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Correcting diastolic dysfunction by Ca²⁺ desensitizing troponin in a transgenic mouse model of restrictive cardiomyopathy

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Abstract

Several cardiac troponin I (cTnI) mutations are associated with restrictive cardiomyopathy (RCM) in humans. We have created transgenic mice (cTnI^{193His} mice) that express the corresponding human RCM R192H mutation. Phenotype of this RCM animal model includes restrictive ventricles, biatrial enlargement and sudden cardiac death, which are similar to those observed in RCM patients carrying the same cTnI mutation. In the present study, we modified the overall cTnI in cardiac muscle by crossing cTnI^{193His} mice with transgenic mice expressing an N-terminal truncated cTnI (cTnI-ND) that enhances relaxation. Protein analyses determined that wild type cTnI was replaced by cTnI-ND in the heart of double transgenic mice (Double TG), which express only cTnI-ND and cTnI R193H in cardiac myocytes. The presence of cTnI-ND effectively rescued the lethal phenotype of RCM mice by reducing the mortality rate. Cardiac function was significantly improved in Double TG mice when measured by echocardiography. The hypersensitivity to Ca²⁺ and the prolonged relaxation of RCM cTnI^{193His} cardiac myocytes were completely reversed by the presence of cTnI-ND in RCM hearts. The results demonstrate that myofibril hypersensitivity to Ca²⁺ is a key mechanism that causes impaired relaxation in RCM cTnI mutant hearts and Ca²⁺ desensitization by cTnI-ND can correct diastolic dysfunction and rescue the RCM phenotypes, suggesting that Ca²⁺ desensitization in myofibrils is a therapeutic option for treatment of diastolic dysfunction without interventions directed at the systemic β -adrenergic-PKA pathways.

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Keywords

Troponin; diastolic dysfunction; RCM; transgenic mice; Ca²⁺ desensitization

Restrictive cardiomyopathy (RCM) along with hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are three major types of myocardial diseases that may lead to progressive heart failure and even death. Among these types of cardiomyopathies, RCM is characterized by the restriction of the amount of blood that can fill the heart. The cardiac myocytes, presenting such dysfunction, are abnormally stiffened due to unknown etiology. Although RCM is not as common as that of HCM or DCM, its prognosis is poor [1] and many patients suffering from this disease die in their childhood [1-3]. The clinical features of RCM are described as biatrial dilation, along with a restricted left ventricle characterized on echocardiography. An elevation of left ventricular end-diastolic pressure with a restricted left ventricular filling is often observed in RCM patients. Most RCM cases are described as idiopathic, i.e. etiology is unknown [4-5].

Recent studies have revealed that RCM is associated with mutations of cardiac Z-line located proteins such as desmin [6] and the thin filament proteins such as cardiac troponin I (cTnI) [7], and troponin T [8]. To date most of the RCM mutations in cTnI have not been investigated in animal models, though they have been characterized in *in vitro* functional studies [9-14]. The data from the *in vitro* reconstituted thin filament assays showed that the RCM cTnI mutations had much greater Ca²⁺-sensitizing effects on force generation.

Recently, the transgenic mouse cTnI^{193His}, corresponding to the human RCM mutant cTnI R192H, has been characterized in our laboratory. The cTnI^{193His} mice present neither significant cardiac hypertrophy nor ventricular dilation. However, they display a phenotype similar to that in human RCM patients carrying the same mutation, which is characterized morphologically by enlarged atria and restricted ventricles and functionally by diastolic dysfunction and diastolic heart failure [15-16].

A physiologically occurring restrictive cleavage of cTnI at its N-terminus (cTnI-ND) has been found to play a role in myocardial adaptation to stress conditions [17]. cTnI-ND, originally discovered in simulated micro-gravity, is found at low levels in normal hearts of all species examined, indicating a broad physiological significance, and is up-regulated under hemodynamic stress [17] and heart failure [18]. To support the novel hypothesis that cTnI-ND is an effect of regulation of myocardial function rather than structural destruction, we recently expressed cTnI-ND in the absence of endogenous cTnI by crossing cTnI-ND transgenic mice [19] with cTnI knockout (cTnI-KO) mice [20]. The transgenic mice (cTnI-ND mice) that contain 100% cTnI-ND in cardiac muscle survived to adulthood with normal baseline life activities [21]. Functional studies using *ex vivo* working heart preparations demonstrated that the cTnI-ND transgenic mouse hearts have desensitized myofibrils to Ca²⁺ and enhanced diastolic function [18-19].

In the present study, we modified the overall cTnI function in RCM cardiac muscle by crossing cTnI^{193His} mice with cTnI-ND mice. Phenotype characterization of the double transgenic mice (Double TG) that express only cTnI-ND and cTnI R193H in the heart

indicated that the presence of cTnI-ND significantly reduced the mortality rate of RCM mice. The hypersensitivity to Ca^{2+} and the prolonged sarcomere relaxation of cTnI R193H myofibrils were completely reversed by the presence of cTnI-ND in RCM hearts. The results demonstrated that Ca^{2+} desensitization by cTnI-ND could correct diastolic dysfunction and rescue the RCM phenotype.

Materials and Methods

This investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996) and was in accordance with the protocols approved by the Institutional Animal Care and Use Committees at Florida Atlantic University.

Transgenic Animals

By crossing cTnI^{193His} with cTnI-KO mice, we produced heterozygous cTnI-KO mice expressing cTnI R193H. Then we crossed these heterozygous cTnI-KO mice containing cTnI R193H with cTnI-ND mice, we obtained double transgenic mice (Double TG) expressing only cTnI-ND and cTnI R193H in cardiac muscle. Two single TG mouse lines (cTnI^{193His} and cTnI-ND) and double transgenic mice (Double-TG expressing cTnI-ND and cTnI R193H) as well as wild type C57BL/6 mice (WT) were used in the study.

Analysis of transcriptional expression and protein replacement

Real-time RT-PCR was performed to determine the cTnI R193H transgene transcriptional levels in WT, cTnI193His single TG, cTnI-ND and double TG mouse hearts using the methods described previously [15]. Transcriptional level of β -actin was used as a control. Cardiac myofibril proteins were examined on SDS-PAGE and Western blotting using two mouse monoclonal antibodies (mAb) TnI-1 against a C-terminal epitope involving R193 and 4H6 recognizing an epitope in the middle region of cTnI polypeptide chain as previously described [16]. The cTnI R193H mutation destroyed the TnI-1 epitope but not the 4H6 epitope, allowing the distinction between cTnI WT and R193H. Both of the two mAbs recognize cTnI-ND. For Western blotting, whole cardiac muscle was homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS to extract myofilament proteins. The samples were resolved by 14% Laemmli SDS-PAGE with an acrylamide:bisacrylamide ratio of 180:1 using a Bio-Rad mini-Protean II system, 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 in Tris-buffered saline and incubated with mAbs diluted in TBS containing 0.1% bovine serum albumin. Antibodies on immunoblots were visualized by enhanced chemiluminescence (ECL detection kit from GE Healthcare). Cardiac TnT (cTnT) was used as an internal control to normalize the protein sample loading. For quantifications, the protein bands were scanned by densitometry and compared among the samples on the same blot.

Western blotting assays were conducted to detect SR Ca^{2+} ATPase pump (SERCA), phospholamban (PLB) and the S16 phosphorylated phospholamban (phospho-PLB) levels in cardiac myocytes isolated from WT, cTnI^{193His}, cTnI-ND and Double TG mice. The

monoclonal antibodies against SERCA2 (1:1500 in dilution) and phospholamban (1:2000 in dilution) and polyclonal antibody against phospho S16 phospholamban (1:750 in dilution) (Abcam Inc., Cambridge, MA) were used and similar procedure was followed as described previously, except that BioTrace PVDF transfer membranes (Pall Life Sciences) were used for SERCA protein transfer in Western blotting assays. Cardiac TnT (cTnT) antibody (CT3, 1:15000 in dilution) was obtained from Dr. Jin laboratory and was used as an internal control for protein sample loading.

Cardiac morphology and histological examinations

For light microscopy, hearts were quickly excised after euthanasia and immersed in 10% formaldehyde solution. The fixed hearts were sectioned into 10 μ m thick slices, stained with hematoxylin and eosin, and then viewed under an Olympus IX71 microscope. Gomori's trichrome staining was also performed to detect fibrosis in the sections.

Cardiac function measurements

Echocardiography measurements on adult mice were performed using a Vevo 770 High-Resolution echocardiograph (VisualSonics, Toronto, ON, Canada) as described previously [15, 16, 22] with the standards established for human echocardiography [7, 23, 24].

Cardiomyocyte isolation and contractility measurements

Mouse cardiomyocytes were isolated using a Langendorff Perfusion Cell Isolation System (ac-7034, Cellutron Life Technology, Baltimore, MD) as described previously [16]. Only the rod-shape cells with clear edges and well-defined sarcomere structure and without sarcolemmal blebs or spontaneous contractions were used for experiments. Mechanical properties of cardiomyocytes were determined using an IonOptix Myocam system (IonOptix Inc., Milton, MA). Cardiomyocytes were loaded into the perfusion chamber mounted on the microscope stage and superfused (1 ml/min, 37°C) with Tyrode solution containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES, at pH 7.4. The cells were field stimulated at 8 volts at a frequency of 0.5 Hz. Sarcomere shortening was measured and 10 contraction cycles were used for analysis. For physiological stress tests, the stimulation frequency was varied from 0.5 to 2 Hz.

Measurement of calcium transients

Freshly Isolated mouse cardiac myocytes were loaded with 2 μ M of Fura-2 AM (Molecular Probes) at room temperature for 20 minutes in Tyrode solution containing (in mM): 131 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 Glucose, 10 HEPES, at pH 7.4 with 1% BSA and 0.02% pluronic F-127 (Molecular Probes). After 30-minute of de-esterification of fura-2 AM, the cells were superfused in Tyrode solution and electrically stimulated at 8 volts at a frequency of 0.5 Hz. For measurement of Ca²⁺ transients, the cells were excited by UV light (360 and 380 nm, alternately). The fura-2 emission at 510 nm was collected with a photomultiplier tube. The kinetics of Ca²⁺ transients was analyzed in conjunction with myocyte mechanical measurements. 10 mM caffeine was used to determine the functional SR Ca²⁺ load in myocardial cells.

Functional studies in skinned papillary muscle fibers

Mouse papillary muscles (~1.2 mm long and 70-100 μm in diameter) were dissected from the left ventricle of freshly excised mouse hearts in relaxing solution and then skinned in relaxing solution containing 1% (vol/vol) Triton X-100 at room temperature for 30 minutes. The skinned fibers were placed in a quartz cuvette and mounted in the Guth Muscle Research System (the sarcomere length was 2.1 μm), and then gradually exposed to solutions with increasing Ca^{2+} concentrations (pCa 8.0–4.0). Data were fitted using the following equation: % change in force = $100 \times [\text{Ca}^{2+}]^n / ([\text{Ca}^{2+}]^n + [\text{Ca}^{2+}_{50}]^n)$, where $[\text{Ca}^{2+}_{50}]$ is the free $[\text{Ca}^{2+}]$ that produces 50% force and n is the Hill coefficient.

Results

By crossing the heterozygous cTnI-KO mice containing cTnI R193H with cTnI-ND mice, we obtained double transgenic mice (Double TG) expressing only cTnI-ND and cTnI R193H in cardiac muscle. PCR-based genotyping screening showed that no wild type cTnI expression was detected in the cardiac muscles of double TG mice (not shown). Real-time RT-PCR data indicated that the transcriptional expression of cTnI R193H transgene were observed only in cTnI^{193His} single TG and double TG mouse hearts, not in WT or cTnI-ND mouse hearts (Figure 1A). The transcriptional levels of actin were very similar between WT and TG mouse hearts (Figure 1B). When normalized to actin expression level, the transcriptional levels of cTnI R193H transgene in cTnI^{193His} single TG was very similar to that in double TG mouse hearts (Figure 1C). The Western blotting data using two specific mAbs indicated that cTnI^{193His} hearts contained about 25% of mutant cTnI (cTnI R193H), which is consistent with our previous report [16]. The single cTnI-ND transgenic hearts contained 100% cTnI-ND in the cardiac muscle (Figure 2A). mAb 4H6 recognizes cTnI-ND but not slow skeletal muscle TnI (ssTnI) [21], precluding re-expression of ssTnI in the adult mouse hearts studied. In the heart of Double-TG mice, endogenous cTnI was replaced by cTnI-ND and only cTnI-ND and cTnI R193H were detected by the mAbs on Western blotting (Figure 2). The protein analyses data are consistent with the transcriptional data, indicating that approximately 22% cTnI R193H were present in myofibrils of the Double TG hearts. (Figure 2B).

Morphological examination of hearts did not show significant ventricular hypertrophy or dilation in RCM cTnI mutants and other TG lines. The ratio of heart weight/body weight (HW/BW, mg/g) was similar in WT and transgenic groups (Table 1). However, a significant biatrial enlargement was observed in cTnI^{193His} hearts compared to WT or cTnI-ND hearts (Figure 3A and Table 1), which is consistent with our previous findings [16]. However, the atrial enlargement became insignificant in Double TG hearts (Figure 3A). No significant myofibril disarray was observed with histological examinations in cardiac muscles from cTnI^{193His} and other transgenic mice (Figure 3A). The absence of fibrosis on cardiac sections was determined by the Gomori trichrome staining (not shown). In cTnI^{193His} mice, the rate of sudden cardiac death (SCD) was significantly high and the survival rate was about 70% for 28 mice observed in a 12-month period (Figure 3B), which is significantly lower compared to WT and cTnI-ND mice (100% survival rate in 25 WT and 22 cTnI-ND mice observed at the same time period, $P < 0.05$ analyzed by the log-rank test). In contrast,

the survived rate improved significantly in 25 Double TG mice (approximately 95%) with no significant difference from WT controls ($P>0.05$). The data suggest that the presence of cTnI-ND in cTnI^{193His} mutant hearts can rescue the RCM phenotypes and significantly reduce the mortality rate of RCM mice.

Cardiac function measurements were performed on experimental animals with high-resolution echocardiography using the B-mode and M-mode observations. Short-axis view and anatomic M-mode were used for a better analysis of left ventricular function in cardiac cycles. Anatomic M-mode provides the ability to obtain anatomically correct left ventricular (LV) measurements. Table 1 summarizes the *in vivo* cardiac function measured with echocardiography on WT and TG mice. There was no significant difference in body weight and heart rate among the transgenic mouse lines compared to the WT mice. The LV posterior wall (PW) thickness was similar in WT and transgenic lines, indicating no significant hypertrophy in cTnI^{193His} mouse hearts, which is consistent with the histological observations. The LV systolic function did not change significantly as shown by similar ejection fraction and fractional shortening in cTnI^{193His} and other mice. However, the end diastolic dimension and volume in cTnI^{193His} hearts were significantly reduced compared to WT and cTnI-ND mice (Table 1). Furthermore, a significantly prolonged IVRT and an increased E/E' were observed with mitral Doppler and tissue Doppler analyses on RCM cTnI^{193His} mice, indicating a diastolic dysfunction and an increased mean left atrial pressure in RCM cTnI^{193His} mice. Both LA and RA dimensions of cTnI^{193His} mice were enlarged at atrial dilation or contraction stages compared to WT mice (Table 1), which is consistent with the gross heart observation. All these overt signs were corrected by the presence of cTnI-ND in Double TG mice in which LV end diastolic dimension, atrial dimension, IVRT and E/E' were back to the levels similar to that observed on WT mice (Table 1).

The function of cardiac muscle was also measured for sarcomere shortening in isolated cardiac myocytes from WT and transgenic mouse hearts. Cardiac myocytes from cTnI^{193His} mice showed a significantly altered pattern of sarcomere shortening, which is characterized by a considerable prolonged relaxation time with no acute change in contraction (Figure 4A). The sarcomere length at end diastolic stage was significantly shortened in RCM cTnI^{193His} cardiac myocytes compared to that in WT cells (Figure 4B). No noticeable difference was seen between TG and WT cardiac myocytes for sarcomere shortening amplitude and time for 90% shortening (Figure 4C and D). However, a significant increase of relaxation time was observed in cTnI^{193His} cardiac myocytes as compared to the WT (Figure 4E), indicating that impaired relaxation is manifested as a prolonged relaxation time in cardiac myocytes carrying the RCM cTnI mutation. The prolonged relaxation was corrected by cTnI-ND in Double TG cardiac myocytes (Figure 4E). We further investigated Ca²⁺ transients in WT and TG cardiac myocytes. Ca²⁺ transient assays using fluorescent fura-2 as an indicator showed a concomitant slowing of the Ca²⁺ transient decay in cardiac myocytes isolated from cTnI^{193His} hearts (Figure 5). In contrast, the cardiac myocytes from cTnI-ND hearts showed an accelerated Ca²⁺ transient decay compared to WT cells (Figure 5A and E). These data are consistent with the report obtained from the working heart experiments performed in the cTnI-ND mice [18, 19]. The slowing of the Ca²⁺ transient decay in RCM cTnI^{193His} cardiac myocytes was effectively corrected by cTnI-ND in Double TG hearts, resuming a similar Ca²⁺ transient pattern to that of the WT (Figure 5A and E).

The Ca^{2+} transient at end diastolic stage, the peak Ca^{2+} transient and the Ca^{2+} transient at contractile stage did not change significantly in RCM cTnI mutant cardiac myocytes compared to that in WT cells (Figure 5B, C and D). The significant slowing of the late Ca^{2+} transient decay in cTnI^{193His} cardiac myocytes was unlikely due to the affected Ca^{2+} loading in SR of cTnI R193H cardiac myocytes, since the Ca^{2+} release from the SR was no significant difference between WT and TG cardiac myocytes after a rapid exposure to 10 mmol/L caffeine (Figure 6). Furthermore, Western blotting data indicate that no significant difference was observed in total expression of Ca^{2+} handling proteins, SR Ca^{2+} -ATPase pump (SERCA2), phospholamban and phosphorylated phospholamban, in all four groups of animals (Figure 6C).

We further performed frequency-dependent contraction and relaxation assays to explore whether the frequency-dependent acceleration of relaxation was retained in cTnI^{193His} cardiac myocytes. Cell shortening and contractility were recorded under electrical stimulations at frequencies of 0.5 to 2.0 Hz. The data showed that WT cardiac myocytes exhibited complete contraction-relaxation cycles showing an accelerated relaxation corresponding to an increased stimulation frequency (Figure 7A). The data are consistent with that reported in other studies [25]. However, RCM cTnI mutant cardiac myocytes could not relax completely by returning to the baseline at the end of diastole (Figure 7A). The impaired diastolic tone was exacerbated in these cells at a higher stimulating frequency (1 Hz or 2 Hz), indicated by EDSL, a distance between end diastolic sarcomere length and the baseline. The damaged diastolic tone was corrected by cTnI-ND in Double TG cardiac myocytes (Figure 7A). The results suggest that the mutant myofibrils in cTnI^{193His} mice cannot produce an acceleration of relaxation in response to an increased frequency of electrical stimulation, manifesting a damaged diastolic reserve in cTnI R193H cardiac myocytes. This defect was reversed by the presence of cTnI-ND in cardiac myocytes from the Double TG mice (Figure 7A and B).

To further test the hypothesis that the increased myofibril sensitivity to Ca^{2+} is the major factor in RCM cTnI^{193His} hearts, which results in impaired relaxation and diastolic dysfunction in RCM, we further carried out the pCa-force measurements in freshly skinned papillary muscle fibers from all groups of transgenic mice versus WT mice under isometric conditions. Figure 8A shows that a significant increase of Ca^{2+} sensitivity in TG R193H fibers compared to that in WT fibers was found in steady-state force development measurements with $\text{pCa}_{50}=5.99\pm0.02$ for TG R193H and $\text{pCa}_{50}=5.88\pm0.03$ for WT ($P<0.05$). In contrast, a significant decrease in Ca^{2+} sensitivity was seen in TG cTnI-ND fibers compared to WT fibers with $\text{pCa}_{50}=5.73\pm0.02$ ($P<0.05$). The pCa_{50} in double TG fibers was 5.88 ± 0.01 and had no significant difference from that of WT fibers ($P>0.05$). These data indicate that a significant hypersensitivity to Ca^{2+} is confirmed in myofibrils isolated from RCM cTnI^{193His} hearts and the presence of cTnI-ND in cardiac muscle can lower the Ca^{2+} hypersensitivity in the heart.

We have further investigated the response to PKA stimulation in cardiac myofibrils from WT and TG mice. After PKA incubation, the pCa-force curve in WT fibers had a rightward shift, indicating a decreased myofibril sensitivity to Ca^{2+} , which is consistent with previous studies (Figure 8B). The response to PKA treatment in cTnI R193H fibers was similar to

that in WT fibers (Figure 8C), indicating that R193H mutation at C-terminus of cTnI does not affect the N-terminal Ser23/24 phosphorylation by PKA. This is consistent with our previous Western blotting results showing that the *in vivo* cTnI Ser23/24 phosphorylation level in RCM cTnI^{193His} mouse hearts after β -adrenergic stimulation was similar to that in WT hearts [16]. However, it was observed that the cTnI R193H fibers had a significant decrease in the cooperativity of the thin filament activation (n_{Hill}) compared to WT (Figure 8). These data demonstrate that the increased myofibril sensitivity to Ca^{2+} in cTnI R193H cardiac myocytes is not related to the PKA mediated TnI phosphorylation.

Discussion

In general, in the case of DCM, heart failure is characterized by a systolic dysfunction (i.e., reduced ejection fraction), whereas HCM and RCM are characterized as having diastolic dysfunction (i.e., impaired relaxation) [26]. However, the mechanism underlying the development of RCM is still unknown. Recently, six cTnI mutations (L144Q, R145W, A171T, K178E, D190G and R192H) have been found to be associated with RCM. Among them, the two mutations K178E and R192H have the worst clinical phenotype [7]. The data from analyzing *in vitro* reconstituted thin filaments showed that the RCM cTnI mutations had high Ca^{2+} -sensitizing effects on force generation [10, 11]. Davis *et al* used an acute genetic engineering method to transfer the RCM mutant cTnI genes into isolated adult rat cardiac myocytes and found that permeabilized myocyte Ca^{2+} sensitivity was increased. RCM cTnI-dependent mechanical tone causing acute remodeling to a quasicontracted state is not elicited by other Ca^{2+} -sensitizing proteins and is a direct correlation of the stiff heart characteristic of RCM *in vivo* [12]. Increased crossbridge cycling kinetics has been observed in skinned cardiac muscle after exchange of C-terminal truncated TnI [27], further supporting the role of cTnI abnormality in impairing diastolic function of the heart.

We have generated TG mice expressing RCM mutant cTnI in the heart to investigate the mechanisms connecting the profound deficits in myofilament function to the development of diastolic dysfunction associated with heart failure. Our *in vivo* studies on the RCM TG mice have demonstrated that impaired relaxation (diastolic dysfunction) without significant systolic function changes is the main manifestation caused by the RCM mutations of cTnI. The significant signs of diastolic dysfunction in the RCM cTnI^{193His} mice are enlarged atria caused by the increased atrial pressure and an increased IVRT, which is the earliest sign detected by echocardiography in RCM [16, 28]. In this study, we also noticed a significant increase of E/E' ratio in cTnI^{193His} TG mice by mitral Doppler and tissue Doppler analyses. The ratio of mitral velocity to early diastolic velocity of the mitral annulus (E/E') is an important indication for mean left atrial pressure [29]. The significant increase of the E/E' ratio in cTnI^{193His} TG mice further indicates an increase of atrial pressure in RCM mice due to a restrictive left ventricle. The *in vivo* echocardiograph data are consistent with the histological examination results on the mutant TG mice. More experiments were performed to explore the mechanism underlying the diastolic dysfunction in RCM cTnI mutant mice. The prolonged relaxation time concomitant with the delayed Ca^{2+} transient decay in isolated cardiac myocytes from RCM cTnI^{193His} mice indicate that relaxation damage in cTnI mutant cardiac muscles plays a key role in the development of RCM disorder in cTnI mutant mice. Caffeine stimulation assays in WT and TG mutant cardiac myocytes indicate that SR

Ca²⁺ loading function is not affected by cTnI mutations in the mutant cardiac myocytes. Davis *et al* reported that there were no significant differences in Ca²⁺ handling protein expression between WT and transgenic cardiac myocytes containing 100% cTnI R193H [12]. To test the hypothesis that the prolonged relaxation time and the delay of Ca²⁺ transient decay in RCM cTnI^{193His} cardiac myocytes are due to the increased myofibril sensitivity to Ca²⁺, we further carried out the skinned myofibril experiments. The pCa-force measurement data reveal a significantly enhanced myofibril sensitivity to Ca²⁺ in cTnI mutant myofilaments that is probably the major cause of the prolonged relaxation resulting in diastolic dysfunction in RCM cTnI^{193His} mice. The Ca²⁺ hypersensitivity of RCM cTnI mutant hearts is unlikely associated with the alteration of cTnI phosphorylation, since our results also show that RCM cTnI mutant fibers have a similar response to PKA stimulation compared to WT fibers. The Ca²⁺ sensitization of myofibrils in RCM cTnI^{193His} mice not only results in impaired relaxation, but also is likely associated with the sudden cardiac death observed in this TG mouse line since myofilament Ca²⁺ sensitization causes susceptibility to cardiac arrhythmia in mice [30]. Furthermore, cardiac TnI R193H showed a decrease in the cooperativity of thin filament activation that could be correlated with the poor prognosis in these patients. It was previously shown in HCM cTnT I79N TG mice that display a large leftward shift in the Ca²⁺ sensitivity of force development also show a decrease in the Hill coefficient [31].

Modulation of myofilament Ca²⁺ sensitivity is a key to regulating cardiac function. For example, PKA-catalyzed phosphorylation of Ser23 and Ser24 in the N-terminal domain of cTnI has been extensively investigated for roles in alteration of myofibril sensitivity to Ca²⁺ [32, 33]. So far, most chemicals and molecules tried in clinical studies or in experimental animals are focused on calcium-sensitizing agents that increase myofibril sensitivity to Ca²⁺ and enhance cardiac contraction [34]. The myosin ATPase inhibitors, BDM and blebbistatin, have been used *in vitro* to desensitize myofilament affinity for Ca²⁺ [12, 30], but the high toxicity of these compounds prevent them from being used in intact animals.

The question that is currently poorly elucidated is how one would find a Ca²⁺ desensitizer which can be applied *in vivo* to lower left ventricular end diastolic pressure and enhance diastolic function. It is of clinical importance to find therapeutic strategies that directly target heart relaxation performance, since up to 40% of heart failure patients have impaired relaxation, known as diastolic dysfunction, with normal systolic performance [35]. It seems that the alteration of calcium response by modification of contractile proteins is a way to correct cardiac dysfunction. Jagatheesan *et al* reported recently that generation of mice with chimeric α -/ β -tropomyosin could rescue tropomyosin mutation-induced HCM [36]. Our present study has revealed a desensitizing regulation of diastolic dysfunction by changing the overall troponin function with an endogenous molecule, cTnI-ND that is produced by a physiologically occurring restrictive cleavage of cTnI to selectively remove its cardiac specific N-terminal extension and retain the conserved core structure intact [17]. Our data indicate that cTnI-ND, conferring a hyposensitivity to Ca²⁺, can correct the Ca²⁺ hypersensitivity in TG cTnI^{193His} cardiac muscle fibers, showing a self-adaptive mechanism of the cardiac muscle in response to physiological or pathophysiological stresses. In particular, the change of myofibril sensitivity to Ca²⁺ by cTnI-ND is not caused by the alteration of cTnI phosphorylation mediated by PKA pathway. Actually a recent study has

demonstrated that cTnI-ND is upregulated in β -adrenergic deficiency as a compensation for cardiac function through increasing relaxation by selectively utilizing an analogous effect of the β -adrenergic signaling pathway [18]. Therefore, the selective N-terminal truncation of cTnI provides us with a promising and novel therapeutic tool for modulation of the overall troponin function and treatment of diastolic dysfunction without interventions directed at the systemic β -adrenergic-PKA pathways.

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Figure 1. Quantification of cTnI R193H transgene transcriptional levels in the hearts of WT, cTnI-ND, cTnI^{R193H} single TG and double TG mice
 Real-time RT-PCR analysis was performed on cardiac RNA from four groups with primers specific for cTnI R193H transgene (A). β -actin expression was used as quantity controls (B). Values were normalized to β -actin control and presented as mean $\pm$ SE from three independent experiments. NS, no statistical significance.

Figure 2. Characterization of the single and double transgenic mice

(A) Western blotting analysis using two specific monoclonal antibodies, mAb 4H6 that recognizes WT, cTnI R193H but not slow skeletal TnI (ssTnI) from soleus muscle and mAb TnI-1 that recognizes WT cardiac and slow skeletal TnI but not cTnI R193H. Both mAbs recognize cTnI with truncated N-terminus (cTnI-ND). Cardiac troponin T (cTnT) was used as protein loading controls. (B) Summary of Western blotting results from 3 separate experiments. WT, wild type; cTnI-ND, transgenic mice containing N-terminal deleted cTnI; cTnI^{R193H}, transgenic mice containing cTnI R193H; Double TG, double transgenic mice containing both cTnI-ND and cTnI R193H.

Figure 3. Cardiac morphological and histological examination and mortality observation in WT and TG mice

(A) Gross heart morphological examination indicates a significant atrial enlargement in cTnI^{193His} hearts compared to WT and cTnI-ND hearts. The morphological change disappeared in double TG hearts. No significant myofibril disarray was detected in TG or WT heart sections stained with hematoxylin and eosin (HE). (B) Kaplan-Meier survival curves for WT (n=25), cTnI-ND (n=22), cTnI^{193His} single TG (n=28) and double TG (n=25) mice. The log-rank test indicates statistically significant differences among the four survival curves ($P < 0.05$). The mortality of cTnI^{193His} mice was significantly reduced by cTnI-ND in double TG mice. WT, wild type; cTnI-ND, transgenic mice containing N-terminal deleted cTnI; cTnI^{193His}, transgenic mice containing cTnI R193H; Double TG, double transgenic mice containing both cTnI-ND and cTnI R193H.

Figure 4. Myocardial contractility measurements

(A) Representative raw sarcomere shortening recordings from WT and TG cardiac myocytes. The rightward shift (prolonged relaxation) caused by RCM cTnI mutation was reversed by cTnI-ND in Double TG cardiomyocytes. No significant change in contraction time was observed in cardiac myocytes among the experimental groups. Summary of end diastolic sarcomere length (B), sarcomere shortening amplitude (C), 90% sarcomere shortening time (D) and 90% sarcomere relaxation time (E) of cardiac myocytes from WT, cTnI^{193His}, cTnI-ND and Double TG mice are shown. Values are expressed as means $\pm$ SE from at least 3 separate experiments ($n = 25-35$ cells per group). Statistical significance was determined by ANOVA followed by post hoc Newman-Keuls (SNK) tests. * $P < 0.05$. WT, wild type mice; cTnI-ND, transgenic mice containing N-terminal deleted cTnI; cTnI^{193His}, transgenic mice containing cTnI R193H; Double TG, double transgenic mice containing both cTnI-ND and cTnI R193H.

Figure 5. Ca^{2+} transient measurements in cardiac myocytes

(A) Representative raw Ca^{2+} transients obtained from WT and TG cardiac myocytes. A significant slowing of Ca^{2+} transient decay was observed in RCM cTnI mutant cardiac myocytes, which was corrected by cTnI-ND in Double TG cardiac myocytes. Summary of Ca^{2+} transient at end diastolic stage (B), peak amplitude of Ca^{2+} transient (C), time to 90% Ca^{2+} peak (D) and time to 90% Ca^{2+} decay (E) in cardiac myocytes from WT, cTnI^{193His}, cTnI-ND and Double TG mice. Values are expressed as means $\pm$ SE from at least 3 separate experiments ($n = 25-35$ cells per group). Statistical significance was determined by ANOVA followed by post hoc Newman-Keuls (SNK) tests. * $P < 0.05$. WT, wild type; cTnI-ND, transgenic mice containing N-terminal deleted cTnI; cTnI^{193His}, transgenic mice containing cTnI R193H; Double TG, double transgenic mice containing both cTnI-ND and cTnI R193H.

Figure 6. Caffeine stimulation assays in WT and TG cardiac myocytes

(A) Representative Ca²⁺ transient recordings in WT or TG cardiac myocytes rapidly exposed to 10 mmol/L caffeine (+ caffeine) compared to Ca²⁺ transients in cells without caffeine stimulation (- caffeine). (B) Summary of SR function evaluation of Ca²⁺ loading in WT and TG cardiac myocytes as measured by Ca²⁺ transient amplitude with or without 10 mmol/L caffeine stimulation. Values are expressed as means $\pm$ SE from at least 3 separate experiments ($n = 20-30$ cells per group). Statistical significance was determined by ANOVA followed by post hoc Newman-Keuls (SNK) tests. (C) Representative Western blotting analysis of cardiac SR Ca²⁺ ATPase pump (SERCA), phospholamban (PLB), and Ser16 phospho-phospholamban (Phospho-PLB) and loading control sarcomeric cTnT for ventricles from wild type (WT) and three TG animals (cTnI^{193His}, cTnI-ND and Double TG mice).

Figure 7. Frequency-shortening responses of WT and TG cardiac myocytes

(A) Representative recordings of sarcomere shortening are shown for cardiac myocytes from WT, cTnI^{193His}, cTnI-ND and Double TG mice stimulated at different frequencies. RCM cTnI mutant cardiac myocytes could not relax completely by returning to the baseline at the end of diastole (red line). The impaired diastolic tone was worse in these cells at a higher stimulating frequency (1 hz or 2 hz), indicated by EDSL, a distance between end diastolic sarcomere length and the baseline. The damaged diastolic tone was corrected by cTnI-ND in Double TG cardiac myocytes. (B) Summary of EDSL, a distance between end diastolic sarcomere length and the baseline obtained from frequency-shortening assays in 8-10 separated experiments. Values are expressed as means $\pm$ SE. Statistical significance was determined by ANOVA followed by post hoc Newman-Keuls (SNK) tests. * $P < 0.05$. WT, wild type; cTnI-ND, transgenic mice containing N-terminal deleted cTnI; cTnI^{193His}, transgenic mice containing cTnI R193H; Double TG, double transgenic mice containing both cTnI-ND and cTnI R193H.

Figure 8. Normalized pCa-force relationship in skinned cardiac muscle fibers

pCa-force relationship was measured in skinned cardiac muscle fibers prepared from hearts of WT and TG mice. A significant leftward shift was observed in muscle fibers from cTnI^{193His} mice and a significant rightward shift is seen in skinned fibers from cTnI-ND mice (A). The hypersensitivity to Ca^{2+} in cTnI^{193His} fibers was reversed by the presence of cTnI-ND in cardiac fibers from Double TG mice (A). Values are expressed as means $\pm$ SE (n = 6-8 per group). pCa-force relationship was measured in skinned cardiac muscle fibers from WT (B) and cTnI^{193His} (C) mice before and after PKA incubation. Values are expressed as means $\pm$ SE (n=6-8). Statistical significance was determined by ANOVA followed by post hoc Newman-Keuls tests. * P < 0.05 compared against WT either before or after PKA. # P < 0.05 comparing in the same mouse heart before and after PKA treatment.

Table 1
***In vivo* cardiac function measurements with echocardiograph on WT and TG mice**

Parameters	Wild type	cTnI ^{193His}	cTnI-ND	Double TG
Body Weight (g)	28.1 ± 1.3	24.9 ± 1.7	31.2 ± 2.0	26.8 ± 3.5
Heart Rate (bpm)	469 ± 20	426 ± 36	455 ± 36	419 ± 29
HW/BW (mg/g)	3.48 ± 0.7	3.88 ± 0.6	3.78 ± 0.4	3.28 ± 0.9
<i>Echo-ECG</i>				
LA end diastole (mm)	1.71 ± 0.01	1.87 ± 0.01 *	1.78 ± 0.17	1.83 ± 0.02
LA end systole (mm)	1.22 ± 0.03	1.44 ± 0.08 *	1.26 ± 0.03	1.18 ± 0.02
RA end diastole (mm)	2.21 ± 0.05	2.34 ± 0.04 *	2.17 ± 0.02	2.12 ± 0.06
RA end systole (mm)	1.74 ± 0.03	2.18 ± 0.05 *	1.87 ± 0.01	1.86 ± 0.06
LV end diastole				
IVS (mm)	0.73 ± 0.1	0.81 ± 0.1	0.89 ± 0.1	0.77 ± 0.1
PW (mm)	0.78 ± 0.1	0.80 ± 0.1	0.88 ± 0.1	0.75 ± 0.1
LVEDD (mm)	3.82 ± 0.1	3.25 ± 0.1 *	3.98 ± 0.1	3.88 ± 0.1
LV volume (μl)	54.8 ± 0.8	42.3 ± 0.7 *	59.3 ± 0.7	60.1 ± 4.9
LV end systole				
IVS (mm)	1.22 ± 0.1	1.47 ± 0.1	1.42 ± 0.2	1.35 ± 0.2
PW (mm)	1.02 ± 0.2	1.11 ± 0.1	1.21 ± 0.2	1.12 ± 0.1
LVEDD (mm)	2.59 ± 0.7	2.01 ± 0.3	2.53 ± 0.1	2.43 ± 0.3
LV volume (μl)	11.0 ± 2.5	10.2 ± 1.4	13.1 ± 1.8	14.7 ± 4.3
LV FS %	49.1 ± 3.6	44.8 ± 3.2	44.8 ± 3.2	43.3 ± 6.5
LV EF %	79.7 ± 4.1	76.2 ± 3.5	76.2 ± 3.5	74.4 ± 6.9
<i>Mitral Doppler</i>				
E (mm/s)	657 ± 54	663 ± 58	580 ± 48	639 ± 56
A (mm/s)	508 ± 47	498 ± 41	387 ± 40	500 ± 49
E/A	1.30 ± 0.2	1.35 ± 0.1	1.50 ± 0.1	1.28 ± 0.1
IVRT (ms)	24.5 ± 1.5	32.1 ± 2.2 *	22.4 ± 1.7	20.1 ± 1.9
IVCT (ms)	15.0 ± 1.2	15.8 ± 1.8	14.1 ± 1.5	13.8 ± 1.2
<i>DTI</i>				
E' (mm/s)	23.5 ± 0.8	17.8 ± 0.5	22.1 ± 0.9	25.4 ± 0.8
A' (mm/s)	17.2 ± 0.4	17.4 ± 0.5	15.3 ± 0.3	21.3 ± 0.6
E'/A'	1.37 ± 0.1	1.02 ± 0.1	1.45 ± 0.2	1.20 ± 0.1
E/E'	27.8 ± 2.5	38.1 ± 2.7 *	26.2 ± 2.2	25.2 ± 2.5

Values are expressed as mean ± SE for 5 wild type, 5 cTnI^{193His}, 5 cTnI-ND and 5 double TG mice at ages of 2-3 months. HW, heart weight; BW, body weight; LA, left atrium; RA, right atrium; EDD, end diastolic dimension; ESD, end systolic dimension; LV, left ventricle; PW, posterior wall thickness of LV; IVS, Intra-ventricular septum; FS, fractional shortening of LV; EF, ejection fraction of LV. IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; E, mitral Doppler E peak velocity; A, mitral Doppler A peak velocity; DTI, Doppler tissue imaging; E', tissue Doppler E' peak velocity; A', tissue Doppler A' peak velocity. Statistical significance was determined by ANOVA followed by post hoc Newman-Keuls (SNK) tests.

* P < 0.05.