ABSTRACT The functional significance of the developmental transition from slow skeletal troponin I (ssTnI) to cardiac TnI (cTnI) isoform expression in cardiac myocytes remains unclear. We show here the effects of adenovirus-mediated ssTnI gene transfer on myofilament structure and function in adult cardiac myocytes in primary culture. Gene transfer resulted in the rapid, uniform, and nearly complete replacement of endogenous cTnI with the ssTnI isoform with no detected changes in sarcomeric ultrastructure, or in the isoforms and stoichiometry of other myofilament proteins compared with control myocytes over 7 days in primary culture. In functional studies on permeabilized single cardiac myocytes, the threshold for Ca\(^{2+}\)-activated contraction was significantly lowered in adult cardiac myocytes expressing ssTnI relative to control values. The tension–Ca\(^{2+}\) relationship was unchanged from controls in primary cultures of cardiac myocytes treated with adenovirus containing the adult cardiac troponin T (TnT) or cTnI cDNAs. These results indicate that changes in Ca\(^{2+}\) activation of tension in ssTnI-expressing cardiac myocytes were isoform-specific, and not due to nonspecific functional changes resulting from overexpression of a myofilament protein. Further, Ca\(^{2+}\)-activated tension development was enhanced in cardiac myocytes expressing ssTnI compared with control values under conditions mimicking the acidosis found during myocardial ischemia. These results show that ssTnI enhances contractile sensitivity to Ca\(^{2+}\) activation under physiological and acidic pH conditions in adult rat cardiac myocytes, and demonstrate the utility of adenovirus vectors for rapid and efficient genetic modification of the cardiac myofilament for structure/function studies in cardiac myocytes.

The thin filament protein troponin I (TnI) plays an essential role in the regulation of striated muscle contraction. One approach to understanding the role of TnI within the contractile apparatus is to study functional differences among TnI isoforms. TnI isoforms are expressed in a muscle lineage-specific and developmentally regulated pattern (1, 2). In cardiac muscle, there are two developmentally regulated TnI isoforms. The slow skeletal TnI (ssTnI) isoform is expressed in fetal myocardium and the cardiac TnI (cTnI) isoform is expressed exclusively in adult myocardium (1, 2). During cardiac development, there are also marked alterations in cardiac contractile function, including an increase in the steepness and a rightward shift in the tension–Ca\(^{2+}\) relationship (1, 3). Thus, there is a correlation between TnI isoform expression and changes in myofilament function. However, the precise role of TnI isoforms in mediating these functional changes remains unclear, because developmental transitions in other contractile protein isoforms occur over the same time interval as the TnI isoform transition.

TnI isoforms are also postulated to influence cardiac myofilament pH sensitivity (4, 5). Contractile function decreases markedly during acute myocardial ischemia (4), and acidosis plays a significant role in this decreased function by reducing myofilament Ca\(^{2+}\) sensitivity (4–7). Solution studies indicate that TnI may play a role in this phenomenon because acidic pH-induced decreases in Ca\(^{2+}\) binding to and/or subsequent conformational changes within troponin C (TnC) are increased in magnitude in the presence of TnI (8). Studies using permeabilized muscle preparations also have implicated TnI in this pH effect. For example, the acidosis-induced shift in myofilament Ca\(^{2+}\) sensitivity is greater in cTnI-expressing adult myocardium than in slow skeletal muscle fibers (9, 10) or fetal/neonatal myocardium (3, 5), which express ssTnI (1, 2). Together, these results suggest that TnI influences the myofilament Ca\(^{2+}\) sensitivity response to acidosis in an isoform-dependent manner. However, direct confirmation that TnI isoforms influence the acidosis-mediated shift in Ca\(^{2+}\) sensitivity is difficult to assess in cardiac myocytes because other contractile protein isoforms also differ across muscle lineages and during myocardial development (11). There are no previous reports documenting the effect of ssTnI alone on Ca\(^{2+}\)-activated tension in adult cardiac myocytes.

Thus, the major goal of the present study is to define the functional role of ectopically expressed ssTnI in adult cardiac myocytes as a means of further understanding the function of TnI isoforms within the contractile apparatus at physiological and acidic pH. Recombinant adenovirus is used to deliver a ssTnI expression cassette into adult cardiac myocytes in primary culture. Western blot and immunohistochemistry analyses of cultured myocytes show rapid and efficient expression and incorporation of ssTnI into the myofilament. Furthermore, ectopic ssTnI expression occurs without any detectable effects on the isoform composition or stoichiometry of other contractile assembly proteins, or on the highly ordered structure of the contractile lattice. Importantly, functional measurements of Ca\(^{2+}\)-activated tension under physiological-activating conditions directly demonstrate, for the first time to our knowledge, that TnI isoforms influence adult cardiac myocyte contractile function. Specifically, we observe a lower threshold for Ca\(^{2+}\) activation in adult cardiac myocytes expressing ssTnI, a phenotype similar to that of fetal/neonatal myocardium (1, 3). Functional studies further show that pH sensitivity is reduced in the adult cardiac myocytes expressing ssTnI.
ssTnI, indicating that TnI isoform expression significantly influences the myofilament response to acidosis.

### METHODS

#### Generation of Adenoviral Vectors

To construct recombinant adenovirus vectors, the plasmids pAdCMVssTnI and pJM17 (12) were cotransfected by calcium phosphate into a HeLa 293 cell line (Fig. 1A). The shuttle plasmid pAdCMVssTnI contained adenovirus serotype 5 sequences 0–1 map units (mu) and 9–16 mu flanking an expression cassette containing the cytomegalovirus (CMV) promoter, the coding sequence for the full-length rat ssTnI cDNA and its 3' untranslated region (13), and the simian virus 40 polyadenylation signal. The pJM17 plasmid was a 0–100 mu derivative of adenovirus serotype 5 containing a partial deletion in the E3 region and a 4.3-kb pBRX insert at 3.7 mu. The insert allowed replication of the plasmid in bacteria but made the viral genome too large to be packaged into the virus capsid (12). After homologous recombination, the expression cassette in pAdCMVssTnI replaced the pBRX insert and the E1 region of the genome (1–9 mu) in pJM17, thus making the recombinant adenovirus, AdCMVssTnI, capable of being packaged but replication defective. The HeK 293 cell line is adenovirus serotype 5-transformed and expresses the E1 region (1–11.3 mu) in trans (12), which allows for replication of the recombinant adenovirus. A similar strategy was used to generate AdCMVcTnI and AdCMVαTnT, which contain the adult rat cTnI and cardiac troponin T (TnT) cDNAs described previously by Murphy et al. (13) and Jin and Lin (14), respectively. To harvest high titer recombinant virus, infected HeK 293 cells were collected and lysed by three cycles of freezing and thawing. After removing cellular debris, the supernatant of the viral lysate was stored at −80°C. Southern blot analysis was used to identify recombinant adenovirus as described by Sambrook et al. (15), and the blot identifying ssTnI is shown in Fig. 1B.

#### Primary Cultures of Rat Ventricular Cardiac Myocytes

Ventricular myocytes were isolated using a protocol adapted from Haworth et al. (16). Briefly, hearts from heparinized, female Sprague–Dawley rats (200 g) were mounted on a modified Langendorff apparatus, perfused for 5 min with Krebs–Henseleit buffer (pH 7.40; 118 mM NaCl, 4.8 mM KCl, 25 mM Hepes, 1.25 mM K2HPO4, 1.25 mM MgSO4, 11 mM glucose) containing 1 mM CaCl2 (KHB-B), followed by KHB lacking added Ca2+ (KHB-B) for 5 min, and then with 60 ml of recirculating KHB-B perfusate containing collagenase (0.5 mg/ml; type II, Worthington) and hyaluronidase (0.2 mg/ml; Sigma) for 15 min. After increasing the [Ca2+] in the digestion solution (1 mM), and continuing the perfusion for 15 min, isolated ventricles were minced into pieces and gently shaken in digestion solution with occasional trituration using silanized pasteur pipets. Undigested ventricular tissue was removed using a 230-μm mesh sieve. The cell suspension was centrifuged, and the [Ca2+] was increased to 1.75 mM after resuspending pelleted cells in KHB-A containing 2% BSA. After centrifuging cells again, they were resuspended in culture media containing serum [DMEM containing 50 units/ml penicillin plus 50 μg/ml streptomycin (P/S), and 5% fetal bovine serum]. Rod-shaped ventricular myocytes were plated onto laminin-coated coverslips in DMEM + P/S + fetal bovine serum for 2 hr and then infected with recombinant adenovirus (~170 plaque-forming units/cell) in serum-free media (DMEM + P/S). Serum-free medium (2 ml) was added 1 hr later and changed every 2–3 days thereafter.

#### Analysis of Protein Composition by Gel Electrophoresis and Immunoblotting

Myofilament proteins were analyzed by collecting 10 to 20 ventricular myocytes on a glass micropipet tip and then transferring the myocytes to microcentrifuge tubes containing 10 μl of sample buffer for separation by gel electrophoresis as described previously (17). For Western blot analysis, glass micropipettes were used to scrape cultured ventricular myocytes from coverslips into 10 μl of sample buffer 1 to 7 days after plating. Samples were separated by gel electrophoresis, and then blotted onto poly(vinylidene difluoride) membrane for 2,000 V h with immunodetection carried out as described by Westfall et al. (18). Gels and blots were scanned with an Arcus II laser densitometer (AGFA-Gevaert NV) and analyzed with Molecular Analyst software (Bio-Rad). TnI, TnT, and tropomyosin (Tm) isoform composition were determined using the primary TI-4 mAb (1:250; ref. 2), JLT-12 mAb (1:200; Sigma), and CH-1 mAb (1:100; Sigma), respectively.

#### Indirect Immunohistochemistry in Single Cardiac Myocytes

A dual-mAb protocol (18) was used to determine the extent of thin filament remodeling resulting from ectopic ssTnI expression within single cardiac myocytes in primary culture. The two primary mAbs used were cardiac-specific TI-1 mAb and TI-4, a mAb recognizing all striated isoforms of TnI (2). Anti-cTnI antibody (TI-1; 1:1,000) binding was detected with goat anti-mouse IgG mAb conjugated to Texas Red (1:100), whereas TI-4 mAb (1:500) binding was detected with fluorescein isothiocyanate-conjugated secondary antibody (1:200). Immunofluorescence was analyzed on a Leitz Aristoplan microscope, and representative cells were photographed using a Bio-Rad MRC 600 confocal microscope.

#### Characterization of Ca2+-Sensitive Tension in Single Cardiac Myocytes

Complete details of the experimental chamber and attachment procedure for mounting single cardiac myocytes and soleus fibers and for permeabilization and measurement of Ca2+-activated tension have been reported elsewhere (9, 17).

#### Statistics

An analysis of variance was used to test for significant differences between groups, with a post hoc Student–Newman–Keuls multiple comparison test to determine significance.

### RESULTS

Before studying the effect of TnI isoforms on contractile function, it was first important to demonstrate that adenovirus-mediated gene transfer leads to incorporation of an exogenous myofilament protein into the contractile apparatus, which has previously been difficult to establish (19). To this end we used two immunological assays to assess transgene expression and incorporation into the myofilaments of adult cardiac myocytes. First, Western blots were used to directly demonstrate ssTnI expression and incorporation into the contractile apparatus of...
AdCMVssTnI-treated adult cardiac myocytes (Fig. 2). In this assay, ssTnI content increased from 30% of total TnI content at 3 days to 76% at 4 days and 97% at 5 days, with high levels of expression maintained for at least 7 days post-AdCMVssTnI infection. This time course closely follows the 3.2-day half-life of TnI in cardiac muscle (20). Thus, from day 4 onward ssTnI was the dominant TnI isofrom in the AdCMVssTnI-treated adult cardiac myocytes. In addition, ssTnI expression on Western blots was not different for permeabilized myocytes and membrane-intact samples, which was evidence that ssTnI was incorporated into the thin filament regulatory complex and was not simply accumulating in the cytoplasmic space. Western blots also demonstrated that the cTnI isofrom was exclusively expressed throughout the culture period in controls (Fig. 2) and in cardiac myocytes treated with nonrecombinant adenovirus (results not shown). Densitometric analysis of the blots revealed that the stoichiometry of TnI expression was unchanged from controls throughout the culture period (Fig. 2). Thus, culture conditions and/or nonrecombinant adenovirus infection alone did not alter TnI isofrom expression and/or remodeling in adult cardiac myocytes.

In the second assay, dual immunostaining with the cTnI-specific TI-1 mAb and TI-4 mAb, which recognizes all striated isoforms of TnI, allowed cTnI to be distinguished from other TnI isoforms within a single ventricular myocyte. Adult cardiac myocytes expressing cTnI stained positive with TI-1 and TI-4 mAbs (Fig. 3 A and C), whereas slow-twitch soleus muscle fibers expressing ssTnI stained positive with TI-4 mAb, but not with TI-1 mAb (18). In fetal mouse ventricular myocytes, which primarily express ssTnI, the area and intensity of TI-4 mAb-associated immunofluorescence was much greater than the fluorescence observed after incubation with TI-1 mAb (18). Taken together, these results indicate that TI-1 mAb fluorescence is specifically associated with cTnI expression in the myofilament.

The TI-1 and TI-4 mAb immunostaining profiles of control rat cardiac myocytes in primary culture were positive and remained invariant throughout the entire 7-day culture period ($n = 500$ myocytes per day for days 1 and 4–7). In addition, confocal image analysis of staining with both antibodies showed the characteristic, periodic immunostaining pattern of striated muscle (Fig. 3 A and C). These results indicate that the cTnI isofrom was present within the cardiac contractile assembly throughout the culture period. In marked contrast, the immunostaining profile of AdCMVssTnI-treated cardiac myocytes progressively changed after gene delivery (Fig. 3 B and D). Cardiac myocytes staining positive for cTnI with TI-1 mAb decreased from 96.4% at 4 days ($n = 1,084$), to 87.6% at 5 days ($n = 1,110$), 39.6% at 6 days ($n = 897$), and 9.9% at 7 days ($n = 1,140$) after ssTnI gene transfer. Importantly, the TI-4 mAb immunostaining pattern in these cardiac myocytes did not change during this time and was indistinguishable from controls (Fig. 3). This is evidence that the endogenous cTnI isofrom was effectively replaced by the exogenous ssTnI gene product. A diffuse or irregular immunostaining pattern would be expected if ssTnI was nonspecifically distributed throughout the cell, a result that was not obtained. To determine whether adenovirus alone influenced TnI isofrom expression, we also examined the immunostaining pattern in myocytes treated with nonrecombinant adenovirus serotype 5 virus ($n = 138$) and found the pattern of staining with both anti-TnI antibodies was unchanged from controls during the entire culture period.

We also tested for possible alterations in the isofrom composition and stoichiometry of other key myofilament proteins. Sarcomere organization, myofilament protein expression, and stoichiometry were unchanged in control ventricular myocytes for at least 7 days in primary culture (unpublished data). These characteristics were also stable in ventricular myocytes treated with adenovirus carrying a reporter gene (unpublished data). In the present study, immunoblot analysis showed that the isofrom expression and stoichiometry of the adult isoforms of TnT and Tm were identical in control and AdCMVssTnI-treated cardiac myocytes (Fig. 2). Further, SDS/PAGE analysis showed normal expression of myosin heavy chain and normal isoforms and conserved stoichiometry of TnC, and myosin light chains 1 (LCl) and 2 (LC2) in AdCMVssTnI-treated cardiac myocytes (Fig. 4 Right).
The effects of TnI isoform composition on Ca$^{2+}$ alterations in the contractile machinery allowed us to examine protein within the cell. Exquisitely regulate the amount of geneic experiments, it appears that cardiac myocytes associated with overexpression of a normal or mutant myofilament protein in the indirect flight muscle of Drosophila melanogaster. Based on our results and those from transgenic experiments, it appears that cardiac myocytes exquisitely regulate the amount/content of each contractile protein within the cell.

The demonstration of TnI remodeling independent of other alterations in the contractile apparatus in the absence of other changes in myofilament composition. Finally, transmission electron micrographs (Fig. 4 Left) demonstrated normal sarcomeric structure in AdCMVssTnI-treated myocytes, indicating that gene transfer did not alter the highly ordered myofibrillar lattice. Taken together, these results provide strong evidence that the cardiac myocyte contractile apparatus is preserved after adenovirus-mediated transfer of the ssTnI cDNA, in agreement with earlier transgenic studies on other myofilament genes. Examination of other electron micrographs up to 5 days postinfection with AdCMVssTnI showed no differences in sarcomeric ultrastructure compared with control myocytes in primary culture. Ventricular myocytes in primary culture were fixed, embedded, and mounted as described previously.

These results are consistent with targeted remodeling of the contractile apparatus of cardiac myocytes at day 6 postinfection because immunofluorescence and Western blot analysis demonstrated marked incorporation of ssTnI at this time point. Representative isometric tension recordings over a range of Ca$^{2+}$ concentrations are shown in Fig. 5A for control and AdCMVssTnI-treated ventricular myocytes. Normalized maximum isometric tension was not different in AdCMVssTnI-treated cardiac myocytes compared with control values (Fig. 5A) or to values of control (lane 2) ventricular myocytes. Gels represent transgenic studies on other myofilament genes. (21, 22) Normalized maximum isometric tension was not different in AdCMVssTnI-treated cardiac myocytes and in single soleus skeletal muscle fibers at pH 7.00 (D) and pH 6.20 (E). Values in B-E are expressed as mean ± SEM. The pC$_{50}$ and n$_{H1}$ (see Results) values are derived from the pCa-P values as described in Methods, and n indicates the number of observations per point. The tension-pCa relationships in controls cultured for 6 days are unchanged from values obtained from acutely isolated myocytes. Thus, control data shown in B are pooled from control myocytes cultured for 6–8 days (n = 11–12 observations/point). Results for AdCMVssTnI-treated single cardiac myocytes in primary cultures (n = 11–14 observations per point). (D) Summary of pC$_{50}$ in control and AdCMVssTnI-treated cardiac myocytes and in single soleus skeletal muscle fibers at pH 7.00 (empty bars) and pH 6.20 (filled bars). (E) Summary of pC$_{50}$, pH 7.00 = pC$_{50}$ at pH 6.20 in control (n = 12) and AdCMVssTnI-treated (n = 11), and AdCMVssTnI-treated (n = 4; day 6) cardiac myocytes and in soleus skeletal muscle fibers (n = 5). Values in B–E are expressed as mean ± SEM. The pC$_{50}$ and n$_{H1}$ (see Results) values are derived from the tension-pCa curves as described in Methods, and n indicates the number of observations per point. The tension-pCa relationship in B and C. Cross indicates significantly different from control at pH 7.00 (D; P < 0.01). Asterisk indicates significantly different (P < 0.001) from control (E) or different from control at pH 6.20 (D).
obtained in acutely isolated myocytes (24). The similar maximum tension values for control and adenovirus-treated myocytes provides functional evidence that myofilament content and sarcomere architecture are maintained after transfer of the ssTnI cDNA into cardiac myocytes.

The tension recordings also used to derive the tension–log[Ca^{2+}] (pCa) relationship, and a summary of this relationship in control and AdCMVssTnI-treated adult single cardiac myocytes is shown in Fig. 5 B and C. A significant change at physiological pH (7.00) was observed in nH (Fig. 5 B and C), which is an index of myofilament cooperativity (ref. 17 and references therein). The nH was significantly lower in AdCMVssTnI-treated (nH = 1.6 ± 0.2; P < 0.05) cardiac myocytes compared with controls (nH = 3.0 ± 0.2) and was similar to the nH in soleus fibers (6, 9), and in fetal (3) and neonatal myocardium (1), which also express ssTnI (1, 2).

Further analysis of the tension-pCa relationship demonstrated that the pCa required to produce 50% of maximal tension (pCa50), a measure of myofilament Ca2+ sensitivity, was increased in AdCMVssTnI-treated cardiac myocytes compared with untreated controls (Fig. 5D). The pCa50 and nH in myocytes treated with nonrecombinant adenovirus (results not shown) were not significantly different from untreated control values. In a separate set of control experiments, cTnI gene transfer had no effect on the tension-pCa relationship after 7 days (control: pCa50 = 5.71 ± 0.04, nH = 2.5 ± 0.4, n = 6; AdCMVcTnI: pCa50 = 5.77 ± 0.02, nH = 2.6 ± 0.4, n = 5, P > 0.05). Thus, the increased pCa50 observed in AdCMV’sssTnI-infected cardiac myocytes relative to control values indicates that TnI isoform expression influences myofilament sensitivity to Ca2+. However, the pCa50 in AdCMVssTnI-treated myocytes was less than in soleus muscles (P < 0.05; Fig. 5D), isoforms of other contractile proteins likely contribute to the myofilament Ca2+ sensitivity differences observed between cardiac and slow skeletal muscles (3, 9). Functionally, the most important conclusion to draw from the increased Ca2+ sensitivity at pH 7.00 is that the threshold for Ca2+ activation is significantly reduced in AdCMV’sssTnI-treated cardiac myocytes compared with controls.

We also examined the influence of TnI isoform composition on the Ca2+-activated tension response to acidosis. Our results show that the pCa50 in cardiac myocytes expressing ssTnI was markedly increased at pH 6.20, relative to control values (Fig. 5D; filled bars). Further, the magnitude of the pCa50 difference between myocytes expressing ssTnI and control myocytes was greater at pH 6.20 (Fig. 5D; filled bars) than the magnitude difference observed at pH 7.00 (Fig. 5D; empty bars). To more clearly show the prominent effect of ssTnI expression on the pH sensitivity of Ca2+-activated tension, the ΔpCa50 was calculated (Fig. 5E) and found to be significantly reduced in the ssTnI-expressing myocytes, such that it was comparable to the ΔpCa50 in slow soleus fibers (Fig. 5E). These results directly show that TnI isoforms play a significant role in determining the sensitivity of the cardiac contractile assembly to acidosis. Acidosis had no apparent effect on nH in control or AdCMV’sssTnI-treated cardiac myocytes (pH 6.20; control nH = 3.6 ± 0.3, AdCMV’sssTnI nH = 2.0 ± 0.2; see above for pH 7.00 data).

In other control experiments, we studied the effect of acidic pH on contractile function using cardiac myocytes infected with AdCMVαTaTnI, an adenoviral vector containing an adult rat cardiac TnT cDNA (14). TnT was chosen because it is another key regulatory protein in the contractile apparatus and has a turnover rate comparable to TnI (20). The ΔpCa50 in myocytes treated with this adenoviral vector was not different from controls (Fig. 5E), indicating that overexpression of an endogenous cardiac isoform does not, in itself, alter myofilament Ca2+ sensitivity.

DISCUSSION

We report here that ssTnI gene transfer results in two significant alterations in the Ca2+-activated mechanical function of adult cardiac myocytes. First, the threshold for Ca2+-activated tension and molecular cooperativity are lowered in these myocytes. Tension development is evident over the pCa range of 7.0–6.5 in ssTnI-expressing cardiac myocytes, whereas over this same Ca2+ range the cTnI-expressing adult cardiac myocyte is fully relaxed. Second, the acidic pH-induced desensitization of Ca2+-activated tension is markedly reduced in ssTnI-expressing cardiac myocytes and is similar to that obtained in slow soleus fibers that also express ssTnI. These effects appear to be specific, as ssTnI gene transfer results in rapid and efficient expression and incorporation of ssTnI into the cardiac myofilament without detectable effects on the stoichiometry or isoforms of other key contractile/regulatory proteins, or on sarcomere ultrastructure. Finally, normalized maximum Ca2+-activated tension is not different between the ssTnI- and cTnI-expressing adult cardiac myocytes, indicating that peak mechanical performance is not altered by ssTnI gene transfer.

The overall effect of the ssTnI-induced changes in cardiac myocyte contractile function at physiological pH is to lower the threshold for Ca2+-activated tension without substantial changes in tension development at high Ca2+ concentrations. A reduction in the threshold for Ca2+-activation may result from altered interactions within a thin filament functional group, with a functional group defined as seven actins, one Tin, and one Tn (25). One possible interaction that could affect the Ca2+-activation threshold is the interaction between TinC and TnI. A stronger interaction between TnC and TinS compared with cTnI would lower the threshold for Ca2+-activated tension development in the adult cardiac myocytes expressing ssTnI. The inhibitory/carboxy terminal region of Tin is one possible domain that could be involved in strengthening the ssTnI-TnC interaction relative to cTnI-TnC. Solution studies indicate this region of Tin is necessary for the full inhibitory actions of Tin (26, 27) and interacts with the amino terminus of TinC that contains the regulatory Ca2+ binding site (27). Thus, isoform differences within this domain could directly affect Ca2+-mediated activation of the thin filament. In addition, other contractile protein isoforms, such as TinP, appear to influence submaximal Ca2+-activation (28, 29). Thus, interactions between Tin1 and other contractile proteins may be involved in the TinS isoform-dependent change in the threshold for Ca2+-activated tension.

The reduced threshold for Ca2+-activation of tension also may help to explain the decrease in cooperativity observed in cardiac myocytes expressing ssTnI. Cycling crossbridges are present in these cardiac myocytes over a range of Ca2+ concentrations, which does not activate the cTnI-expressing cardiac myocytes. Both Ca2+ and crossbridge binding to actin cause cooperative activation of the thin filament, in part by influencing the activation of neighboring functional groups (30). Brandt and colleagues (31) have suggested that cooperative activation of the thin filament decreases when both of the activating ligands, Ca2+ and attached crossbridges, are present. Thus, the presence of cycling crossbridges at low Ca2+ in cardiac myocytes expressing ssTnI may be one possible explanation for the observed decrease in cooperativity.

Our results provide direct evidence to support the hypothesis that ssTnI isoform expression also plays an important role in the reduced pH sensitivity observed in fetal/neonatal cardiac (3, 5) and slow skeletal (9, 10) muscle compared with adult cardiac myocytes. The acidosis-induced decrease in myofilament Ca2+ sensitivity is considerably lessened in cardiac myocytes expressing ssTnI rather than cTnI and is not different from the shift observed in slow skeletal muscle. The molecular mechanism by which TnI isoforms confer varied pH
sensitivity of contraction in cardiac muscle is not known. One possibility is that the interactions made between TnC and ssTnI are less affected by acidic pH compared with those between TnC and cTnI.

An important implication of our findings is that the increases in the threshold for Ca\(^{2+}\) activation, cooperativity, and sensitivity to pH found during normal cardiac development are due, at least in part, to the developmental transition in TnI isoforms. These structural and functional changes within the myofilament are also likely to be important for the function of the developing heart expressing ssTnI. The reduced threshold for myofilament Ca\(^{2+}\) activation may enhance myofilament force development during cardiac maturation, when intracellular Ca\(^{2+}\) handling systems are still maturing (32). In addition, relaxation may be slowed slightly in myocardium expressing ssTnI compared with relaxation rates observed in the presence of cTnI. Finally, one of the most important physiological functions of ssTnI in the maturing heart may be to preserve contractile function during periods of acidosis (5). The decreased developed tension and reduced rates of tension development and relaxation induced by acidosis in the intact heart are less severe in fetal and neonatal hearts compared with adults (5, 33), and this adaptation may be important for preserving cardiac function during transient periods of acidosis, such as during the birth process (34).

In addition to demonstrating the functional role of ssTnI in cardiac myocytes, our results show that adenovirus-mediated gene transfer can be used to rapidly and specifically remodel both myofilament structure and function in the highly differentiated adult cardiac myocyte. Future studies can now be designed using adenovirus-mediated gene transfer to investigate the functional domain(s) within TnI, which affect myofilament Ca\(^{2+}\) sensitivity, cooperativity, and pH sensitivity in adult cardiac myocytes. In addition, this approach may now be useful as a general model for studying the function(s) of other key myofilament proteins. Recently, Marian and colleagues (19) delivered a mutant myosin heavy chain into adult cardiac myocytes, although they were unable to structurally or functionally distinguish expression of the mutant myosin from endogenous myosin expression. Thus, the success of this approach critically depends on the protein isoform studied and its turnover rate within the contractile assembly. In this regard, structure/function studies may be more complicated when studying myosin, myosin light chain, or actin, myofilament proteins that all have longer endogenous turnover rates than TnI (20). Additional studies will be needed to determine whether adenovirus-mediated gene delivery can be used to deliver other contractile protein isoforms and/or mutants as a means of elucidating the functional role of these proteins.

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