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Myofilament Incorporation Determines the Stoichiometry of Troponin I in Transgenic Expression and the Rescue of A Null Mutation

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Abstract

The highly organized contractile machinery in skeletal and cardiac muscles requires an assembly of myofilament proteins with stringent stoichiometry. To understand the maintenance of myofilament protein stoichiometry under dynamic protein synthesis and catabolism in muscle cells, we investigated the equilibrium of troponin I (TnI) in mouse cardiac muscle during developmental isoform switching and in under- and over-expression models. Compared with the course of developmental TnI isoform switching in normal hearts, the postnatal presence of slow skeletal muscle TnI lasted significantly longer in the hearts of cardiac TnI (cTnI) knockout (cTnI-KO) mice, in which the diminished synthesis was compensated by prolonging the life of myofilamental TnI. Transgenic postnatal expression of an N-terminal truncated cTnI (cTnI-ND) using α-myosin heavy chain promoter effectively rescued the lethality of cTnI-KO mice and shortened the postnatal presence of slow TnI in cardiac muscle. cTnI-KO mice rescued with different levels of cTnI-ND over-expression exhibited similar levels of myocardial TnI comparable to that in wild type hearts, demonstrating that excessive synthesis would not increase TnI stoichiometry in the myofilaments. Consistently, haploid under-expression of cTnI in heterozygote cTnI-KO mice was sufficient to sustain the normal level of myocardial cTnI, indicating that cTnI is synthesized in excess in wild type cardiomyocytes. Altogether, these observations suggest that under wide ranges of protein synthesis and turnover, myofilament incorporation determines the stoichiometry of troponin subunits in muscle cells.

Keywords

myofilament; troponin; stoichiometry; cardiac troponin I knockout mice; cardiac muscle

Skeletal muscle fibers and cardiac myocytes contain abundant myofibrils consisting of myosin and actin filaments that are precisely assembled in the form of sarcomeres [1]. Muscle cells have high capacities of protein synthesis and catabolism [2]. On the other hand, the highly organized contractile machinery in skeletal and cardiac muscles requires the assembly of myofilament proteins with a stringent stoichiometry. The maintenance of myofilament protein stoichiometry is important during muscle and heart development or remodeling in aging, disuse, malnutrition, cachexia, and myocardial hypertrophy or dilated cardiomyopathy.

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Muscle contraction is regulated by Ca^{2+} through the troponin complex in the actin thin filaments. Troponin I (TnI[1]) is a subunit of the troponin complex [3]. Amphibian, avian and mammalian species have evolved with three TnI isoforms in cardiac, fast and slow skeletal muscle fibers [4]. Physiological and pathophysiological switches of troponin isoforms occur during muscle and heart development and adaptation as rapid processes [5-8], consistent with the short half-life of troponin subunits in muscle cells (3-4 days, [9]). Embryonic hearts express exclusively slow skeletal muscle TnI (ssTnI) that is down-regulated during postnatal development while cardiac TnI (cTnI) is up-regulated [6,10]. Deletion of the cTnI gene (*Tnni3)* from mouse genome resulted in postnatal lethality when ssTnI in the cardiac muscle of homozygote mice decreases to below a critical level [11].

Interestingly, over-expression of myofilament proteins, including TnI, in transgenic mouse hearts using the strong α-myosin heavy chain (MHC) promoter resulted in competitive replacement of endogenous protein with no increase in stoichiometry [12]. Although this phenomenon has been observed in numerous studies, the cellular mechanism has yet to be established. The high tolerance of muscle cells to the over-expression of TnI [13] and troponin T (TnT) [14] indicates an effective removal of excessive troponin subunit proteins. On the other hand, a single copy of cTnI gene in heterozygotes of *Tnni3*-deleted mice could sustain cardiac function [11]. Similarly, a single copy of slow skeletal muscle TnT gene can sustain skeletal muscle function in the heterozygotes of a recessive nemaline myopathy found in the Amish [15,16]. Therefore, the syntheses of TnI and TnT might already be in excess in normal diploid muscle cells.

To understand the regulation of troponin stoichiometry in muscle cells under dynamic protein synthesis and turnover, we investigated in the present study the equilibrium of ssTnI and cTnI in cardiac muscle during developmental isoform switching and the slower turnover rate of ssTnI in cardiac muscle of cTnI knockout (cTnI-KO) mice in the absence of cTnT synthesis. Rescuing the postnatal lethality of the cTnI-KO mice with transgenic expression of an N-terminal truncated cTnI (cTnI-ND) accelerated the turnover of ssTnI and cTnI-ND alleles with different levels of over-expression produced similar normal levels of TnI in the cTnI-KO cardiac muscle. These experiments suggest that myofilament incorporation determines the stoichiometry of troponin subunits in muscle cells.

MATERIALS AND METHODS

Genetically Modified Mouse Lines

A cTnI-KO mouse line was generated as described previously by homologous recombination-mediated deletion of the entire *Tnni3* gene in embryonic stem cells [11]. Due to postnatal lethality of homozygous *Tnni3* deletion, the *Tnni3* knockout allele was maintained in heterozygous lines.

Two lines of transgenic mice (Line #7 and line #27) over-expressing an N-terminal truncated cTnI (cTnI-ND) under an α-MHC promoter [17] were generated previously [18]. cTnI-ND was originally found as a product of restricted proteolytic modification that selectively removes the cTnI-specific N-terminal extension from the conserved core structure [19]. cTnI-ND is present at low levels in normal adult ventricular muscle and is up-regulated in myocardial adaptation to haemodynamic changes [19] and in β-adrenergic deficient hearts [20]. Transgenically expressed cTnI-ND effectively incorporates into the cardiac myofilaments to produce functional effects [18].

[1]***Abbreviations used are:*** TnI, troponin I; cTnI, cardiac TnI; cTnI-ND, N-terminal truncated cTnI; cTnI-KO, cTnI gene deletion; mAb, monoclonal antibody; MHC, myosin heavy chain; PAGE, polyacrylamide gel electrophoresis; ssTnI, slow skeletal muscle TnI; TnT, troponin T.

Production of Double Transgenic Mouse Lines

cTnI-KO/cTnI-ND double transgenic mouse lines were generated by crossing single transgenic mouse lines cTnI-ND#7 and cTnI-ND#27 with cTnI-KO heterozygous mice (cTnI-KO$^{+/-}$). Genotype screening of the offspring was done by polymerase chain reaction (PCR) on genomic DNA isolated from tail biopsies as described previously [11,18]. The lack of intact cTnI and the expression of cTnI-ND were confirmed by Western blotting of total cardiac muscle protein extracts as described below.

All animal procedures were approved by the Institutional Animal Care and Use Committee 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.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Immediately after isolation, cardiac muscle samples from ventricular apex were homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS using a high speed mechanical tissue homogenizer to extract total muscle proteins. The protein extracts were immediately heated at 80°C for 5 min to inactivate tissue proteases. The samples were resolved using 14% SDS-PAGE in Laemmli buffer system with an acrylamide:bisacrylamide ratio of 180:1 using a Bio-Rad mini-gel system. The protein bands resolved on the gel were visualized using Coomassie Blue R250 staining.

The protein bands from duplicate gels were transferred to nitrocellulose membranes using a Bio-Rad Lab semidry electrotransfer apparatus. The blotted nitrocellulose membranes were blocked with 1% bovine serum albumin in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and incubated with an anti-TnI C-terminus monoclonal antibody (mAb) TnI-1 [21] or an anti-cardiac TnT mAb CT3 [14] diluted in Tris-buffered saline containing 0.1% bovine serum albumin. The subsequent washes, incubation with alkaline phosphatase-labeled anti-mouse IgG second antibody (Sigma), and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate reaction were carried out as described previously [8].

Data Analysis

Densitometry analysis of SDS-gels and Western blots was performed on images scanned at 600 dots/inch using the NIH Image software version 1.61. Quantitative data were documented as mean ± SEM. The linear range of Western blot quantification of TnI and TnT was determined on serial dilutions of mouse cardiac muscle samples (Fig. 1). Statistical significance of differences between the mean values was analyzed by unpaired two-tail Student's *t* test.

RESULTS

Higher Level and Longer Presence of ssTnI in Postnatal cTnI-KO Mouse Hearts

The ssTnI gene is expressed at high levels in normal embryonic cardiac muscle and down-regulated during postnatal heart development, concurrent with the up-regulation of cTnI [10] (Fig. 2A, left panel). In comparison, higher levels of ssTnI were found in neonatal cTnI-KO mouse hearts with prolonged presence during postnatal development when cTnI was absent (Fig. 2A, right panel).

Level of TnI in Cardiac Muscle Determined the Survival of cTnI-KO Mice

Previous large-scale breeding documented that almost all of the cTnI-KO mice died at 18 days of age [11]. Interestingly, our experiments with early genotyping and removal of wild type and heterozygote littermates reproducibly prolonged the life of the cTnI-KO pups to 20-22 days. In Western blots using mAb TnI-1 that recognizes cTnI and ssTnI with similar affinities [21],

we compared the level of total TnI (cTnI and/or ssTnI) in the hearts of cTnI-KO pups at their natural death with that in the hearts of wild type mice of similar age. As predicted, cTnI-KO mouse hearts showed significantly lower levels of total TnI than that in wild type hearts. However, the levels of total TnI in the ventricular muscle of cTnI-KO pups at death were very similar despite the different ages of survival (Fig. 2B). Therefore, the decreased level of TnI in the heart to below a critical level, rather than the age *per se*, determined when death would occur. It is worth noting that the SDS-gel in Fig. 2B showed that the cardiac muscle tissues collected from dead cTnI-KO pups had no visible protein degradation, verifying the sample integrity.

A possible explanation for the prolonged survival when the non-KO pups were removed early is that a better nutrition from eliminating milk-competing littermates might have helped to preserve ssTnI in the cardiac myofilaments of cTnI-KO mice by reducing the rate of overall protein degradation [22]. Another possibility is that decreased physical competition had reduced the level of stress in the pups and thereby decreased the stress on the heart to reduce the turnover rate of myofilament proteins.

TnI Insufficiency Reduced the Amount of Cardiac Myofilaments

Using mAb TnI-1 that recognizes a single epitope at the C-terminus of each TnI polypeptide, the two dimensional quantification of the Western blot intensity can evaluate the number of TnI molecule independent of the molecular weight of the TnI variants. Densitometry quantification of the Western blots by normalization to the level of actin measured from the accompanying SDS-gels determined that the ssTnI level in the cardiac muscle of cTnI-KO mice at their natural death was approximately 37% of the level of total (slow and cardiac) TnI in wild type cardiac muscle at similar age (Fig. 2C). The level of ssTnI in the ventricular muscle of cTnI-KO mice reflects the minimal proportion of troponin-regulated contractile units required for sustaining a surviving level of cardiac function.

However, when normalized to the amount of total muscle proteins the level of TnI in the cTnI-KO cardiac muscle at death is only approximately 25% of that in wild type controls (Fig. 2D). Western blot using anti-cardiac TnT mAb CT3 showed a decrease in the amount of cardiac TnT to approximately 53% of the wild type level when normalized against the total proteins (Fig. 2D). SDS-gel densitometry analysis further showed that the levels of actin and MHC against other proteins in the extracts were both decreased in the cTnI-KO mouse hearts to approximately 70-75% of the wild type level when death occurred (Fig. 2D). This TnI < TnT < actin/myosin pattern suggests that the primary reduction of TnI caused reduction of troponin complex and in turn reductions of both thin and thick myofilaments in the cTnI-KO mouse cardiac muscle. The differentially decreased levels of TnI and actin in cTnI-KO mouse cardiac muscle versus total proteins were consistent with the 37% saturation of TnI in the thin filaments (Fig. 2C).

Transgenic Over-Expression of cTnI-ND Competitively Replaces Endogenous cTnI

To investigate the maintenance of TnI stoichiometry under over-syntheses, we examined the hearts of two transgenic mouse lines, #7 and #27, in which cTnI-ND was over-expressed using strong α-MHC promoter. The Western blots in Fig. 3 show the TnI isoform transition during postnatal development of the transgenic mouse hearts. In comparison with that in wild type mouse heart at the same age (Fig. 2A), the levels of endogenous ssTnI and cTnI were significantly lower in the transgenic mouse hearts when the transgenic expression of cTnI-ND was up-regulated. As a result, the amount of total TnI was maintained at physiological level, therefore, the various levels of transgenic over-synthesis competitively replaced endogenous TnI without increasing TnI stoichiometry in the cardiac muscle.

Although identical transgene construct was used [18], the developmental regulation and level of cTnI-ND expression were different between the two transgenic mouse lines. The Western blot profiles in Fig. 3 showed that the expression of cTnI-ND appeared earlier in line #7 than that in line #27 (detectable 1 day after birth versus not detectable until 7 days after birth) during postnatal development. Nonetheless, line #27 produced a higher level of cTnI-ND incorporation in the cardiac muscle than that of line #7, indicating a higher level of transgene expression. The likely reason for the different developmental timing and level of expression may be due to different chromosomal integration sites of the transgene DNA, although the transgene copy number might also affect the level of expression [23].

The normal timing of the postnatal down-regulation of ssTnI and up-regulation of cTnI was maintained in the cTnI-ND transgenic mouse hearts, indicating that the α-MHC promoter-directed transgenic expression and the over-expression level of cTnI-ND did not affect the developmental regulation of endogenous ssTnI and cTnI genes.

Transgenic Expression of cTnI-ND Rescued the Postnatal Lethality of cTnI-KO Mice

We successfully crossed the cTnI-ND #7 and cTnI-ND #27 transgene alleles to the homozygous cTnI-KO background. Independent Mendelian segregations were seen for the *Tnni3* KO allele and the cTnI-ND transgene alleles, indicating their locations on different chromosomes. Despite the later expression of cTnI-ND by transgene allele #27 and the lower level of expression by transgene allele #7 (Fig. 3), both alleles successfully rescued the postnatal lethality of cTnI-KO mice.

The cTnI-KO/cTnI-ND double transgenic mice survived to adulthood with normal baseline life activities and are fertile. Adult hearts of cTnI-KO/cTnI-ND did not have signs of hypertrophy or myopathy. The effective rescue of cTnI-KO mice by cTnI-ND convincingly demonstrated that cTnI-ND with a selective deletion of the N-terminal extension of cTnI [19] preserves the core function of TnI, confirming that this restricted proteolysis modification of cTnI seen in normal cardiac muscle and accelerated in myocardial adaptations is not a destruction but a functional regulation [18,20].

Total Level of TnI Remained Constant under Over-Syntheses

SDS-gel and Western blot quantifications examined the level of myocardial TnI in the over-expression and rescue mouse models. The data in Fig. 4A showed that the adult hearts of single transgenic mouse cTnI-ND line #7 and cTnI-ND line #27 produced different levels of competitive replacement of endogenous cTnI (approximately 50% and 80%, respectively, Fig. 4B), reflecting different levels of over-synthesis. However, the levels of total TnI in the two single transgenic lines were both similar to that in the wild type hearts when normalized to the level of actin or total cardiac muscle proteins (Fig. 4C).

Despite the different expression levels of transgene alleles #7 and #27, the levels of cTnI-ND in the hearts of the two double transgenic mouse lines were both similar to the wild type control when normalized to the level of actin or total cardiac muscle proteins (Fig. 4D). These data indicate that the production of cTnI-ND by the two transgene alleles was both sufficient or in excess for saturated incorporation into the myofilaments in the absence of endogenous cTnI competition. The constant level of TnI under different levels of over-synthesis suggests that the saturated level of myofilament incorporation determines the normal stoichiometry of TnI in cardiac muscle.

Single Copy of Tnni3 Is Sufficient to Sustain Normal Stoichiometry of cTnI in Cardiac Muscle

Heterozygote *Tnni3* KO mice survival without detectable phenotypes [11]. The Western blot quantification in Fig. 5 showed that the level of cTnI in cTnI-KO+/− heterozygote mouse hearts

was similar to that in the wild type hearts when normalized to the level of actin or total myocardial proteins. This result demonstrated that a single copy of cTnI gene produces sufficient amounts of cTnI protein to saturate the myofilaments, indicating that cTnI may be normally synthesized in excess in wild type cardiomyocytes with diploid *Tnni3*.

DISSCUSION

Consistent with previous studies [11,24], the cTnI gene knockout mouse model demonstrated that TnI is critical to the function of cardiac muscle and the decrease of total TnI to below 40% of the normal level in the actin filament resulted in death of cTnI-KO mice (Fig. 2). When the relative levels of TnI, TnT, actin and MHC were examined in the cTnI-KO hearts, the decrease of TnI appeared as a leading cause. The higher level of TnT relative to that of TnI in the cTnI-KO mouse cardiac muscle indicates some incorporation of TnT into the thin filament in the absence of TnI, which is consistent with the fact that TnT alone binds tropomyosin with high affinity [4,25]. However, the level of TnT relative to actin in the cTnI-KO hearts was lower than normal, suggesting that troponin complex is required for a normal level of thin filament incorporation.

In addition to the loss of myofilaments due to the insufficiency of TnI, the below saturation level of TnI in the cardiac thin filaments might be directly responsible for the impaired relaxation of the cTnI-KO mouse hearts, demonstrated by the severe diastolic dysfunction and diastolic heart failure followed by sudden death [11,24]. While the decrease in myofilaments would cause a decrease in contractile power, the presence of unregulated thin filaments due to the low stoichiometry of TnI in the thin filaments is anticipated to cause impaired diastolic function [24].

The fact that a single copy of cTnI gene could maintain the normal stoichiometry of TnI protein in cardiac muscle (Fig. 5) suggests that the production of TnI from diploid expression in wild type cardiac myocytes is likely in excess. This excessive synthesis of troponin subunits is plausible in preventing the harmful low stoichiometry. On the other hand, a significant amount of surplus TnI protein would need to be degraded in normal muscle cells. The high capacity synthesis and rapid removal of non-myofilament-associated TnI are consistent with the previously finding of a pool of free TnI and the rather short half life (3-4 days) of troponin subunits in the cardiac muscle [9].

The observation that over-expression of TnI in transgenic mice did not increase the protein stoichiometry in cardiac muscle is also consistent with a high baseline capacity of proteolysis for the maintenance of normal muscle structure and function. These posttranslational mechanisms in which myofilament proteins are competitively incorporated into the myofilaments and non-myofilament-incorporated proteins are rapidly degraded would help to understand various transgenic mouse models that over-express dominant mutations or isoforms of troponin subunits using the α-MHC promoter to effectively replace the endogenous protein at physiological stoichiometry for functional studies [12,13,18,20,23,26].

The non-accumulation of over-expressed TnI in cardiac myocytes is in agreement with a previous finding of effective clearance in skeletal muscle cells to eliminate a mutant slow skeletal muscle TnT that has low affinity to incorporate into myofilaments [16,27]. The high capacity of striated muscle cells in degrading non-myofilament-incorporated troponin subunits is also consistent with the long standing knowledge that muscle protein degradation is an early response to catabolic conditions [28].

A previous study found that the transcriptional timing of ssTnI gene did not change in cTnI-KO mouse hearts in which ssTnI mRNA disappeared after 12 days of age [29]. Therefore, the higher level and longer lasting of ssTnI protein in the hearts of cTnI-KO mice (still significant

at 18-22 days of age versus non-detectable at 14 days of age in wild type hearts, Fig. 2) indicate higher thin filament incorporation and delayed turnover of ssTnI protein in the absence of replacement by cTnI. This observation also suggests that the ssTnI incorporated in the cardiac myofilaments was protected from degradation.

The previous study also showed that thyroid hormone levels affected the postnatal expression of ssTnI by altering the transcription of ssTnI gene [29]. The observations that hyperthyroidism shortened and hypothyroidism prolonged the presence of ssTnI mRNA in postnatal cardiac muscle cTnI-KO mice support a role of metabolic equilibrium in regulating the level of myofilament proteins. By early removal of wild type and heterozygous littermates, we were able to prolong the life of cTnI-KO pups from 18 days to 20-22 days. This observation suggests that better nutritional conditions and/or less stress on cardiac function maybe able to increase the translational synthesis of myofilament proteins and/or reduce the demand on muscle protein degradation for systemic supply of amino acids.

The effect of protein metabolism on the fate of myofilament proteins may provide a rapid posttranslational mechanism in the development and physiological or pathological adaptation of heart and skeletal muscles. The concept that the incorporation and dissociation of troponin subunits into or from myofilaments are based on a competitive equilibrium helps to understand the regulation and function of troponin isoforms [5-8,10,16,30]. The finding that TnI stoichiometry in muscle cells is determined by myofilament incorporation also helps to understand how normal stoichiometry of myofilament proteins is maintained. Although TnI deficiency caused decay of myofilaments in cTnI-KO mouse hearts, the decreases in actin and myosin were much less than that of troponin (Fig. 2D), indicating an indirect effect. Therefore, troponin is unlikely the primary regulator for the number of myofilaments in muscle cells during myogenesis and muscle remodeling.

In summary, the present study investigated a posttranslational mechanism for the regulation of myofilament proteins. The maintenance of TnI stoichiometry by myofilament incorporation under an equilibrium of excessive synthesis and high capacity degradation of non-surplus, disassociated or defective proteins provides an effective protection of the highly organized structure of striated muscle.

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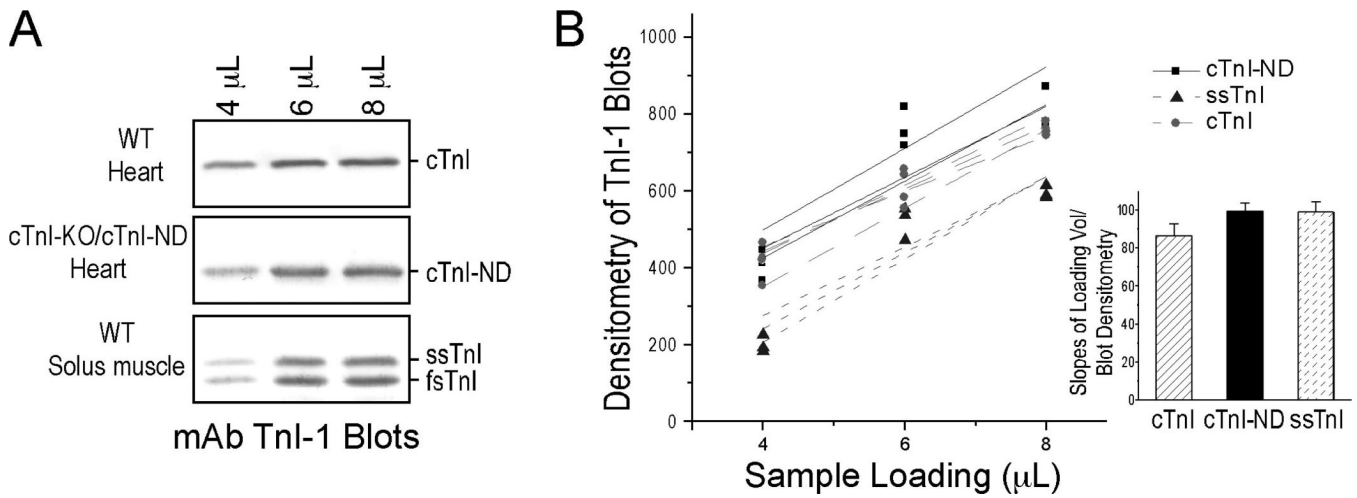

Fig. 1. Quantification of TnI variants using mAb TnI-1 Western blot densitometry

(A) Serially increased amounts of ssTnI in soleus muscle (n=3) and cTnI (n=4) or cTnI-ND (n=3) in cardiac muscles of wild type or cTnI-KO/cTnI-ND mice were examined using mAb TnI-1 Western blot. (B) Two-dimensional densitometry data of the mAb TnI-1 Western blots were analyzed by linear fitting against the amounts of loading. The bar figure showed similar quantitative slopes for the three TnI variants. This range of loading was used in subsequent Western blot quantifications in the present study. Values were presented as mean ± SEM by Student's t test.

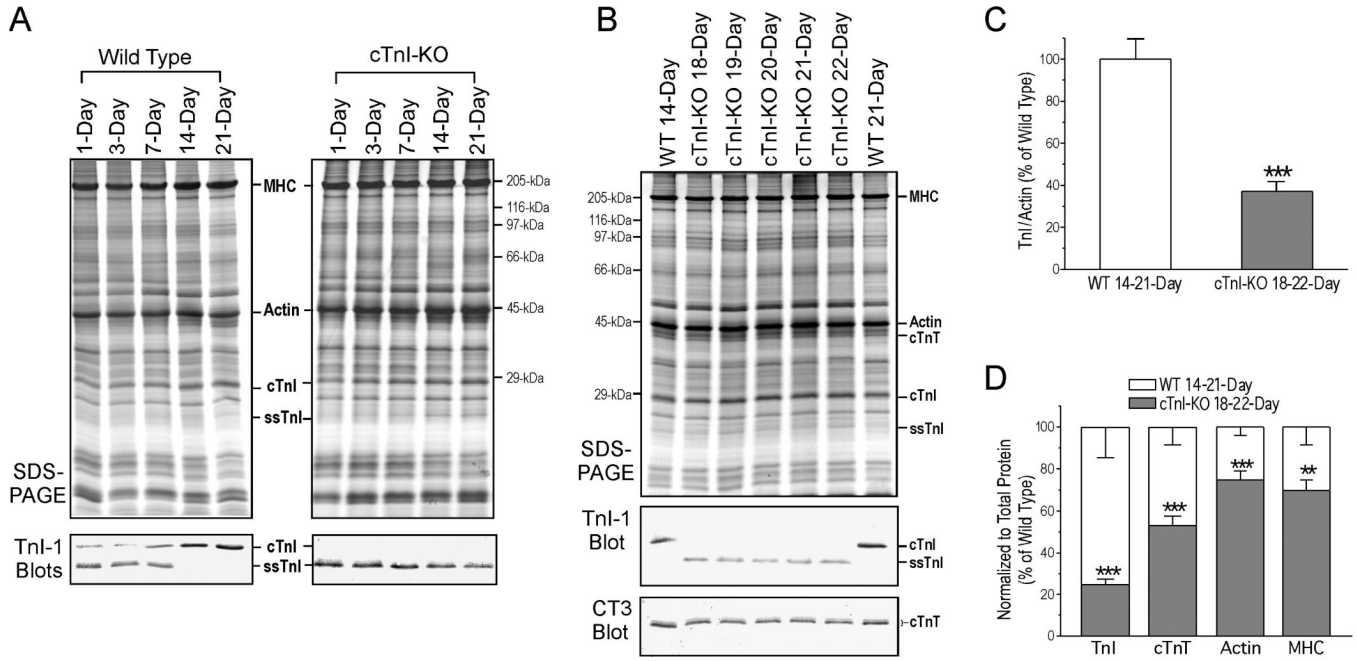

Fig. 2. ssTnI in cTnI-KO mouse cardiac muscle during postnatal development

(A) Higher level and longer lasting of ssTnI in cTnI-KO mouse heart. The SDS-PAGE and Western blots compared the developmental profiles of TnI isoform expression in wild type and cTnI-KO mouse hearts. Cardiac muscle samples were collected at 1, 3, 7, 14 and 21 days after birth and examined by SDS-PAGE and Western blot using mAb TnI-1 that recognizes both cardiac and skeletal muscle TnI. cTnI and ssTnI are found concurrently in day 1 to day 7 postnatal hearts of wild type mice. ssTnI decreases to undetectable 14 days after birth while the level of cTnI increases to become the sole TnI isoform in adult hearts. Only ssTnI was detected in cTnI-KO mouse hearts at levels higher than that in age-matched wild type hearts. The developmental decrease of ssTnI in cTnI-KO mouse hearts was slower in comparison to that in the wild type mouse hearts. (B) The mAb TnI-1 Western blot showed that the level of ssTnI in the cardiac muscle of cTnI-KO mice was significantly lower when death occurred in comparison to the levels of cTnI in wild type (WT) mouse hearts at similar age. Despite the different days of survival, the ssTnI levels at death were similar. The mAb CT3 Western blot showed that the level of cardiac TnT (cTnT) in the cTnI-KO mouse hearts was also decreased though to a less extent. (C) Densitometry quantification determined that the total TnI versus actin in the hearts of cTnI-KO mice at death was at approximately 37% of the normal level. (D) Densitometry analysis of the SDS-PAGE and Western blots further showed that the level of TnI versus total myocardial protein in the cTnI-KO mouse hearts decreased to 25% of the wild type level. The levels of cardiac TnT, actin and MHC versus total myocardial protein also decreased in the cTnI-KO hearts to 53%, 75% and 70%, respectively, of the wild type levels. Values are mean ± SEM, n = 6 in wild type and n = 7 in cTnI-KO groups. **, P<0.01; ***, P<0.001, compared with wild type by Student's *t* test.

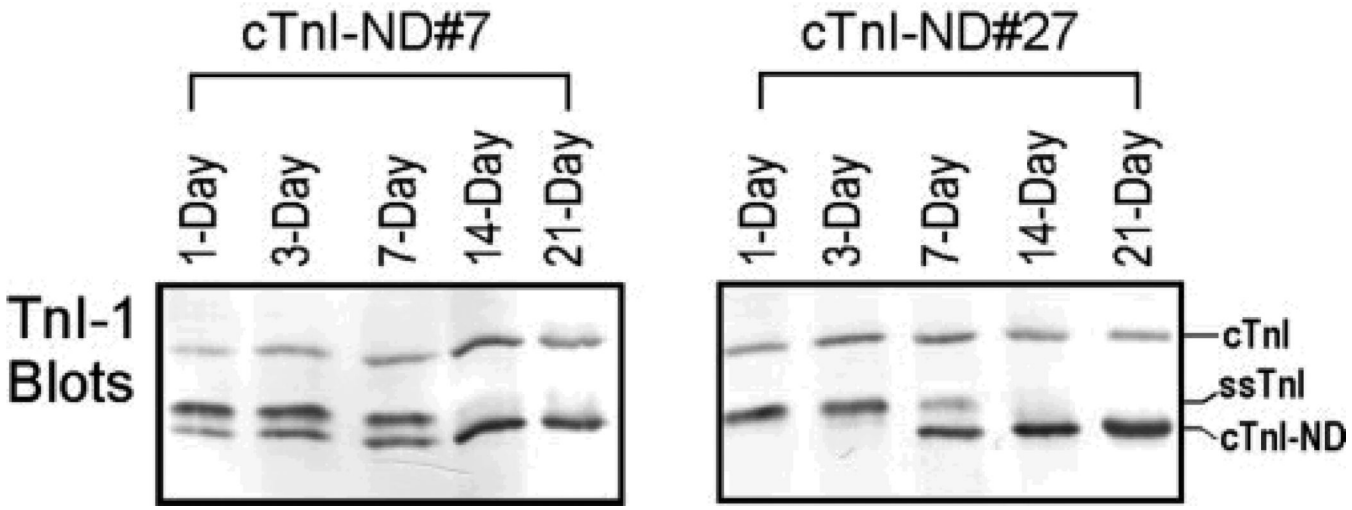

Fig. 3. Developmentally up-regulated expression of cTnI-ND in transgenic mouse hearts

The Western blots using mAb TnI-1 examined the α-MHC promoter-directed postnatally up-regulated expression of cTnI-ND in the hearts of two transgenic mouse lines. The expression of cTnI-ND in line #7 was early in postnatal hearts whereas the expression in line #27 was not detected until 7 days after birth. The levels of ssTnI and endogenous cTnI were decreased when cTnI-ND was over-expressed. The timing of developmental down-regulation of ssTnI and up-regulation of endogenous cTnI in the cTnI-ND transgenic mouse hearts was the same as that in wild type hearts (Fig. 2A).

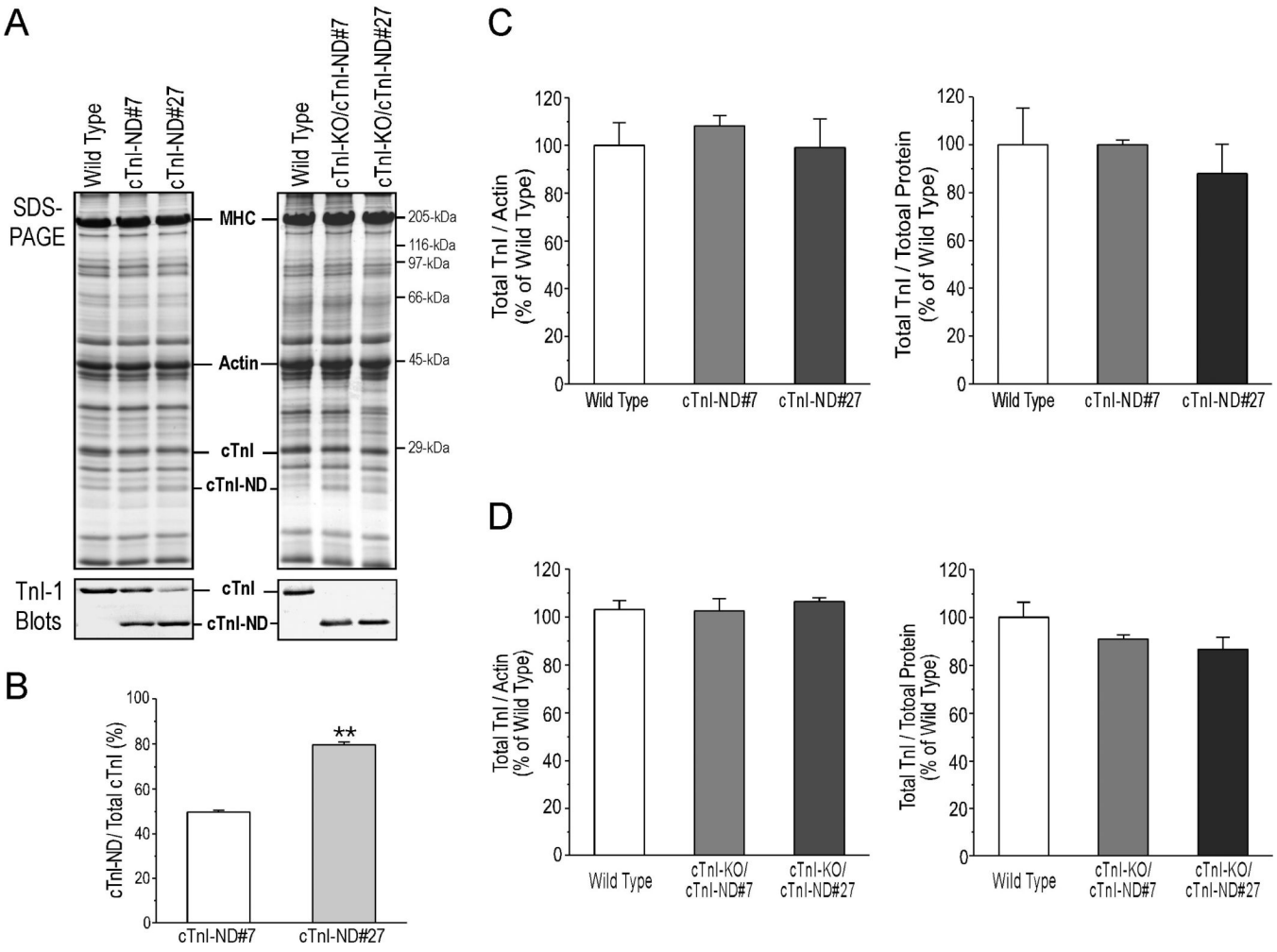

Fig. 4. Similar levels of total TnI in transgenic over-expression, rescued cTnI-KO, and wild type mouse hearts

(A) Hearts from 3 months old cTnI-ND line #7, cTnI-ND line #27, cTnI-KO/cTnI-ND line #7 and cTnI-KO/cTnI-ND line #27, and wild type mice were examined with SDS-PAGE and Western blot using mAb TnI-1. (B) Densitometry analysis of the SDS-gels and Western blots showed that in the single transgenic mouse hearts cTnI-ND replaced a higher portion of endogenous cTnI in line #27 than that in line #7, indicating different levels of over-expression. (C) Densitometry analysis showed that the total levels of TnI (endogenous and transgene-expressed) normalized to actin or total myocardial protein were similar in the two single transgenic mouse lines, which were also similar to the wild type control. (D) Densitometry analysis further showed that the levels of TnI-ND normalized to actin or total myocardial protein were similar in cTnI-KO/cTnI-ND double transgenic mouse lines #7 and #27 and comparable to that of intact cTnI in wild type hearts. Values are mean ± SEM, n = 3 in each group. **, $P<0.01$ compared with cTnI-ND line #7 by Student's *t* test.

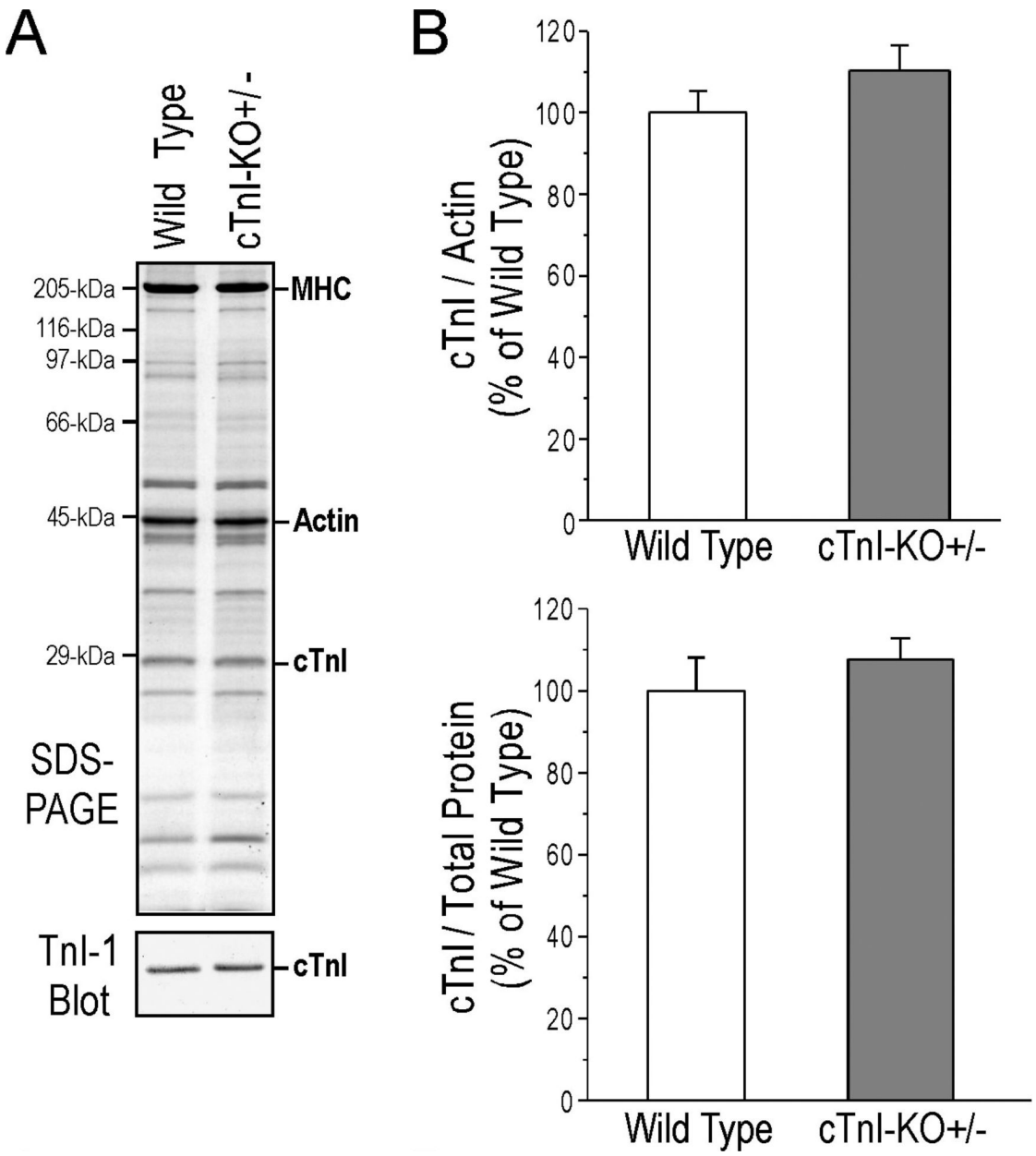

Fig. 5. Normal level of cTnI in the hearts of cTnI-KO+/− heterozygote mice

(A) SDS-PAGE and Western blot showed that the level of cTnI in cTnI-KO+/− heterozygote mouse hearts was similar to that in wild type hearts. (B) Densitometry analysis confirmed the observation that the level of cTnI normalized to actin or total protein was not decreased in cTnI-KO+/− hearts. n = 3 for wild type and n = 4 for cTnI-KO+/− groups.