Knock-In Mouse Model of Dilated Cardiomyopathy Caused by Troponin Mutation

Cheng-Kun Du, Sachio Morimoto, Kiyomasu Nishii, Reiko Minakami, Mika Ohta, Naoto Tadano, Qun-We Li, Yuan-Yuan Wang, Dong-Yun Zhan, Misato Mochizuki, Satomi Kita, Yoshikazu Miwa, Fumi Takahashi-Yanaga, Takahiro Iwamoto, Iwao Ohutsuki, Toshiyuki Sasaguri

Abstract—We created knock-in mice in which a deletion of 3 base pairs coding for K210 in cardiac troponin (cTn/T found in familial dilated cardiomyopathy patients was introduced into endogenous genes. Membrane-permeabilized cardiac muscle fibers from mutant mice showed significantly lower Ca²⁺ sensitivity in force generation than those from wild-type mice. Peak amplitude of Ca²⁺ transient in cardiomyocytes was increased in mutant mice, and maximum isometric force produced by intact cardiac muscle fibers of mutant mice was not significantly different from that of wild-type mice, suggesting that Ca²⁺ transient was augmented to compensate for decreased myofilament Ca²⁺ sensitivity. Nevertheless, mutant mice developed marked cardiac enlargement, heart failure, and frequent sudden death recapitulating the phenotypes of dilated cardiomyopathy patients, indicating that global functional defect of the heart attributable to decreased myofilament Ca²⁺ sensitivity could not be fully compensated by only increasing the intracellular Ca²⁺ transient. We found that a positive inotropic agent, pimobendan, which directly increases myofilament Ca²⁺ sensitivity, had profound effects of preventing cardiac enlargement, heart failure, and sudden death. These results verify the hypothesis that Ca²⁺ desensitization of cardiac myofilament is the absolute cause of the pathogenesis of dilated cardiomyopathy associated with this mutation and strongly suggest that Ca²⁺ sensitizers are beneficial for the treatment of dilated cardiomyopathy patients affected by sarcomeric regulatory protein mutations. (Circ Res. 2007;101:185-194.)

Key Words: dilated cardiomyopathy ■ troponin □ mutation ■ calcium sensitivity ■ knock-in mouse

Dilated cardiomyopathy (DCM) is a disorder of cardiac muscle characterized by cardiac enlargement and systolic dysfunction and accounts for more than 10,000 deaths annually by heart failure and sudden death in the United States. DCM is known to result from nongenetic insults, such as viruses, alcohol, toxins, and immunologic injury; however, recent genetic studies have revealed that mutations in genes for cytoskeletal (dystrophin, desmin, α-sarcoglycan), nuclear envelope (tafazzin and lamin A/C), and sarcomeric (cardiac actin, β-cardiac myosin heavy chain, α-tropomyosin, cardiac myosin-binding protein C, titin/connectin, cardiac troponin [cTn/T, cTnI, and cTnC] proteins are important causes of DCM, and the incidence of the inherited DCM is thought to be 20% to 35%. In a previous in vitro reconstitution experiment, selective displacement of endogenous cTnT in rabbit membrane-permeabilized (skinned) cardiac muscle fibers with human cTnT with a DCM-causing deletion mutation ΔK210 was found to decrease the Ca²⁺ sensitivity of force generation, in direct opposition to Ca²⁺ sensitization caused by mutations associated with hypertrophic cardiomyopathy. This study led us to propose that decreased Ca²⁺ sensitivity of cardiac myofilaments might be a primary functional defect triggering the pathogenesis of DCM associated with the deletion mutation ΔK210 in cTnT.
To test this hypothesis in vivo and to determine the exact cause of the pathogenesis of DCM associated with the deletion mutation ΔK210 in cTnT, in the present study, we created mutant mice in which this mutation was just knocked in to the endogenous mouse TNNT2 gene using embryonic stem cell technology. Analyses of these knock-in mice demonstrated that decreased myofilament Ca$^{2+}$ sensitivity is the absolute cause of DCM associated with the mutation ΔK210 in cTnT and also suggested that Ca$^{2+}$ sensitizers, such as pimobendan, might be beneficial for the treatment of DCM patients affected by sarcomeric regulatory protein mutations.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Generation of a Knock-In Mouse Model by Gene Targeting
Gene targeting was performed using a targeting vector including a 13.8-kb cTnT gene fragment with the loxP-neo cassette on intron 12 and a deletion of 3 base pairs coding for K210 on exon 13. After germline transmission of the properly targeted ES cell clone, knock-in mice were obtained by crossing with a Cre expressing transgenic mouse strain to create a ΔK210 mouse strain. Lower left, Southern blot analysis of genomic DNA from wild-type (WT), neo-ΔK210 (+/neo-ΔK210), and ΔK210 (+/ΔK210) mouse strains. BspHI-digested digested DNAs were hybridized with a 5' probe. Lower right, PCR-mediated genotyping of wild-type, TNNT2+/neo-ΔK210, and TNNT2+/ΔK210 mice. Product lengths are 0.45, 0.76, and 0.52 kb for wild-type, neo-ΔK210, and ΔK210 alleles, respectively.

Preparation of Skinned Cardiac Muscle Fibers and Force Measurements
A small fiber (~200 μm in diameter) was dissected from the skinned left ventricular papillary muscle, and isometric force was measured as described previously.

Preparation of Intact Cardiac Muscle Fibers and Force Measurements
The left ventricular papillary muscle was mounted horizontally in a thermostatically controlled chamber, and isometric force evoked by a bipolar electrical field stimulation was measured with a semiconductor strain gauge.

Fura-2 Loading and Simultaneous Measurements of Fluorescence and Sarcomere Length
Cardiomyocytes were loaded with fura-2 acetoxymethyl ester, and contraction was evoked by bipolar electrical field stimulation. [Ca$^{2+}$],
and sarcomere length were simultaneously monitored using a fluorescence and contractility recording system (IonOptix) as described previously.\textsuperscript{14}

**Results**

We created knock-in mice in which 3 base pairs coding for the amino acid residue K210 in cTnT were deleted from their endogenous gene $\text{TNNT2}$ using gene-targeting technology (Figure 1). These knock-in mice have the same genomic gene condition as DCM patients affected by the deletion mutation $\text{K210}$ in cTnT. Consistent with the previous study showing that this mutation in cTnT has a $\text{Ca}^{2+}$-desensitizing effect on cardiac myofilaments in vitro,\textsuperscript{10} skinned cardiac muscle fibers prepared from mutant mice showed a decrease in $\text{Ca}^{2+}$ sensitivity of force generation, as was evident from a rightward shift of the force/pCa relationship (Figure 2A). The pCa value at half-maximal force generation ($p\text{Ca}_{50}$, index of $\text{Ca}^{2+}$ sensitivity) was statistically significantly lower in mutant mice than in wild-type mice, being smaller in homozygotes ($\text{TNNT2}^{\Delta K210/\Delta K210}$) than in heterozygotes ($\text{TNNT2}^{+/\Delta K210}$) (Figure 2B). On the other hand, the maximum force-generating capabilities (Figure 2C) and Hill coefficient values (an indicator of cooperativity or steepness of curve) (data not shown) were not significantly different between wild-type and mutant mice. Phosphorylation levels of cTnI at Ser22/23, known to influence myofilament $\text{Ca}^{2+}$ sensitivity, were also not significantly different between wild-type and mutant mice (Figure 2D). No significant differences in the phosphorylation levels of serine and threonine residues in cTnI and cTnT, which were reported to affect the myofilament activation level and/or $\text{Ca}^{2+}$ sensitivity,\textsuperscript{15} were detected between wild-type and mutant mice by using anti-phosphoserine and anti-phosphothreonine antibodies (data not shown). These results demonstrate that the deletion mutation $\Delta K210$ in cTnT in vivo does have a $\text{Ca}^{2+}$-desensitizing effect on force generation in cardiac muscle without changing the maximum force-generating capability and cooperativity, confirming the results of previous in vitro reconstitution studies.\textsuperscript{10,16,17} It should be noted that the phosphorylation levels of cTnI and cTnT in skinned fibers could not reflect these levels in vivo,

![Figure 2. Mechanical properties of skinned cardiac muscle fibers. A, Force–pCa relationships in skinned cardiac muscle fibers prepared from hearts of wild-type (WT) (○; 2 to 9 months old; $n=16$), $\text{TNNT2}^{+/\Delta K210}$ (●; 2 to 9 months old; $n=16$), and $\text{TNNT2}^{\Delta K210/\Delta K210}$ (□; 2 to 3 months old; $n=5$) mice. B, $\text{Ca}^{2+}$ sensitivities ($p\text{Ca}_{50}$) of force–pCa relationships in skinned cardiac muscle fibers. C, Maximum force-generating capabilities of skinned cardiac muscle fibers. D, Phosphorylation levels of cTnI in skinned cardiac muscle fibers. Data represent the means±SE for 3 fibers from different mice. Statistical significance was determined by ANOVA followed by post hoc Tukey’s multiple comparison test.](http://circres.ahajournals.org/issue)
Figure 3. Ex vivo assessment of cardiac function of knock-in mice. A, Isometric force generated by electrical stimulation at 3 Hz in intact left ventricular papillary muscle fibers. Force transient curves represent averaged data of 5 fibers from different hearts. Wild type (WT), 4 months old; TNNT2^{ΔK210}, 4 months old; TNNT2^{ΔK210/ΔK210}, 3 months old. B, Peak amplitudes and rates of isometric force development and relaxation. Data represent the means±SE of parameters determined on 5 intact left ventricular papillary muscle fibers from different hearts. C, Ca^{2+} transients induced by electrical stimulation at 3 Hz in left ventricular cardiomyocytes. Ca^{2+} transient curves represent averaged data of 8 cardiomyocytes prepared from 3 hearts. Wild type, 4 months old; TNNT2^{ΔK210}, 4 months old; TNNT2^{ΔK210/ΔK210}, 2 to 3 months old. D, Peak amplitudes and rates of Ca^{2+} transients in left ventricular cardiomyocytes. Data represent the means±SE of parameters determined on 8 cardiomyocytes from 3 hearts. E, Sarcomere length (SL) changes induced by electrical stimulation at 3 Hz in left ventricular cardiomyocytes. Curves represent averaged data of 8 cardiomyocytes prepared from 3 hearts. Wild type, 4 months old; TNNT2^{ΔK210}, 4 months old; TNNT2^{ΔK210/ΔK210}, 2 to 3 months old. F, Resting sarcomere length and dynamic parameters of sarcomere length changes. Data represent the means±SE of parameters determined on 8 cardiomyocytes from 3 hearts. Statistical significance was determined by ANOVA followed by post hoc Tukey’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.001.
because skinned fibers were prepared and stored without denaturation in ATP-containing relaxing solution, mimicking the intracellular environment. Further thorough analysis is necessary to reach a conclusion regarding alterations in sarcomeric protein phosphorylation in vivo.

The decreased $\text{Ca}^{2+}$ sensitivity of cardiac myofilament in knock-in mice is expected to lead to some reduction in the maximum force-generating capability of intact cardiac muscle; however, intact cardiac muscle fibers from mutant mice showed no significant decrease in maximum isometric force.
per cross-sectional area compared with those from wild-type mice (Figure 3A and 3B). Intact cardiac muscle fibers from TNNT2H9004K210/H210 mice showed slightly higher rates of isometric force development and relaxation, as was evident from a decrease in the time to peak and a steeper rise and fall in force (Figure 3B). Figure 3C shows Ca\(^{2+}\) transients measured in fura-2–loaded cardiomyocytes. The peak amplitude was found to be significantly increased in mutant mice compared with wild-type mice, being greater in TNNT2H9004K210/H210 than in TNNT2H9004K210 mice (Figure 3D). The peak rates of increase and decrease in cytoplasmic Ca\(^{2+}\) in mutant mice are faster than those in wild-type mice, consistent with the faster kinetics of isometric force development and relaxation observed in TNNT2H9004K210/H210 mice. Resting sarcomere length of cardiomyocytes in mutant mice was significantly longer than that in wild-type mice, consistent with decreased myofilament Ca\(^{2+}\) sensitivity (Figure 3E and 3F). No significant differences were observed in the number and shape of mitochondria in cardiomyocytes between wild-type and mutant mice, strongly suggesting that the change in peak Ca\(^{2+}\) sensitivity and maintain the force-generating capability of cardiac muscle; however, fractional sarcomere shortening and peak velocity of sarcomere shortening of isolated cardiomyocytes were significantly decreased in TNNT2H9004K210/H210 mice, indicating that a defect of dynamic contractile performance was still present in cardiomyocytes of these mutant mice (Figure 3F). Sarcoplasmic reticulum Ca\(^{2+}\) content assessed by caffeine-induced Ca\(^{2+}\) release was significantly increased in mutant mice, suggesting that sarco-plasmic reticulum Ca\(^{2+}\) pump (SERCA2a) activity was enhanced in mutant mice (supplemental Figure II). Whereas protein expression levels of ryanodine receptor, sarcoplasmic reticulum Ca\(^{2+}\) pump, and phospholamban (PLB) were not significantly different between wild-type and mutant mice, significant increases in the phosphorylation of ryanodine receptor and PLB catalyzed by cAMP-dependent protein kinase were observed in TNNT2H9004K210/H210 mice, suggesting that an increase in the intracellular cAMP level might be responsible for the augmented Ca\(^{2+}\) transient in mutant mice (supplemental Figure III).

Despite preserved maximum isometric force-generating capability in intact cardiac muscle, knock-in mice developed markedly enlarged hearts characteristic of DCM in a mutant-gene dosage-dependent manner, and histological examination of their cardiac sections showed significant interstitial fibrosis with normal cellular organization (Figure 4A). Cardiomyocyte apoptosis was significantly increased in the mutant mice, suggesting that apoptosis-inducing stress such as ischemia occurs in the myocardium of mutant mice (Figure 4B). Echocardiography demonstrated that left ventricular end-diastolic dimension was significantly increased in mutant mice, and left ventricular ejection fraction, an index of cardiac systolic function, was significantly reduced in mutant mice, with TNNT2H9004K210 being more severely affected than TNNT2H9004K210 (Table 1). No significant differences were detected in ventricular wall thickness (interventricular septal thickness and left ventricular posterior wall thickness), heart rate, and blood pressure between wild-type and mutant mice (Table 1). There were no significant differences in in vivo basal hemodynamic parameters between wild-type and mutant mice, but left ventricular end-systolic pressure of TNNT2H9004K210 mice was significantly lower than that of wild-type mice after administration of isoproterenol (Table 2).

Ex vivo analyses of isolated work-performing heart preparations showed that cardiac pump function was impaired in mutant mice (Figure 4C). These results indicate that the deletion mutation \\Delta K210 in cTnT causes cardiac enlargement with marked ventricular dilation and systolic dysfunction in mice, closely recapitu-lating the phenotypes of human DCM patients.\(^18,19\)

Mutant mice showed high mortality in a mutant-gene, dosage-dependent manner (Figure 4D). TNNT2H9004K210/H210 mice showed a particularly high incidence of sudden death in their growth periods from 1 to 3 months old. Surface ECG showed that TNNT2H9004K210/H210 mice commonly had electrophysiological abnormality in the heart with long QT, which might be involved in their frequent sudden death (Table 1). Telemetric ECG recordings showed that mice died by abruptly developing repetitive Torsade de Pointes several hours before death, which ultimately degenerated into ventricular fibrillation (Figure 4E).

The expression of β-cardiac myosin heavy chain (β-MyHC) isoform in ventricular myocardium, known to be upregulated in heart failure,\(^20–22\) was found to be markedly increased in TNNT2H9004K210/H210 mice at 2 months old (Figure 4F). A significant increase in the expression of β-MyHC was also detected in TNNT2H9004K210 mice at 3 and 5 months old but not at 2 months old, indicating that mutant mice undergo heart failure, with homozygous mice being affected from much earlier stages of life.

Oral administration of pimobendan, a Ca\(^{2+}\) sensitizer, known to directly increase the Ca\(^{2+}\) sensitivity of myofilament, was found to reduce the heart size of mutant mice, as was evident from a statistically significant decrease in the heart to body weight ratio (Table 3). Echocardiography showed that pimobendan also improved left ventricular systolic function while reducing the left ventricular end-diastolic dimension (Table 3). The expression of β-MyHC polypeptide isoform in ventricular myocardium was also markedly decreased in mice treated with pimobendan (Figure 5A).

Oral administration of pimobendan (30 mg/kg per day) was also found to markedly improve the life span of TNNT2H9004K210/H210 mice (Figure 5B). Pimobendan is a so-called inodilator with inhibitory action on phosphodiesterase 3 as well as Ca\(^{2+}\)-sensitizing action on cardiac myofilaments.\(^23\) Oral administration of a specific phosphodiesterase 3 inhibitor amrinone had no effect on the survival of TNNT2H9004K210/H210 mice at 30 mg/kg per day and markedly increased the mortality of TNNT2H9004K210/H210 mice at 100 mg/kg per day (Figure 5C); amrinone had no direct effect on the Ca\(^{2+}\) sensitivity of force generation in skinned cardiac muscle fibers (supplemental Figure IV). Echocardiography and blood pressure measurements indicated that amrinone had a weaker positive inotropic effect and a stronger vasodilating effect on TNNT2H9004K210/H210 mice compared with pimobendan (Figure 5D). Amrinone also had a much stronger effect on the...
phosphorylation of PLB catalyzed by cAMP-dependent protein kinase (Figure 5E). These results strongly suggest that pimobendan exerts its preventive effects on cardiac enlargement, heart failure, and sudden death as a Ca²⁺ sensitization. Intraperitoneal administration of β-adrenergic agonist isoproterenol or antagonist propranolol increased the mortality of TNNT2<sub>K210/K210</sub> mice even at a low dose (supplemental Figure V). The dramatic preventive effects of pimobendan on the development of pathology in TNNT2<sub>K210/K210</sub> mice provide strong evidence that decreased myofilament Ca²⁺ sensitivity is the absolute cause of the pathogenesis of DCM associated with the mutation ΔK210 in cTnT and suggest that Ca²⁺ sensitizers that antagonize the decrease in Ca²⁺ sensitivity of cardiac myofilaments are potential therapeutic drugs. Cardiomyocytes isolated from pimobendan-treated TNNT2<sub>K210/K210</sub> mice no longer showed enhancement of the Ca²⁺ transient, indicating that pharmacological intervention with pimobendan can prevent or reverse functional remodeling involving the enhancement of intracellular Ca²⁺ handling (supplemental Figure VIA). Cardiomyocytes of pimobendan-treated TNNT2<sub>K210/K210</sub> mice still had a defect of dynamic contractile performance (supplemental Figure VIB), and intact cardiac muscle fibers prepared from pimobendan-treated mice developed significantly lower isometric force than those from wild-type mice (supplemental Figure VIC). Skinned cardiac muscle fibers from pimobendan-treated mice still had decreased Ca²⁺ sensitivity of force generation, indicating that pharmacological intervention with pimobendan cannot remove the intrinsic defect from myofilaments (supplemental Figure VII).

### TABLE 1. Summary of Echocardiography, Blood Pressure, and ECG Data

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TNNT2&lt;sup&gt;ΔK210&lt;/sup&gt;</th>
<th>TNNT2&lt;sup&gt;ΔK210ΔK210&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, wk</strong></td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>No. of mice</strong></td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>BW, g</strong></td>
<td>20.9±1.1</td>
<td>28.5±0.6</td>
<td>21.9±0.6</td>
</tr>
<tr>
<td><strong>HR, bpm</strong></td>
<td>5.4±0.1</td>
<td>4.8±0.1</td>
<td>6.1±0.2</td>
</tr>
<tr>
<td><strong>LVESP (mm Hg)</strong></td>
<td>98.2</td>
<td>137.3±2.6</td>
<td>138.6±6.4</td>
</tr>
<tr>
<td><strong>LVEDD, mm</strong></td>
<td>1.63±0.17</td>
<td>1.95±0.09</td>
<td>2.82±0.25*</td>
</tr>
<tr>
<td><strong>LVEDD, mm</strong></td>
<td>3.10±0.25</td>
<td>3.38±0.06</td>
<td>4.00±0.22</td>
</tr>
<tr>
<td><strong>LVPWT, mm</strong></td>
<td>0.67±0.09</td>
<td>0.85±0.06</td>
<td>0.82±0.04</td>
</tr>
<tr>
<td><strong>EF, %</strong></td>
<td>48.0±1.2</td>
<td>43.3±2.6</td>
<td>30.0±3.4***</td>
</tr>
<tr>
<td><strong>HR, bpm</strong></td>
<td>263.0±14.8</td>
<td>220.8±11.8</td>
<td>329.8±19.9</td>
</tr>
<tr>
<td><strong>SBP, mm Hg</strong></td>
<td>92.2</td>
<td>103.5±3.2</td>
<td>96.1±14.1*</td>
</tr>
<tr>
<td><strong>DBP, mm Hg</strong></td>
<td>6.2</td>
<td>6.9±2.9</td>
<td>12.5±2.9</td>
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<tr>
<td><strong>PR, ms</strong></td>
<td>21±0.1</td>
<td>56±4</td>
<td>42±3</td>
</tr>
<tr>
<td><strong>QRS, ms</strong></td>
<td>12±1</td>
<td>12±1</td>
<td>12±1</td>
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<tr>
<td><strong>QT, ms</strong></td>
<td>35±2</td>
<td>37±3</td>
<td>42±2</td>
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</table>

Data represent the means±SE. Statistical significance was determined by ANOVA followed by post hoc Tukey’s multiple comparison test. WT indicates wild-type; BW, body wt; HR, heart rate; bmp, beats per minute; LVESP, LV end-systolic pressure; LVEDD, LV end-diastolic dimension; LVPWT, LV posterior wall thickness; EF, fractional shortening; SBP, systolic blood pressure; DBP, diastolic blood pressure. *P<0.05, **P<0.01, ***P<0.001 vs age-matched wild type; †P<0.05, ††P<0.01, †††P<0.001 vs age-matched TNNT2<sup>ΔK210</sup>.

### TABLE 2. Summary of In Vivo Hemodynamic Parameters

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TNNT2&lt;sup&gt;ΔK210&lt;/sup&gt;</th>
<th>TNNT2&lt;sup&gt;ΔK210ΔK210&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (wk)</strong></td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>No. of mice</strong></td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>263.0±14.8</td>
<td>220.8±11.8</td>
<td>329.8±19.9</td>
</tr>
<tr>
<td><strong>LVESP (mm Hg)</strong></td>
<td>98.2±3.1</td>
<td>84.3±6.2</td>
<td>81.6±4.1</td>
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<tr>
<td><strong>LVEDD (mm Hg)</strong></td>
<td>6.7±14.10</td>
<td>6.7±5.3</td>
<td>8.8±1.9</td>
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<tr>
<td><strong>dP/dt&lt;sub&gt;max&lt;/sub&gt;, mm Hg/sec</strong></td>
<td>6153±304</td>
<td>4905±271</td>
<td>4866±726</td>
</tr>
<tr>
<td><strong>dP/dt&lt;sub&gt;min&lt;/sub&gt;, mm Hg/sec</strong></td>
<td>-4810±90</td>
<td>-4804±152</td>
<td>-4829±570</td>
</tr>
<tr>
<td><strong>Isoproterenol (0.1 mg/kg, IP)</strong></td>
<td>566.3±55.9</td>
<td>664.6±6.2</td>
<td>637.6±35.9</td>
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<tr>
<td><strong>LVESP, mm Hg</strong></td>
<td>135.3±1.0</td>
<td>103.5±3.2</td>
<td>96.1±14.1*</td>
</tr>
<tr>
<td><strong>LVEDD, mm Hg</strong></td>
<td>14.1±3.5</td>
<td>13.9±6.0</td>
<td>12.5±2.9</td>
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<tr>
<td><strong>dP/dt&lt;sub&gt;max&lt;/sub&gt;, mm Hg/sec</strong></td>
<td>9678±269</td>
<td>9778±511</td>
<td>7491±922</td>
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<td><strong>dP/dt&lt;sub&gt;min&lt;/sub&gt;, mm Hg/sec</strong></td>
<td>-8825±248</td>
<td>-7197±480</td>
<td>-6211±1284</td>
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</table>

Data represent the means±SE. Statistical significance was determined by ANOVA followed by post hoc Tukey’s multiple comparison test. LVESP indicates LV end-systolic pressure; LVEDD, LV end-diastolic pressure; dP/dt<sub>max</sub>, maximum rate of LV pressure change; dP/dt<sub>min</sub>, minimum rate of LV pressure change.

*P<0.05 vs wild type.
**TABLE 3. Summary of Heart Weight and Echocardiography Data in Mutant Mice With Vehicle or Pimobendan Treatment**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vehicle-Treated</th>
<th>Pimobendan-Treated</th>
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<tr>
<td></td>
<td>TNNT2&lt;sup&gt;ΔK210&lt;/sup&gt;</td>
<td>TNNT2&lt;sup&gt;ΔK210/ΔK210&lt;/sup&gt;</td>
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<tr>
<td>Age, wk</td>
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<td>8</td>
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<td>No. of mice</td>
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<td>5</td>
</tr>
<tr>
<td>BW, g</td>
<td>30.7±0.8</td>
<td>19.9±1.4</td>
</tr>
<tr>
<td>HW, mg</td>
<td>162.9±8.1</td>
<td>220.6±28.4</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>5.3±0.1</td>
<td>11.3±1.5</td>
</tr>
<tr>
<td>Echocardiography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>ND</td>
<td>407±27</td>
</tr>
<tr>
<td>WST, mm</td>
<td>ND</td>
<td>0.66±0.07</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>ND</td>
<td>4.26±0.24</td>
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<tr>
<td>LVEDD, mm</td>
<td>ND</td>
<td>5.06±0.19</td>
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<td>LVPWT, mm</td>
<td>ND</td>
<td>0.60±0.08</td>
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<td>FS, %</td>
<td>ND</td>
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<tr>
<td>EF, %</td>
<td>ND</td>
<td>38.6±3.4</td>
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Pimobendan was administered to TNNT2<sup>ΔK210</sup> mice at a dose of 10 mg/kg and TNNT2<sup>ΔK210/ΔK210</sup> mice at a dose of 100 mg/kg orally once daily for 4 weeks from 20 and 4 weeks of age, respectively. ND indicates not determined. Data represent the means±SE. *P<0.05, **P<0.01 vs vehicle-treated mice with the same genotype (unpaired t test).

**Discussion**

The deletion mutation ΔK210 in the TNNT2 gene has so far been identified in 4 unrelated families. In a family reported by Kamisago et al., this mutation caused frequent sudden death in patients with mild heart failure before 30 years of age, including 2 infants and 3 young adults. In another family reported by Kamisago et al., this mutation caused frequent death attributable to congestive heart failure in young patients before 20 years of age, including 2 girls (17 and 19 years old), with marked dilatation of ventricles and increased interstitial fibrosis and a boy (15 years old) with a markedly enlarged heart (~400g). Early-onset phenotype with a high incidence of sudden death and/or heart failure was also reported in a family reported by Hanson et al.. In a family with 4 affected individuals reported by Mogensen et al., 3 patients died or underwent cardiac transplantation in their second or third decade of life; a patient with mild heart failure died suddenly at 36 years, a patient died of heart failure at 26 years, and a patient with very severe heart failure underwent cardiac transplantation at 22 years (weight of the explanted heart, >450 g). The knock-in mice developed enlarged hearts and heart failure and showed a high incidence of premature sudden death, closely recapitulating the clinical phenotypes of human patients; however, it should be noted that this mutation in knock-in mice causes a mutant-gene, dosage-dependent phenotype of DCM. This finding indicates that the DCM phenotype caused by the deletion mutation ΔK210 in cTnT is inherited in a semidominant, additive manner, at least in mice, but not only in a true dominant manner, as implied by a limited number of human DCM patients, which strongly suggests that homozygous patients would exhibit a more severe phenotype than heterozygous patients.

Skinned cardiac muscle fibers prepared from mutant mice showed a significant decrease in the Ca<sup>2+</sup> sensitivity of force generation with no change in maximum force-generating capability. Because intact cardiac muscle is known to be never activated beyond the half-maximal level, the decrease in Ca<sup>2+</sup> sensitivity is expected to cause a reduction in the force generation of the myocardium even if there is no change in the maximum force-generating capability; however, intact cardiac muscle fibers showed no significant reduction in isometric force generated per cross-sectional area. Analyses using a Ca<sup>2+</sup> indicator, fura-2, revealed a significant increase in the peak amplitude of Ca<sup>2+</sup> transient in cardiomyocytes of mutant mice, which could account for the preserved isometric force-generating capability of intact cardiac muscle fibers. Despite the preserved isometric force-generating capability of intact cardiac muscle fibers, mutant mice developed marked cardiac enlargement, heart failure, and frequent sudden death, strongly suggesting that global functional defect of the heart caused by decreased myofilament Ca<sup>2+</sup> sensitivity could not be fully compensated by augmentation of the Ca<sup>2+</sup> transient. In this regard, it should be noted that the 2 isoforms of MyHC can produce a similar level of premature sudden death and/or heart failure, closely recapitulating the clinical phenotypes of human patients; however, it should be noted that this mutation in knock-in mice causes a mutant-gene, dosage-dependent phenotype of DCM. This finding indicates that the DCM phenotype caused by the deletion mutation ΔK210 in cTnT is inherited in a semidominant, additive manner, at least in mice, but not only in a true dominant manner, as implied by a limited number of human DCM patients, which strongly suggests that homozygous patients would exhibit a more severe phenotype than heterozygous patients.
Figure 5. Effects of pimobendan on knock-in mice. A, Protein expression levels of β-MyHC in ventricular myocardium of TNNT2<sup>K210/K210</sup> mice treated with pimobendan. From 30 days of age, pimobendan (100 mg/kg) or vehicle only (methylcellulose) was administered to TNNT2<sup>K210/K210</sup> mice orally once daily for 4 weeks. Data represent the means ± SE for 3 mice. *P < 0.05 vs control mice treated with vehicle only (unpaired t test). B, Effects of pimobendan on survival of TNNT2<sup>K210/K210</sup> mice. From 30 days of age, pimobendan (3 mg/kg, n = 6; 30 mg/kg, n = 12) or vehicle only (methylcellulose, n = 11) was administered to TNNT2<sup>K210/K210</sup> mice orally once daily. Kaplan–Meier survival curves indicate that mice treated with pimobendan 30 mg/kg per day have significantly longer life spans than mice treated with vehicle only (logrank test, P = 0.001). C, Comparison of effects of pimobendan and amrinone on survival of TNNT2<sup>K210/K210</sup> mice. From 30 days of age, pimobendan (30 mg/kg, n = 5; 100 mg/kg, n = 5), amrinone (30 mg/kg, n = 6; 100 mg/kg, n = 8), or vehicle only (methylcellulose, n = 6) was administered to TNNT2<sup>K210/K210</sup> mice orally once daily. D, Comparison of effects of pimobendan and amrinone on ejection fraction and blood pressure of TNNT2<sup>K210/K210</sup> mice. Pimobendan (30 mg/kg, n = 6; 100 mg/kg, n = 5), amrinone (30 mg/kg, n = 4; 100 mg/kg, n = 3) or vehicle only (methylcellulose, n = 3) was administered to 5- to 8-week-old TNNT2<sup>K210/K210</sup> mice orally, and ejection fraction (EF) and systolic blood pressure (SBP) were measured 2 to 3 hours later. Data represent the means ± SE. E, Comparison of effects of pimobendan and amrinone on PLB phosphorylation in the left ventricular myocardium from 5- to 8-week-old TNNT2<sup>K210/K210</sup> mice. Immunoblot analysis was performed with anti-PLB monoclonal antibody (Abcam) and anti–phospho-PLB-Ser16 polyclonal antibody (Abcam) to obtain relative phosphorylation levels of PLB. Data represent the means ± SE (n = 4). Statistical significance was determined by ANOVA followed by post hoc Tukey’s multiple comparison test in D and E. *P < 0.05, **P < 0.01, ***P < 0.001.
transient to offset the reduced power output of myocardium until it decompensates.

Mutant mice showed a high incidence of death, and two-thirds of TNNT2K210R mice did not survive to 3 months of age (Figure 4D). In most cases, they died suddenly, without showing overt congestive heart failure symptoms, such as decreased spontaneous movement activity and dyspnea, to at least a day before their death. Instantaneous death was frequently observed by mild chest compression, suggesting that their hearts are very susceptible to fatal arrhythmias caused by mechanical stimuli. Preliminary experiments suggest that cardiomyocytes of mutant mice have a longer action potential duration and greater tendency to develop early afterdepolarization than wild-type mice, which might be responsible for their frequent sudden death (data not shown). Further studies are required to clarify the molecular mechanisms of sudden death, probably involving electrophysiological abnormality of the heart.

Finally, pinobendan had marked effects of preventing cardiac enlargement, heart failure, and sudden death of TNNT2K210R mice exhibiting a particularly malignant phenotype. The results demonstrate that decreased myofilament Ca$^{2+}$ sensitivity is the absolute causal functional defect triggering the pathogenesis of DCM associated with the mutation ΔK210 in cTnT and suggest that Ca$^{2+}$ sensitizers, such as pinobendan, might be beneficial for the treatment of DCM patients affected by mutations of the sarcomeric regulatory proteins troponin and α-tropomyosin, which have been demonstrated to have a common functional consequence of decreasing myofilament Ca$^{2+}$ sensitivity in vitro.10,16,17,27 The present study also demonstrated that cardiac myofilament Ca$^{2+}$ sensitivity, and thus its primary regulator, troponin complex, has an extremely important role in maintaining the physiological structure and function of the heart.

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Disclosures

None.

References

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Cheng-Kun Du, Sachio Morimoto, Kiyomasa Nishii, Reiko Minakami, Mika Ohta, Naoto Tadano, Qun-Wei Lu, Yuan-Yuan Wang, Dong-Yun Zhan, Misato Mochizuki, Satomi Kita, Yoshikazu Miwa, Fumi Takahashi-Yanaga, Takahiro Iwamoto, Iwao Ohtsuki and Toshiyuki Sasaguri

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Expanded Materials and Methods

Generation of a Knock-In Mouse Model by Gene Targeting

Fragments from mouse TNNT2 genomic DNA (Genbank accession No. AB052890) were assembled into a targeting vector. The vector consisted of a BamHI (intron 2)-BamHI (intron 12) 9.3 kb cTnT gene fragment, a BglII-BamHI 1.7 kb fragment including the loxP-neo cassette, and a BamHI (intron 12)-NheI (in the intron downstream of exon 16) 2.8 kb cTnT gene fragment, which were assembled into the BamHI-XbaI site of the pBluescript KS(-) plasmid (Stratagene). The targeting vector had a deletion of three base-pairs coding for K210 in cTnT on exon 13. An XhoI-SalI fragment of Diphtheria toxin A was inserted into the SalI site flanking the vector backbone. The targeting vector was linearized by digestion with SalI prior to electroporation, and gene targeting was performed using CCE embryonic stem cells with a G418 concentration of 250 µg/ml. After germline transmission of the properly targeted clone, the neo drug selection marker was removed by crossing with a CAG-Cre transgenic mouse strain. Southern blotting was used to confirm the favorable targeting event using a NdeI-BglII 0.7 kb cTnT gene fragment as a probe. Genotypes were also determined by PCR analysis of genomic DNA isolated from tail biopsies using three primer mixtures: 5’-CCTAAGCCCCAGACCTATGC, 5’-GGTTTCTCCCCGTCCC, and 5’-CTCGTGCTTTACGGTATGC.

The experimental protocol was reviewed by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University, and carried out under the control of the Guideline for Animal Experiments in the Faculty of Medicine, Kyushu University, and The Law (No. 105) and Notification (No. 6) of the Japanese Government.
Preparation of Skinned Cardiac Muscle Fibers and Force Measurements

Ventricles dissected from the heart were skinned with 0.5% Brij-58 and a small fiber (about 200 µm in diameter) was dissected from the left ventricular papillary muscle, and isometric force was measured as described previously. Fiber samples were subjected to immunoblot analyses, and relative phosphorylation levels of cTnI were obtained by normalizing the optical densities of cTnI bands probed with anti-cTnI (phosphorylated in serine 22/23) antibody 5E6 (ab8291; Abcam, UK) to those probed with anti-cTnI antibody 4C2 (HyTest, Finland).

Preparation of Intact Cardiac Muscle Fibers and Force Measurements

The left ventricular papillary muscle was mounted horizontally in a thermostatically controlled chamber with a capacity of 0.2 ml and perfused with oxygenated Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 0.5 mM EDTA-Na₂, 10 mM HEPES, 11 mM D-glucose) at 37 °C at a flow rate of 1.5 ml/min. Isometric force evoked by a bipolar electrical field stimulation of 5 ms duration with a voltage about 30% above threshold was measured with a semiconductor strain gauge (AE801; Aksjeselskapet Micro-Elektronikk, Norway). After 30 min of equilibration with stimulation at 0.15 Hz, the muscle was gently stretched to the length of maximum systolic force, and isometric forces were measured at an electrical stimulation rate of 3 Hz. Ten consecutive force transients were averaged for wave form analysis.

Isolation of Cardiomyocytes

The heart was perfused at 37 °C in Langendorff mode at a constant perfusion pressure of 65 mmHg with oxygenated Krebs–Henseleit solution containing 50 mM BDM and 50 µM CaCl₂ for 10 min, and then with oxygenated Krebs–Henseleit solution (without EDTA) containing 50 mM BDM, 50
µM CaCl₂ and 420 U/ml collagenase type 2 (Worthington Biochemical Corp., USA) for about 30 min. Left ventricles were placed into 5 ml of oxygenated Krebs–Henseleit solution (without EDTA) containing 50 mM BDM and 100 µM CaCl₂. After mincing with scissors, cells were mechanically dispersed by repetitive suction and ejection with a 10 ml plastic pipette. The cell suspension was filtered through nylon gauze, and the cell pellet was resuspended in RPMI-1640 Medium (Sigma-Aldrich, Japan) supplemented with 10% FBS, plated onto laminin-coated glass coverslips and incubated for 15-60 min at 37 °C with 5 % CO₂.

**Fura-2 Loading and Simultaneous Measurements of Fluorescence and Sarcomere length**

The cardiomyocyte-attached coverslip was mounted on a thermostatically controlled chamber with a capacity of 1 ml, which was located on the stage of an inverted microscope and perfused with oxygenated Krebs–Henseleit solution at 37°C at a flow rate of 1.2 ml/min. Cardiomyocytes were loaded with fura-2 AM (0.5 µM) for 10 min at 37°C in oxygenated Krebs-Henseleit solution. Contraction was evoked by bipolar electrical field stimulation of 5 ms duration with a voltage about 30% above the threshold determined at 0.15 Hz via platinum wire electrodes. [Ca²⁺], in a single cardiomyocyte stimulated at 3 Hz was monitored as the ratio of fluorescence emitted at 510 nm with alternating excitation at 340 and 380 nm, collected through a 40× objective lens at 4 ms per ratio, using a fluorescence and contractility recording system (MyoCam and Photo-Multiplier system with Galvo-Driven HyperSwitch Dual Excitation Light Source; IonOptix, USA) as described previously⁵, and 10 consecutive Ca²⁺ transients were averaged for waveform analysis. Sarcomere length (SL) was simultaneously monitored at 240 Hz, and 10 consecutive SL transients were averaged for waveform analysis.
**Echocardiography, Electrocardiography (ECG), Blood Pressure Measurements and Cardiac Catheterization**

Transthoracic echocardiography (M-mode) and surface ECG were measured after sodium pentobarbital administration (50 mg/kg, i.p.) using a 14-MHz linear array probe with a diagnostic ultrasound system Nemio SSA-550A (Toshiba, Japan) and an ECG processor SP-2000 (Softron, Tokyo, Japan), respectively. Blood pressure was measured in conscious mice using a computerized noninvasive tail-cuff system BP-98A (Softron, Japan). Long-term ECG recordings in conscious mice were carried out using a telemetry system with subcutaneous radio transmitter model TA 10ETA-F20 (Data Sciences International, USA). For invasive hemodynamics in mice, a 1.4F Millar catheter (Millar Instruments, USA) was placed into the left ventricle through the right carotid artery to monitor real-time heart rate, arterial and ventricular pressure after administration of ketamin/xylazine (100mg/10mg/kg, i.p.).

**Myosin Isoform Contents**

MyHC isoforms in the left ventricular myocardium were separated on an SDS-PAGE gel according to the method of Rundell et al. 6, and relative β-isoform expression (% of total MyHC) was determined by an optical densitometric scan using Phoretix gel analysis software (Phoretix International, UK).

**Drug Administration**

Pimobendan (UD-CG 115 BS) was supplied by Nippon Boehringer Ingelheim Co., Ltd. (Japan). Amrinone was purchased from Sigma Chemical Co. (USA). Drugs suspended in 0.25 % methylcellulose solution were administered orally once daily at doses of 10-100 mg/kg while control mice received vehicles only.
References


Supplementary Figure I. Electron microscopy of left ventricular myocardium from 8-week-old wild-type (A) and $TNN^T2^{K210/K210}$ (B) mice. Scale bars, 9.5 µm
Supplementary Figure II. Sacoplasmic reticulum (SR) Ca\(^{2+}\) contents in left ventricular cardiomyocytes isolated from 4-month-old wild-type (WT), 4-month-old *TNNT2\(^{+/-}\Delta K210\) and 2-month-old *TNNT2\(^{Delta K210/Delta K210}\) mice. Cardiomyocytes were loaded with fura-2 AM, and the peak amplitude of Ca\(^{2+}\) transients induced by rapid application of 20 mM caffeine after steady-state stimulation at 1 Hz was used as an index of SR Ca\(^{2+}\) content. Peak amplitude of caffeine-induced Ca\(^{2+}\) transients in mutant mice is normalized to that in wild-type mice. Data represent the means ± SE for cardiomyocytes (n=12) from 3 hearts. Statistical significance was determined by ANOVA followed by post-hoc Tukey’s multiple comparison test.
Supplementary Figure III. Protein expression and phosphorylation levels of sarcoplasmic reticulum Ca\(^{2+}\)-pump (SERCA2a), ryanodine receptor (RyR2) and phospholamban (PLB) in the left ventricular myocardium from 8-week-old mice. Immunoblot analysis were performed with anti-SERCA2a monoclonal antibody (Abcam), anti-RyR2 polyclonal antibody (Chemicon International), anti-phosphoRyR2-pSer2809 polyclonal antibody (kindly donated by Dr. Andrew R. Marks), anti-PLB monoclonal antibody (Abcam), anti-phosphoPLB-Ser16 polyclonal antibody (Abcam), or anti-phosphoPLB-Thr17 polyclonal antibody (Santa Cruz Biotechnology). Protein was measured with bicinchoninic acid assay reagent and an identical amount of protein was loaded onto each lane of SDS-PAGE gel. Data represent the means ± SE (n=3). *P<0.05, **P<0.01 vs. WT (paired t-test).
Supplementary Figure V. Direct effects of pimobendan and amrinone on Ca\textsuperscript{2+} sensitivity of skinned muscle fibers prepared from \textit{TNNT2} \textsuperscript{K210/K210} mice. Data represent the means ± SE of determination on 4 fibers.
Supplementary Figure V. Effects of isoproterenol and propranolol on survival of $TNNT2^{K210/0\kappa K210}$ mice. From 30 days of age, isoproterenol or propranolol (0.1 mg/kg, n=6) was administered to mice intraperitoneally once daily.
Supplementary Figure VI. Effects of pimobendan treatment on the Ca\(^{2+}\) handling and dynamics of cardiomyocytes and mechanical contraction of intact cardiac muscle fibers in \(TNNT2^{ΔK210/ΔK210}\) mice. Pimobendan was administered at a dose of 100 mg/kg orally once daily for 4 weeks from 4 weeks of age. A, Ca\(^{2+}\) transients induced by electrical stimulation at 3 Hz in left ventricular cardiomyocytes. Curves represent averaged data of 10 cardiomyocytes prepared from 2 hearts. B, Sarcomere length (SL) changes induced by electrical stimulation at 3 Hz in left ventricular cardiomyocytes. Curves represent averaged data of 10 cardiomyocytes prepared from 2 hearts. C, Isometric force generated by electrical stimulation at 3 Hz in intact left ventricular papillary muscle fibers. Curves represent averaged data of 3 fibers from different hearts. WT and \(TNNT2^{ΔK210/ΔK210}\) mice were 8-week-old. Data represent the means ± SE. Statistical significance was determined by unpaired t-test.
Supplementary Figure VCa²⁺ sensitivity of skinned cardiac muscle fibers prepared from pimobendan-treated TNNT2 △K210/△K210 mice. Pimobendan was administered to mice at a dose of 100 mg/kg orally once daily for 4 weeks from 4 weeks of age. Data represent the means ± SE of determination on 6 fibers.