Developmental Control of Titin Isoform Expression and Passive Stiffness in Fetal and Neonatal Myocardium

Sunshine Lahmers, Yiming Wu, Douglas R. Call, Siegfried Labeit, Henk Granzier

Abstract—Developmental changes in contractile behavior are known to occur during fetal and postnatal heart development. In this study, we examined whether adaptations take place in titin. A range of species was used to evaluate titin isoform expression and altered function during cardiac muscle development. A novel titin exon microarray that allows all 363 titin exons to be monitored simultaneously was used for transcript studies. Results reveal expression of fetal titin isoforms, characterized by additional spring elements both in the tandem Ig and PEVK region of the molecule. At the protein level, the fetal cardiac isoform predominates in fetal and neonatal myocardium and gradually disappears during postnatal development with a time course that varies in different species. Passive myocardium, contrary to previous reports, was found to be less stiff in the neonate than in the adult. This can be explained by the unique spring composition of fetal cardiac titin expressed by the neonate. Changes in titin expression are likely to impact functional transitions and diastolic filling behavior during development of the heart. (Circ Res. 2004;94:505-513.)

Key Words: diastole ▪ compliance ▪ filling ▪ connectin ▪ microarray

Large changes in hemodynamic load occur during the course of cardiac development and are known to be associated with changes in contractility due to alterations in isoform expression patterns of sarcomeric proteins. Whether changes occur in passive tension of the myocardium is less well established. Research performed decades ago revealed that passive stiffness is highest in fetal myocardium and progressively decreases with age. To our knowledge, this earlier work has not been followed up. Furthermore, the molecular basis of the adaptations in passive stiffness during cardiac development is also unknown, except that the total amount of collagen in fetal and adult myocardium appears unaltered. Considering that titin is a major source of passive stiffness in adult myocardium, we investigated titin’s role in the developmental regulation of passive stiffness. Titin is a giant protein that spans the half sarcomere with an I-band segment that functions as a molecular spring, the elastic properties of which define the passive mechanical properties of the cardiac myocyte. Titin is encoded by a single gene containing in humans 363 exons that are differentially spliced in the adult heart, creating the stiff N2B (short molecular spring) and more compliant N2BA (long molecular spring) isoforms. These isoforms can be coexpressed in the same sarcomere, allowing passive stiffness to be adjusted anywhere in-between that of stiff sarcomeres that express only N2B titin and compliant sarcomeres that express N2BA titin. Recent work revealed that differential splicing is subject to regulatory mechanisms that control entry to either N2B or N2BA splice-pathways. For example, the adult canine myocardium coexpresses N2B and N2BA titins at a similar level, but in response to long-term tachycardia the expression ratio shifts toward more prominent N2B expression, giving rise to increased passive muscle stiffness.

Considering that diastolic dysfunction is a high risk factor for perinatal mortality, we decided to study the role of titin in normal fetal and postnatal development in both human and animal model systems. We found that neonatal myocardium expresses a high level of an unusually large cardiac titin isoform that we named fetal cardiac titin. Ultrastructural studies showed that fetal cardiac titin is incorporated in the sarcomere, and passive mechanics revealed that fetal cardiac titin is responsible for low myocardial stiffness. During postnatal development, fetal cardiac titin is replaced by stiffer titin isoforms, giving rise to increased passive myocardial stiffness; we propose that this plays an important role in adjusting diastolic function during development.

Materials and Methods

Muscle Specimen

Left ventricular (LV) myocardium was collected from rabbits (Western Oregon Rabbit Company, Philomath, Ore), rats (Simonsen Laboratories, Inc, Gilroy, Calif), mice (Jackson Laboratories, Inc, Bar Harbor, Maine), and pigs (Swine Center, Washington State University, Pullman, Wash). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC).
SDS-PAGE and Western Blot Analysis of Titin

SDS-PAGE and Western blotting were as described. During the course of this work, a SDS-agarose gel electrophoresis system was published, and in later experiments this system was used.

Immunoelectron Microscopy

LV myocardial muscle strips in relaxing solution were stretched, immunolabeled, and processed for electron microscopy (EM) as described.

Transcript Studies

Total RNA was isolated and converted to biotinylated cDNA. Commercially available total RNA was used for all human transcript studies and total RNA was isolated from left ventricular myocardium from animal models (see the expanded Materials and Methods section available in the online data supplement at http://circres.ahajournals.org). An oligonucleotide array containing 385 probes was developed representing all of titin’s human gene exons, and normalization, positive, and negative controls (see online data supplement). Biotinylated target was hybridized to the array and quantified by a fluorescence-based detection system. The median intensity within the spot area was determined and normalized to the median intensity value of a constitutively expressed titin exon (exon 5 for all species other than rat and pig, which use exon 7 due to sequence dissimilarity in these species). A 5-bp mismatch probe for exon 5 (5MM) was used for evaluation of array specificity. All probes with normalized intensity less than the mismatch probes were considered to have no significant hybridization. Each exon probe was printed in duplicate and their median intensity was determined. The decrease in N2BA titin was less fast in the adult N2BA bands than in the fetal N2BA bands, with a mobility that is higher than that of the major N2BA isoform in the neonate. This suggests that the N2BA titins expressed by fetal/neonatal tissue are distinct from those in adult tissues. Because the slow migrating N2BA titins are expressed already in the fetus, we henceforth refer to them as fetal cardiac titins.

We also studied neonatal titin expression by SDS-agarose gels and Western blots in pig, a species that expresses a large level of N2BA titin in adult myocardium (as does human). Porcine neonatal LV (day 1) expresses a modest level of N2B titin and a high level of N2BA titin. The bands identified as N2B titin and N2BA titin in adult tissue are on Western blots relatively sharp, whereas the N2BA band of neonatal myocardium is relatively broad (Figure 2A, middle and bottom). The latter is most likely due to the presence of N2BA subsisforms in the neonatal tissues that are not well resolved after they have been transferred to the PVDF membrane. Agarose gels of neonatal myocardium indeed reveal a minor N2BA band that comigrates with the N2BA band of adult pig and adult bovine myocardium (Figure 2A, BLV), and thus, is likely to represent adult N2BA titin. In addition, there are two lower mobility N2BA bands, present at high levels. These are likely to represent fetal N2BA isoforms. We also determined the expression level of fetal (summing the two subsisforms) and adult N2BA isoforms as a function of age. The fetal isoforms rapidly decreased during the first few weeks of neonatal development and then more slowly, with a small amount still evident in 4-month-old animals (Figure 2B). Fitting results with a double exponential equation revealed a T1/2 of disappearance of fetal isoforms of ≈18 days. The adult N2BA isoform increased with age, its expression level was equal to that of the fetal isoforms in 1-month-old animals, and it was the only detectable N2BA isoform at 6 months (Figure 2B).

To determine whether the fetal N2BA isoforms are incorporated in the sarcomere, we performed immunoelectron microscopy (IEM) with an antibody to the C-terminal end of the unique sequence of the N2B element (N2B-Uc). This antibody labels, at a given sarcomere stretch, an epitope that is closer to the Z-disc in N2BA titins than in N2B titin, the so-called UC4 and UC6 epitopes, respectively (for details, see Trombitas et al). Results reveal normal sarcomeric structure in porcine neonatal myocardium with a strong epitope near the Z-disc (UC4) and a more spotty, sometimes barely visible, epitope toward the A-band (UC6; Figure 2C, top). This is consistent with gel and Western blot results and supports that the sarcomere of neonatal tissues expresses high levels of...
N2BA titin (strong near Z-disc epitope) and a low level of N2B titin (weak near A-band epitope). Adult tissues (6 months) show two approximately equal epitopes (Figure 2C, bottom), reflecting equal expression of N2B titin and N2BA titin.

Close inspection of micrographs revealed that the near Z-disc epitope (UC_A) in neonatal tissue is a doublet (UC_A(1) and UC_A(2), see Figure 2C, middle). This finding is consistent with the presence of N2BA subisoforms in neonatal myocardium. We measured the distance between the epitopes and the middle of the Z-disc, as a function of SL. The near A-band epitope derived from the N2B isoform (UC_B) were indistinguishable in the neonatal and adult tissues, suggesting that the N2B isoform is the same in both tissue types (Figure 2C, right, UC_B). The near Z-disc epitopes behaved differently, and the epitope closest to the Z-disc (UC_A(1)) increased less steeply with sarcomere stretch than UC_A(2), of the neonate and UC_A of the adult (Figure 2C, right). Slopes were significantly different (P<0.01). This suggests that the neonatal sarcomere incorporates a subisoform (giving rise to UC_A(1)) that is distinct from N2BA titin in the adult myocardium.

**Transcript Studies**

To test whether titin’s exon composition is developmentally regulated, we developed a titin microarray on which all 363 exons found in humans are represented (see online data supplement). The array was validated by determining the exon composition of adult human cardiac and soleus cDNAs, two tissues where titin has been sequenced. For soleus, the array detected all 313 exons present in the soleus cDNA (data library accession X90569). Of the remaining 50 exons that have not been detected in the soleus, 35 exons were absent on the microarray and 15 were positive. These 15 exons are from the central PEVK region where sequence
identity is high due to genomic duplication (during evolution LINE repeat insertions occurred) and thus cross-reactivity may explain the unexpected positives. Alternatively, these exons may have been missed when sequencing soleus cDNA. As a lower estimate, in soleus, the array appropriately characterized 96% of all 363 exons (concordant presence and absence, respectively, both by sequencing and array hybridization, 313/363 = 96%). For cardiac muscle, previous RT-PCR studies identified the cardiac-specific expression of the Z-disc sequence Zr6 (exon 11) and N2B (exon 49). The array confirmed that exons 11 and 49 are cardiac-specific (Figure 3A, middle and right), and furthermore demonstrated that these two exons are the only cardiac-specific exons in adult titin. Proximal Ig and PEVK exons were downregulated in adult cardiac muscle (relative to skeletal), as predicted by RT-PCR studies. Thus, tests indicate that the microarray is a powerful tool for rapidly determining the exon composition of titin isoforms.

Using commercially available total RNA (see Materials and Methods), we compared the titin exon repertoire expressed in human adult and fetal (16 to 22 weeks) cardiac muscle (Figure 3B). We found that 23 exons were greater than 5-fold upregulated (P < 0.01) in the fetal transcript (Figure 3B, left shows examples). Of these 23 upregulated exons all except one (the A-band exon 325) code for immunoglobulin (Ig)-like domains and PEVK elements found in titin’s elastic I band region (Figure 3B, right). Thus, the array data identified a fetal-specific exon subset and confirmed that fetal myocardium expresses a novel titin isoform, predicted to be more compliant than adult cardiac titin (see Discussion).

We studied the exon composition of the animal models used in Figures 1 and 2 by cross-hybridizing nonhuman cDNAs to the microarray. Because the microarray was designed for human titin, we tested whether it could be used to evaluate exon expression in animals. cDNA was isolated from soleus muscle, under the assumption that the exon compositions are identical to that of human soleus. (The available sequence of rabbit soleus titin supports this.) The human array probes successfully cross-hybridized and appropriately characterized (see earlier analysis) 77% of the exons expressed in soleus (the array detected 286 out of 363 exons predicted to be present in human soleus and absent were 50). Because the array appropriately identifies 77% to 93% of the exons in animal species, we conclude that the array has great utility for evaluating differential exon expression in nonhuman species.

RNA was isolated from neonatal LV (d0) and adult LV from mouse, rat, rabbit, and pig. Similar to what was seen for human transcripts, all species revealed upregulation of ~20 exons in neonatal myocardium (Figure 4A shows an example). The upregulated exons fall in (1) the previously described I27 to I79 splice region and (2) the PEVK region (Figures 4B through 4E). (No species has more than two additional differentially expressed exons outside the Ig and PEVK segments; results not shown.) We also examined the exon expression profile of fetal rat myocardium and found it to be largely identical to that of neonatal rat myocardium (results not shown).

**Passive Stiffness**

The presence of additional spring elements in fetal cardiac titins is predicted to result in a low titin-based stiffness. To test this, mechanical experiments were performed on skinned
muscles dissected from the LV wall of neonatal and adult pig. To measure passive tension, muscles were stretched from their slack length to a predetermined SL, with a velocity of 1 length/sec (the estimated in vivo stretch rate; see online data supplement). Total passive tension was significantly higher in adult than neonatal myocardium (Figure 5A). No significant differences in either slack sarcomere length (SL) or maximal active tension were found (Table). Determining the collagen-based and titin-based passive tension (see Materials and Methods) revealed that the higher total tensions of adult tissue is due to titin at all SLs (Figure 5B), with an increase in collagen-based tension at SLs longer than 1.95 μm (Figure 5C). We also converted passive tension to passive stiffness (slope of tension–SL relation) and determined average stiffness within the SL range of 1.95 to 2.25 μm. Results (Table) reveal that total passive stiffness in the adult tissue is about double (209%) of that in the neonate, and furthermore, that the majority of this increase is due to titin (79%), with a smaller but significant role for collagen (accounting for 21% of the stiffness increase). We also determined passive stiffness after a 1-minute hold (see Materials and Methods), and again found that passive stiffness was significantly higher in adult myocardium, with the majority of the difference due to titin (results not shown). Thus, measurements made during physiological stretch and after a 1-minute hold both reveal that adult myocardium has significantly higher passive stiffness than neonatal myocardium, and that the majority of this difference is due to titin.

**Discussion**

To study the exon composition of fetal cardiac titin, we developed a titin exon microarray that allows all 363 exons of the human titin gene (see Materials and Methods). RNA isolated from human adult skeletal (HAS) and human adult cardiac (HAC) muscles was reverse-transcribed, biotinylated, and hybridized to the exon microarray. Left, Differential hybridization between HAS (top) and HAC (bottom) cDNAs on the whole array level (note large blocks of exons that are only positive in skeletal muscle). Middle, Examples of constitutively expressed exons (5 and 7), cardiac-specific exons (11 and 49), and skeletal muscle–specific exons (156 and 210). 5MM indicates a 50-mer from exon 5 including 5-bp mismatches as a control for hybridization stringency. Right, Scatter plot of intensities of HAC versus HAS exons. Exons 11 and 49 are cardiac-specific. Downregulated exons (green) all fall in the proximal Ig and PEVK region, which have been shown to be skeletal muscle–specific.10,18 B, Differential expression of titin exons in human fetal cardiac (HFC) versus human adult cardiac (HAC) muscle. Left top, Examples of differentially expressed exons from a single microarray experiment. Two rows per sample show duplicate spots and reveal reproducibility. Exon 50 is an example of a constitutively expressed exon. Left, bottom, Domain organization of I-band region of largest N2BA cardiac isoform known,10 with Ig domains in red and PEVK in yellow. Many of the exons that are upregulated in fetal myocardium have not been previously described in cardiac transcripts, as indicated by insertion arrows. Right, Bar graph shows mean and SEM values of differentially expressed exons. All exons listed are in the fetal transcript significantly upregulated (P<0.01) by at least 5-fold (when compared with the adult tissue) and represent proximal Ig and PEVK exons.
the human titin gene to be rapidly evaluated. The array appropriately characterizes >96% of human soleus exons and therefore is a powerful tool for determining the exon composition of titin isoforms. Human transcript studies of fetal and adult myocardium revealed >5-fold upregulation of 23 titin exons in fetal myocardium. The combined molecular mass encoded by these exons is 118 kDa, explaining why the fetal isoforms can be detected as a separate slow migrating band on high-resolution gels. Of these upregulated exons, 22 code for spring elements found in the extensible I-band region of titin. Many of the exons that we identified in human fetal myocardium (Figure 3B, left) have not previously been identified in cardiac transcripts and include proximal Ig exons (I32, I34, I36, I38, I40, and I42), as well as PEVK exons (PEVK 67, PEVK 75, PEVK 83, and PEVK 85). The Ig exons have been described as skeletal-specific, and the PEVK exons were not known to be expressed in adult cardiac or skeletal muscle. We also used the human exon array to analyze titin transcripts expressed in mouse, rat, rabbit, and pig. Consistent with the high degree of conservation of the titin sequence in vertebrates, tests revealed that the array appropriately characterized ~80% to 90% of exons in soleus cDNA of species used in our work. Transcript studies revealed upregulation of titin exons in fetal and neonatal myocardium in all animal species. Consistent with findings from human cDNA, the overwhelming majority of upregulated exons fall in the I27 to I79 splice region as well as the PEVK region (Figures 4B through 4E). In the neonatal transcript, all species have upregulation of exons in the 52 to 76 exon region, which are not included in the largest known adult N2BA transcript. Together the microarray studies indicate that fetal and neonatal myocardium expresses novel titins specific for the developing myocardium. Because these isoforms are already present in the fetal myocardium and are downregulated in the adult, we named the isoforms fetal cardiac titin.

IEM used an antibody to the C-terminal end of the N2B element, which in the sarcomere is found closer to the Z-disc in N2BA titins than in N2B titin and, therefore, can be used to isoform type titin within the sarcomere. Neonatal porcine myocardium was characterized by strong near Z-disc epitopes and much weaker near A-band epitopes (Figure 2C), indicating that N2BA titin dominates. Because fetal cardiac titin has a longer extensible region than adult N2BA titin (see later), its binding site is expected to be closer to the Z-disk than that
of adult N2BA titin. This is consistent with the presence in the neonatal sarcomere of a near Z-disc doublet, with the epitope closest to the Z-disc (UC1) having a distance to the Z-disc that increases less steep with sarcomere stretch than the adult N2BA epitope does (Figure 2C). Thus, IEM provides evidence that fetal cardiac titin is incorporated in neonatal sarcomeres.

Fetal cardiac titin is characterized by the presence of a large number of additional exons that code for Ig domains that comprise the so-called middle tandem Ig segment of N2BA titin and exons that encode PEVK repeats. Some of these fetal PEVK repeats are included in a skeletal muscle PEVK fragment (TP1) that on the basis of in vitro studies has been proposed to function as a protein interaction site. Thus, expression of high levels of fetal titin could potentially enhance titin’s role in mediating protein interactions. The fetal-specific exons are also expected to greatly impact titin’s biomechanics. We calculated that as a result of the additional exons, the overall contour length of the extensible region of fetal cardiac titin is 150 nm longer than that of adult N2BA titin and 495 nm longer than N2B titin (see online data supplement). A given sarcomere stretch will therefore result in a comparatively low fractional extension in fetal titin, and hence in a low passive tension.

Using previously determined molecular characteristics, we simulated the force-sarcomere length relation of single fetal cardiac titin and N2B titin molecules (see online data supplement). Results show that fetal titin is much more compliant than N2B titin (Figure 6). Hence, titin-based stiffness is expected to be much less in neonatal than adult myocardium. We tested this in experiments on muscle strips isolated from porcine LV myocardium. Passive stiffness was more than double (209% increase) in adult than neonatal tissues, with most of the difference due to titin and a minor role for collagen (Figure 5 and Table).

### Table 1. Mechanical Properties of Porcine LV Wall Muscle

<table>
<thead>
<tr>
<th></th>
<th>Neonate*</th>
<th>Adult*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle length, mm</td>
<td>1.86±0.14</td>
<td>2.02±0.26</td>
<td>0.6</td>
</tr>
<tr>
<td>Slack SL, μm</td>
<td>1.88±0.02</td>
<td>1.90±0.02</td>
<td>0.52</td>
</tr>
<tr>
<td>Cross-sectional area, mm²</td>
<td>0.042±0.01</td>
<td>0.045±0.01</td>
<td>0.88</td>
</tr>
<tr>
<td>F_{max†} nN/mm²</td>
<td>42.3±6.8</td>
<td>57.3±4.8</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Neonate, 1 day old; adult, 6 months old. †F_{max} indicates maximal active tension (pCa 4.0) at SL 2.2 μm. ‡Passive stiffness, average slope of passive tension—sarcomere strain relation (strain, ΔSL/SLslack) in SL range of 1.95 to 2.25 μm. Values are mean±SEM (n=6 in each case).

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Passive mechanics of neonatal (1 day) and adult (6 months) pig LV myocardium. A, Total passive tension. B, Titin-based passive tension. C, Collagen-based passive tension. Inset shows mean tensions at three select SLs. Note significantly higher total passive tension and titin-based passive tension in adult. Shown are mean and SEM (n=6); for clarity SEM are only shown at selected SLs.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Predicted force developed by single titin molecule as function of SL. Titin develops restoring force below the slack SL (1.9 μm) and passive force above slack SL. Curves marked “neonate” and “adult” are the average forces per titin molecule in a sarcomere with 3.5 fetal titins per N2B titin molecule (expression ratio of d1 neonatal porcine myocardium) and in a sarcomere that contains an equal number of N2BA and N2B molecules (as in adult porcine myocardium), respectively.
Some of the titin-based stiffness increase could be due to a larger fractional area of myofibrils in adult tissue. Measurement of the fractional area of myofibrils in the porcine myocardium using electron microscopy revealed 37±5% for the neonate (day 1) and 49±6% in adult (6-months) tissue, or an increase of 32%. This relatively modest increase in fractional myofibrillar area is consistent with the maximal active tension that we measured with an increase in the mean tension of 35% when going from neonate to adult and is insufficient to account for the observed doubling of the titin-based passive stiffness. Thus, we consider it likely that replacement of (compliant) fetal titin with (stiff) N2B titin during neonatal development is a major factor that underlies the increase in titin-based stiffness during postnatal development.

Increases in passive tension during postnatal development have been reported recently in skeletal muscle fibers, although the underlying mechanism was not addressed. Our findings are in contrast, however, to results of previous studies of passive myocardial stiffness that reported decreased passive stiffness during fetal and neonatal development. Two main differences between the previous work and the present study stand out. First, the previous work used intact myocardium, and it is possible that under the experimental conditions that were used, actomyosin-based tension in fetal and neonatal myocardium did not fully decay to zero during the diastolic interval. Considering the high calcium-sensitivity of fetal and neonatal myocardium, a basal level of active tension may in intact fetal/neonatal myocardium augment low intrinsic passive stiffness of the myocardium. Second, in the present work, sarcomere length (SL) was measured on-line, using laser-diffraction, and passive tensions were plotted against SL. In the previous work, muscle length was measured and length changes were expressed relative to the muscle length at which twitch force was maximal (L_{max}). Thus, if the SL at which L_{max} occurs were to decrease during development an apparent reduction in passive stiffness would result (a small SL decrease could have a large effect on stiffness).

Based on our work, we propose that fetal myocardium has high intrinsic compliance due to the dominating expression of fetal cardiac titin and that during postnatal development, compliance decreases as a result of replacement of fetal titin by N2B titin. This proposal is overall consistent with known developmental changes in blood velocities during diastolic filling. Doppler echocardiography allows ventricular filling to be monitored noninvasively and reveals early (E-wave) and late (A-wave) diastolic filling. The amplitude of the A-wave depends on the strength of atrial contraction and ventricular compliance. The A-wave is relatively constant throughout development, suggesting that the increase in strength of atrial contraction that occurs during development is accompanied by a reduction in ventricular compliance. This inferred reduction in ventricle compliance during development is consistent with our mechanical studies (Figures 5 and 6) and warrants in future work direct measurement of chamber compliance during development. The amplitude of the E-wave (early filling) is dependent on active myocardial relaxation and ventricular recoil. The E-wave has been shown to greatly increase in amplitude during perinatal development, with most rapid changes occurring during the first postnatal week. It is likely that the developmental increase in E amplitude is due to faster relaxation (faster calcium uptake by the SR and lower calcium sensitivity of myofilaments), and due to enhanced ventricular recoil. Because titin develops restoring force in short sarcomeres, titin can contribute to ventricular recoil. Titin’s restoring force is predicted to be lowest for fetal cardiac titin (because its extensible region is longest) and highest for N2B titin (Figure 6). Thus titin-based ventricular recoil is predicted to increase throughout postnatal development (because expression shifts toward N2B titin), consistent with the developmental increase in early diastolic filling velocity. In summary, our work revealed significant changes in titin isoform expression and titin-based stiffness during postnatal development, and it is likely that these changes play important roles in adaptations in ventricular diastolic function of the developing heart.

Acknowledgments

This work was supported by grants HL61497/62881 (NIH), La668/6-2/7-1 (DFG), and Animal Health Research Center, Pullman, Wash. We are grateful to Caroline Benoist, Nick King, Melissa Krug, Mark McNabb, and Honghui Zhou for superb technical assistance.

References


Developmental Control of Titin Isoform Expression and Passive Stiffness in Fetal and Neonatal Myocardium
Sunshine Lahmers, Yiming Wu, Douglas R. Call, Siegfried Labeit and Henk Granzier

*Circ Res.* 2004;94:505-513; originally published online January 5, 2004;
doi: 10.1161/01.RES.0000115522.52554.86
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/94/4/505

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2004/03/01/94.4.505.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
Methods online.

Muscle specimen. Left ventricular myocardium was collected from the following adult animals: New Zealand white rabbits, Sprague Dawley rats, Black-6 mice, and Yorkshire pigs; animals were ~4, ~9, ~6, and ~6 months old, respectively. Tissues were also collected from neonatal animals, and their ages are indicated in the Results section. All animal procedures and tissue harvesting protocols were performed in concordance with NIH and Washington State University IACUC guidelines. Full thickness left ventricular myocardium was processed as follows: (1) stored within 5 minutes in RNAlater (Ambion, Austin, TX) at -20°C for archival storage and subsequent RNA isolation; (2) quick frozen in liquid nitrogen and stored at –80 °C for subsequent use for gel electrophoresis and western blotting experiments; (3) added to relaxing solution (RS, containing in mmol/L: 40 mM BES; 10 EGTA; 6.6 Mg-acetate; 5.88 NaATP; 15 creatine-phosphate; 46 K-propionate; 1.0 DTT; leupeptin: 0.04 mmol/L and E64: 0.01 mmol/L; pH 7.0) containing Triton-X100 (1%). After ~6 hours, the preparations were washed with RS (without Triton X-100) and kept on ice until use for mechanical experiments (within 30 hours).

SDS-PAGE and western blot analysis of titin. Myocardial samples were pulverized to a fine powder and then rapidly solubilized and analyzed by SDS-PAGE using 2-7 % acrylamide gradient gels (for details see¹). We determined the ratio of N2BA and N2B titins and the total titin:myosin heavy chain (MHC) ratio as previously explained². During the course of this work, a SDS-agarose gel electrophoresis system for separating mega-dalton sized proteins was published³ and in some of our later experiments this system was also used to study titin expression. The methods that were followed were as published ³ and gels were coomassie-blue stained. Western blotting experiments used affinity purified anti-titin antibodies specific to constitutively expressed titin sequences
(Z1-Z2, T12, M7-M9) and differentially expressed sequences (N2B (anti-126/127), anti-I55-57, and anti-N2A). The location of the binding sites of these antibodies in titin are shown in Fig. 1B. (For additional information, see\textsuperscript{2,4}.)

**Immunoelectron microscopy.** Skinned myocardial samples while in relaxing solution were stretched to different lengths, fixed, immuno-labeled, embedded and processed for IEM essentially as described\textsuperscript{5}. Briefly, the muscle samples were fixed in 3% formaldehyde/PBS solution for 20 min, then washed and blocked in 1% BSA/PBS solution for one hour. After dilution of antibodies to the appropriate concentrations (typically \(~50 \mu g/ml\)), the samples were labeled with first the primary and then secondary antibodies for 24 hours each. The samples were fixed in glutaraldehyde and osmium-tetroxide solutions, and embedded in araldite. Sections were cut using a Leica microtome, and stained with 2% of potassium permanganate and lead citrate. EM negatives were taken on a JEOL 1200 electron microscope and used to measured epitope positions (relative to mid-Z-disc).

**Transcript studies  1. Isolation of RNA.** Total RNA was isolated from left ventricular myocardium using Rneasy Fibrous Tissue Kit (Qiagen, Valencia, CA). Commercially available total RNA (Stratagene, La Jolla, CA) was used for all human transcript studies. RNA concentration and quality was assessed spectrophotometrically and by 1.2% formaldehyde agarose gel electrophoresis. RNA aliquots were stored at -80°C. 2. Labeling of cDNA. Total RNA was converted to biotinylated cDNA using a random octamer, biotinylated d-CTP and the Labelstar kit (Qiagen, Valencia, CA). 3. Array. A 50-mer oligonucleotide array containing 385 probes was used representing all of titin’s
human gene exons (see\textsuperscript{6}), and normalization, positive and negative controls. A 50-mer array was chosen to optimize sensitivity and specificity of the array\textsuperscript{7}. A 5µM 25-mer biotin probe was used as a positive control for detection chemistries. Human β-actin (5’GAGAAGATGACCAGATCATGTTTGGAGACCTTCAACACCCAGCCATGTA3’) and GAPDH (5’CACCAGGGCTGCTTTTAACTCTGGTAAAGTTGATATTGGTTGCCATCAAAT3’) were used as housekeeping genes and as positive controls for the reverse transcriptase reaction. A five basepair mismatch for titin exon 5 (5MM) was included as a negative control. All probes were printed in duplicate at 40µM in 1x print buffer (0.01% SDS, 0.1M Na\textsubscript{2}HPO\textsubscript{4}, 0.2M NaCl, pH 11.6) on epoxy-silane coated Teflon\textsuperscript{®} masked slides (Erie Scientific, Portsmouth, NH)\textsuperscript{8} using a MicroGrid II arrayer (BioRobotics Ltd. Cambridge, UK). All slides were baked for 1hr at 130°C in a vacuum oven. Oligonucleotide probes were selected to obtain a goal Tm of 80 (+/-5), GC content of 50% (+/-5) and to minimize dimmer-and hairpin-formation. \textbf{4. Array hybridization}. Biotinylated target was mixed with 2x hybridization buffer (1M NaH\textsubscript{2}PO\textsubscript{4}, 2mM EDTA, 2%SDS, 2%BSA, pH 4.8), heat denatured and hybridized to the oligonucleotide array in a humidified chamber at 55°C for 18 hrs. Detection chemistries and signal amplification were achieved using the TSA™ biotin System (PerkinElmer, Boston, MA), with modification as published\textsuperscript{9}. \textbf{5. Detection}. The fluorescence intensity was measured using \textit{arrayWoRx}\textsuperscript{eTM} Auto biochip reader (Applied Precision, LLC, Issaquah, Washington). \textbf{6. Data Analysis and normalization}. The data was analyzed with \textit{sofiWoRx} Tracker (Applied Precision) and GeneSifter (VizX Labs LLC, Seattle, WA). All probe intensities were normalized to a constitutively expressed titin exon (exon 5: 5’GTGAAGAAGAAGTACCTGCTAAAAGACAAACAGTTGTTTGCAGCTGCT3’, or exon 7: 5’GTCCGTGTCTCCAGCAAGAATCTCCACATCCCCCATCGTGCTGTTA3’), to account for variation in efficiency of conversion to biotinylated cDNA, variation in target loading and overall upregulation of transcription. The mismatch probe for exons 5 (5MM) and 4 (4MM) was used for evaluation of array specificity evaluation (5MM:}
5′GTGAAGCAGTAAGTTAAAATTACAAAGACAATTCCTTCGACTGCT3′, 4MM: 5′CTGAGACTGCACCAGCAACTTCCTTCAACGTCTGCAGACCATGACCCTG3′.) The median intensity within the spot area was determined and normalized to the median intensity value of the chosen constitutively expressed titin exon. All probes with normalized intensity less than the mismatch probe were considered to have no significant hybridization. Because each exon probe is printed in duplicate the median intensity for each exon was averaged. We performed a minimum of three independent experiments per muscle type. Results from these experiments were then used to determine the mean ±SEM for each exon. Exons identified as up/down regulated had 5 fold or greater change of their mean values for human targets and 3 fold or greater for targets from other species (these values are within the range typically used in microarray studies). Results from different muscle types were compared with a two-tailed t-test for samples of unequal variance and p<0.05 was used as criterion for statistical significance.

**Passive tension measurements.** Skinned LV myocardial samples were dissected into small muscle strips the ends of which were attached to aluminum clips. The clips were used to mount the specimen to a mechanical apparatus, allowing control of muscle length, and measurement of force and sarcomere length (using an on-line using laser-diffraction system). The muscles were stretched to a sarcomere length (SL) of ~2.2 µm and were then activated with pCa 4.0 solution, to determine the maximal active force, after which the muscles were relaxed and brought back to their slack length. Passive force was measured with preparations present in relaxing solution and stretching them at a constant velocity (1 length/sec) from their slack length to a predetermined amplitude, followed by 1 minute hold, a release at the same constant speed, and then a 20-min period of rest at the slack length. (Note that the stretch velocity was chosen because it represents the
estimated average stretch rate during diastole. This calculation assumes that diastole lasts 200 msec (based on a heart rate of 120 Hz), and that during diastole the sarcomeres extend by 0.4 µm. The preparations were stretched to a range of SL amplitudes with the maximal amplitude staying below the SL limit for reversible passive mechanics (see\textsuperscript{12}). Collagen also develops passive force in stretched myocardium\textsuperscript{1,12} and to determine the collagen-based force, muscles were incubated for 20 min with RS+0.6 mol/L KCl, followed by a 20 min incubation in RS+1.0 mol/L KI. These solutions depolymerize the thick and thin filaments and thereby remove titin's anchors in the sarcomere. We assumed that the passive force decrease that results from extraction is titin-based and the force that remains following extraction is due to collagen\textsuperscript{12}. Force was converted to tension by dividing measured forces by the cross-sectional area of the muscle at slack length (area determined as explained in\textsuperscript{12}).

**Calculations of force per titin molecule.** The elastic region of cardiac titin in the sarcomere was modeled as three serially linked WLCs with different contour and persistence lengths: the tandem Ig segments, the PEVK segment, and N2B-Us. The contour length of the tandem Ig segments was calculated from their combined number of Ig domains (the Ig domains of the N2B element were included) multiplied by a domain spacing of 4.5 nm in a completely extended chain. (Note that this assumes that all domains are folded.) For the N2B isoform the number of Ig domains was 40, for the adult N2BA isoform 64\textsuperscript{13} and for the fetal cardiac titin isoform 72 (based on the 8 additional Ig domains that are expressed in the fetal isoform). The persistence length of the native tandem Ig segment was set as 13.5 nm based on the recent study by
Tskhovrebova and Trinick\textsuperscript{14}. The contour lengths of the PEVK domain and N2B-Us were obtained from their number of amino acid residues times the maximal residue spacing in an unfolded polypeptide (0.38 nm). The number of PEVK residues in human titin N2B was 188 PEVK residues. For adult N2BA titin the number of PEVK residues was 800 \textsuperscript{13} and for fetal N2BA titin 1126 (additional residues based on the number of residues encoded by the additional PEVK exons). The persistence length of the PEVK was assumed to be 1.4 nm\textsuperscript{15}. The number of N2B-Us residues was 572 for all isoforms and the persistence length was 0.65 nm\textsuperscript{15}.

Because the tandem Ig, PEVK, and N2B-Us segments are connected in series, they bear equal forces. Therefore, for a given force, the extension of each segment (\(z_{\text{Ig}}\), \(z_{\text{PEVK}}\), and \(z_{\text{N2B-Us}}\)) can be calculated. We may then calculate for that force the total extension of the titin elastic segment (\(z_{\text{Ig}} + z_{\text{PEVK}} + z_{\text{N2B-Us}}\)). By adding the total length of non-extensible sarcomeric components (assumed to be the same as the slack sarcomere length), the sarcomere length can be calculated, and the predicted force-sarcomere length relation constructed (results are shown in Fig. 6).

References


