (n = 9)	Transgenic (12 mo) (n = 5)	Transgenic (18 mo) (n = 7)
Isovolumic contraction time, ms	20.1±2.6	30.3±1.7[b]	23.6±2.3[e]
Ejection time, ms	61.8±4.1	62.0±5.5	68.2±2.2[c]
Isovolumic relaxation time, ms	19.8±2.5	23.5±1.8	22.9±1.9
Diastolic filling time, ms	77.4±9.0	71.3±7.0	73.3±6.3
Mitral valve E wave-to A wave ratio	1.32±0.1	1.42±0.4	2.17±0.4[c]
End-diastolic LV posterior wall thickness, mm	0.84±0.1	0.80±0.1	0.67±0.1
End-systolic LV posterior wall thickness, mm	1.09±0.1	0.90±0.1	1.14±0.2
End-diastolic dimension, mm	3.67±0.5	3.72±0.3	3.96±0.2
End-systolic dimension, mm	2.36±0.4	2.77±0.5	3.11±0.3[d]
Fraction of shortening, %	34.1±5.8	25.7±7.1	21.4±4.4[d]
Baseline ejection fraction, %	63.2±7.9	46.4±13[a]	43.5±7.6[d]
Dobutamine-stimulated ejection fraction, %	85.8±3.3[f]	55.5±1.5[g]	64.5±3.5[g]

Data are means ± SE. LV, Left ventricle. Results demonstrate age-progressive decreases in cardiac function as reflected by changes in both systolic and diastolic parameters. The systolic malfunction could not be completely corrected by dobutamine stimulation. LV dilation was detected in the 18-mo-old transgenic mice. [a]$P < 0.05$ and [b]$P < 0.01$ vs. wild-type control. [c]$P < 0.05$ and [d]$P < 0.01$ vs 12-mo-old transgenic and wild-type controls. [e]$P < 0.05$ vs. 12-mo-old wild-type control. [f]$P < 0.001$ vs. baseline level; [g]$P < 0.001$ vs. the wild-type group under the same treatment.

Table 3. *In vivo cardiac function*

Dobutamine-Induced Changes	Baseline Parameters		Dobutamine-Induced Changes, %	
	Wild Type (n = 5)	Transgenic (n = 10)	Wild Type (n = 5)	Transgenic (n = 10)
Heart rate, beats/min	450±12	527±25	+18.6±3.0	+16.3±3.4
LVP_{max}, mmHg	142.6±10.3	116.3±6.9*	+10.8±4.0	+23.0±5.7
LVP_{min}, mmHg	3.8±1.5	4.55±6.3	NA	NA
+dP/dt	8,238±762	9,575±781	+80.6±9.2	+50.9±7.0*
−dP/dt	8,450±200	8,150±601	+43.2±10.6	+41.5±9.1

Values are means ± SE. Mice were 8–10 mo old. LVPmax, maximal LV pressure; LVPmin, minimal LV pressure; ±dP/dt, pressure development over time; NA, not analyzed. Results indicate decreased systolic function with reduced response to β-adrenergic stimulation. *$P < 0.05$ vs. wild-type control.

pressing fast TnT as an experimental model of cardiac muscle containing two classes of nonmutant TnT. The in vivo and ex vivo measurements demonstrated that the chronic presence of two classes of TnT in the transgenic mouse cardiac muscle decreased heart function in an age-progressive manner. The changes cannot be corrected by β-adrenergic stimulation, suggesting a decreased functional potential in the cardiac muscle. The hypothesis that TnT heterogeneity has a negative effect on cardiac function is supported by the following observations.

Effects of TnT isoforms on muscle contractility. The structure-function relationship of TnT is the basis for understanding the functional effects of cardiac TnT heterogeneity. Cardiac and skeletal muscle TnTs are conserved homologous proteins. Their main structural difference is in the NH_2-terminal region that is a modulator for the conformation and function of TnT (3, 25, 42). Troponin T isoforms different in the NH_2-terminal region have been found to produce different sensitivity of the myofilaments to Ca^{2+} activation (7, 32). Reconstituted thin filaments or muscle strips containing alternatively spliced cardiac TnT isoforms showed differences in the activation of actomyosin ATPase (38) and force development (13). The presence of different TnT isoforms may also have an indirect effect on cardiac muscle function. For example, the expression of fast TnT in the transgenic mouse cardiac muscle has been shown to affect TnI phosphorylation, reducing the β-adrenergic potential of the cardiac muscle (30). These evidences indicate that TnT isoform contents affect the Ca^{2+} activation of muscle contraction.

Consistently, the incorporation of fast skeletal muscle TnT into the transgenic mouse cardiac muscle altered the overall contractility (16). Unlike the protective effect of overexpression slow TnI in the adult transgenic mouse heart to completely replace cardiac TnI (40), the decreased cardiac function due to the presence of fast TnT together with cardiac TnT provides an evidence for the negative effect of co-presence of two functionally different TnT isoforms in the thin filament regulatory system.

TnT heterogeneity interrupts the synchronized action of cardiac muscle and decreases cardiac function. In contrast to the skeletal muscles that normally contain mixed fast and slow fibers (7), the contractile cardiac myocytes in the adult ventricles are homogenous in cell type. The myocardium is known to be an electrophysiological syncytium that undergoes near uniformed membrane action potential changes during cardiac cycles (9). This synchronized activation of the cardiac muscle is critical to the effectiveness of the rhythmic pumping function of the heart. Beyond the electrical syncytium at the organ level, it is logical to propose that the activation of contraction and relaxation downstream of the depolarization of plasma membrane; i.e., the activation of thin filaments by the rising of intracellular Ca^{2+}, would also need to be synchronized. Con-

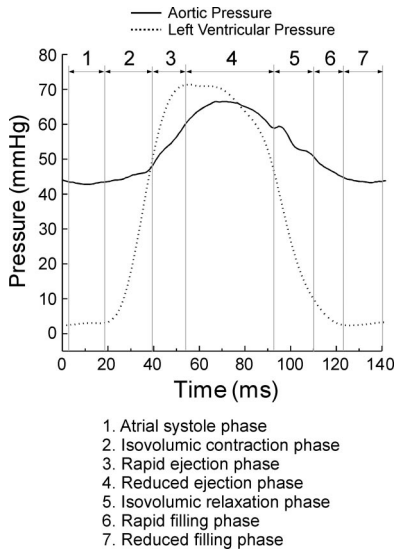

Fig. 6. Ex vivo measurement of mouse cardiac function. Isolated working heart preparations were examined to evaluate cardiac function of the TG mice in the absence of neurohumoral influences. LVP and aortic pressure developments were simultaneously recorded using an F1.4 Milar catheter and a pressure transducer, respectively. Representative traces for the LVP and aortic pressure are shown. The segments of the pressure curves used in the analysis of cardiac function are outlined.

Table 4. *Ex vivo cardiac function*

Parameters	Wild Type (n = 4)	Transgenic (n = 5)
Heart rate	300±19	315±13
Stroke volume, μl/g	118±8	84±4†
Cardiac output, ml·min^{-1}·g^{-1}	34.9±2.8	26.8±1.7*
Mean aortic pressure, mmHg	50.8±0.4	51.3±0.4
LV developed pressure, mmHg	68.1±1.5	61.4±1.6*
Stroke work, ml·mmHg·g^{-1}	5.93±0.42	4.35±0.20†
Pressure work, J·min^{-1}·g^{-1}	0.24±0.02	0.18±0.01*
LV +dP/dt, mmHg/s	3,592±38	2,989±156*
LV −dP/dt, mmHg/s	3,358±108	2,708±95†
Time to peak LVP, ms	33.7±1.1	38.3±1.8
Time to 75% LV relaxation, ms	39.1±2.0	39.8±1.7
Kinetic work, J·min^{-1}·g^{-1}	1.35±0.16	0.85±0.04*

Values are means ± SE. Data were collected from hearts isolated from 15- to 18-mo-old mice. *$P < 0.05$, †$P < 0.01$ vs. wild-type control. Results indicate decreased intrinsic cardiac function in the absence of systemic neurohumoral influences and vascular compensation.

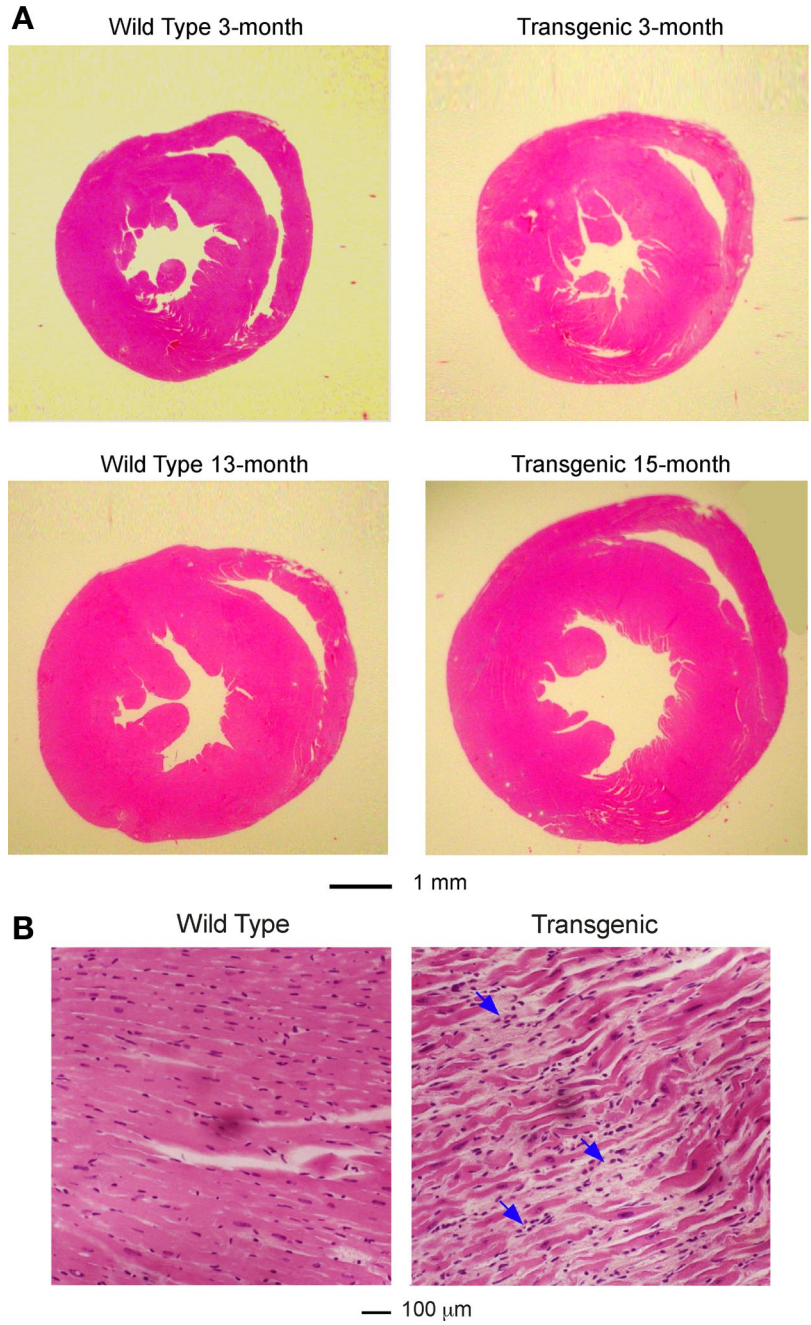

Fig. 7. Ventricular morphology and myocardial degeneration in TG mouse heart overexpressing fast skeletal muscle TnT. *A*: hematoxylin and eosin staining of cross ventricular sections detected LV enlargement in the old (15 mo) but not young (3 mo) TG mouse hearts, consistent with the heart weight-to-body weight ration measurements (Table 1). *B*: hematoxylin and eosin-stained cross sections of LV free wall of representative old WT (13 mo) and TG (15 mo) mice detected fibrosis (the arrows) in the TG mouse myocardium overexpressing fast skeletal muscle TnT as a result of myocardial degeneration.

sistently, the adult cardiac muscle normally expresses only one type of tropomyosin, TnI, TnC, and TnT isoforms.

In our experimental system, the exogenous fast TnT was uniformly expressed in the transgenic mouse ventricular myocytes and incorporated into myofilaments (Fig. 4). The integration of the endogenous cardiac TnT and the transgene-encoded fast TnT in the cardiac muscle thin filaments would produce two classes of troponins slightly differing in their Ca^{2+} responses, and this protein level heterogeneity in the transgenic cardiac muscle would disrupt the normally synchronized action of the myocardium. Indeed, our functional characterization supports that heterogeneity at the thin filament level is sufficient to interfere with the synchrony of cardiac muscle function.

We have shown previously that cardiac muscle preparations from the same lines of transgenic mice had altered force-pCa curves in which the expression of fast TnT resulted in a high cooperativity (16). During the activation and relaxation of cardiac muscle, the two classes of troponin will be activated at slightly different Ca^{2+} concentrations. Compared with the normal cardiac thin filament containing only one class of TnT, the thin filament containing two different TnTs will be activated over a wider range of Ca^{2+} concentrations due to the only partial overlapping of two different TnT activation curves, corresponding to a widened time period. This desynchronization-caused lagging effect will lower the contractile force of the cardiac muscle at peak activation, corresponding to a decreased peak force output of the ventricular muscle. The

peak ventricular pressure is critical for the heart to eject blood against the threshold of arterial pressure to produce cardiac work. A flattened ventricular pressure curve would severely decrease cardiac output. Therefore, this mechanism renders the TnT heterogeneity a dominant negative effect on cardiac function as shown by the decreased systolic function of the transgenic mouse hearts (Tables 3 and 4).

The decreased pump function due to myocardial TnT heterogeneity is also harmful to the heart by chronically causing hypertrophy and myocardial degeneration. Although there is a transient period during perinatal heart development in which both embryonic and adult cardiac TnTs are expressed (18, 23), this period is short and unlikely to produce the chronic negative effects. Transgenic mouse heart containing <5% of a COOH-terminal truncated cardiac TnT that is known to cause human familial hypertrophic cardiomyopathy showed neonatal lethality (37). This highly dominant effect of a very small proportion of the mutant TnT present in the contractile machinery indicates that the loss of function might not be the sole pathogenic factor, supporting the hypothesis that heterogeneity of the thin filament regulatory system could result in significant negative effect on cardiac function. This mechanism may also confer a negative effect when the small amount of cardiac TnT-4 is expressed in the hypertrophic and failing hearts (Fig. 2), although it might have originally expressed as a compensatory adaptation.

It is worth noting that the adult bovine cardiac muscle "normally" expresses two alternatively spliced cardiac TnT differing by the inclusion or exclusion of four amino acids in the NH_2-terminal region encoded by exon 4. Although functional difference was detected between the two bovine cardiac TnT (38), this difference is significantly smaller than that produced by the alternative splicing of exons 7 (8), 6, and/or 5 as seen in dilated cardiomyopathy turkeys and dogs (4, 5). Although domestic bovines are not reported for a high instance of heart failure in their limited life spans, it cannot be precluded that the presence of two classes of TnT in adult bovine myocardium can be completely tolerated in older individuals to compare with the midlife on set of most human cardiomyopathies. In addition to the hypothesis that the slow rate of bovine heart might grant a higher tolerance to the desynchronizing effect of contractile heterogeneity, the extent of TnT heterogeneity in the cardiac muscle may be a key factor to determine the functional outcome and penetration of disease phenotype. Our approach of expressing a chicken fast TnT in mouse heart served as an extreme example to experimentally demonstrate the negative impact of nonmutant TnT heterogeneity on cardiac muscle function.

Functional value of TnT heterogeneity. As mentioned above, skeletal muscles normally contain mixed fibers with fast and slow TnT and multiple alternatively spliced isoforms (7, 20, 41). In contrast to the rhythmic contraction of cardiac muscle, skeletal muscle act by various degrees of tetanic contraction that is fused from multiple twitch contractions. Whereas a synchronized peak contraction would be most effective in the cardiac function of pumping blood against the afterload, less synchronized wider twitch contraction would more readily fuse to produce functional tetanic force in skeletal muscles. Therefore, the different evolutions of cardiac and skeletal muscle TnT isoform expression actually reflect the functional nature of the two types of striated muscles.

Nonetheless, a potentially functional value of TnT heterogeneity that confers a prolonged contractile peak force of the ventricular muscle may exist to underlie the evolutionary fixation of chronic pathogenic cardiac TnT alternative splicing pathways in several avian and mammalian species (4, 5). A likely hypothesis is that the longer duration of peak contraction could increase cardiac output by elongating the ejection time. However, this advantage would be based on a nonproportionally higher energetic cost leading to the chronic development of hypertrophy and degeneration. This short-term benefit in young adult animals may confer a reproductive selection value for the fixation of these genetic variations, whereas the natural selection against postreproductive individuals through the chronic development of cardiomyopathy and heart failure reduced the competition for resources to add to the selection value.

In summary, the present study demonstrated that the coexpression of two classes of TnT in transgenic mouse heart to generate heterogeneity in the cardiac thin filament regulatory system produced decreased cardiac function, myocardial hypertrophy, and degeneration. The results support a hypothesis that the presence of more than one classes of TnT in adult ventricular muscle causes heterogeneity in the Ca^{2+} response and desynchronizes the myocardial contraction with chronically pathological effects.

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