Published in final edited form as:

Anat Rec (Hoboken). 2014 September ; 297(9): 1596–1603. doi:10.1002/ar.22964.

Expression of myotilin during chicken development

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Abstract

Several missense mutations in the Z-band protein, myotilin, have been implicated in human muscle diseases such as myofibrillar myopathy, spheroid body myopathy, and distal myopathy. Recently, we have reported the cloning of chicken myotilin cDNA. In this study, we have investigated the expression of myotilin in cross-striated muscles from developing chicken by qRT-PCR and *in situ* hybridizations. *In situ* hybridization of embryonic stages shows myotilin gene expression in heart, somites, neural tissue, eyes and otocysts. RT-PCR and qRT-PCR data, together with *in situ* hybridization results point to a biphasic transcriptional pattern for *MYOT* gene during early heart development with maximum expression level in the adult. In skeletal muscle, the expression level starts decreasing after embryonic day 20 and declines in the adult skeletal muscles. Western blot assays of myotilin in adult skeletal muscle reveal a decrease in myotilin protein compared with levels in embryonic skeletal muscle. Our results suggest that *MYOT* gene may undergo transcriptional activation and repression that varies between tissues in developing chicken. We believe this is the first report of the developmental regulation on myotilin expression in non-mammalian species.

Keywords

chick embryos; myotilin; Z-bands; skeletal muscle; heart

INTRODUCTION

Myotilin is one of a group of Ig-domain-containing proteins in muscle that bind to actin and Z-band proteins [Otey et al., 2009]. Myotilin binds several Z-disc proteins: actin, alpha-actinin, FATZ, ZASP and filamin, (Salmikangas et al., 1999; Gontier et al., 2005; van der Ven et al., 2000; Salmikangas et al., 2003; von Nandelstadh et al., 2005), although it is not known whether myotilin binds simultaneously to these multiple partners in live muscle cells, or if binding is regulated. Mutations of myotilin in Limb-Girdle Muscular Dystrophy

(LGMD) and myofibrillar myopathies attest to the importance of myotilin for muscle function. Mice null for the *MYOT* gene have muscle that is normal in appearance and activity (Salmikangas et al. 1999; Frank et al., 2006; Moza et al. 2007).

We have recently reported the cloning and sequencing of the myotilin cDNA from chicken (Wang et al., 2011). In the present study, RT-PCR, qRT-PCR, *in situ* hybridization, and western blot analyses have been used to evaluate myotilin expression in chicken heart and skeletal muscle during development. For western blot and immunohistochemical analyses, affinity purified polyclonal antibodies were raised against a 14-mer myotilin peptide that is conserved in various species (Wang et al., 2011). In chicken, as in human and mouse (Mologni et al., 2001), the myotilin transcript is expressed in a variety of tissues during development. In cardiac muscle cells the levels of expression of both myotilin transcripts and protein are increased from embryonic to adult stages. However, in skeletal muscle there is a major reduction of *MYOT* transcription from embryonic to adult stages with a concomitant decrease in total myotilin protein. These results suggest transcriptional and/or translational controls are important for myotilin expression in skeletal muscle during development in chicken. To the best of our knowledge this is the first report on the expression of myotilin during development in avians.

MATERIALS AND METHODS

Isolation of RNA from embryonic and adult chicken hearts & skeletal muscles and adult chicken hearts

RNA was isolated from frozen chicken tissues following published protocols (Zajdel et al., 2003; Wang et al., 2007, 2008). Adult chicken heart and skeletal muscle were procured from Cobblestone Valley Farm, Preble, and NY13141.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

RNA from heart and skeletal muscle was isolated from adult and embryonic chicken. The reference gene for Real-Time qRT-PCR was GAPDH. iQ[®] SYBR green supermix (BioRad) and a Bio-Rad iCycler thermocycler were used according to the manufacturer's protocol as described earlier [Thomas et al., 2010]. The primers used to amplify myotilin.5 were:

qPCR Myot F (+) 5'-ATACAGCCAGACTCGAATGC-3' and

qPCR Myot R (-) 5'-GAATCAGGAGGCAGATCCTT-3'.

The primers were designed to amplify a product 129bp in length that was located in two different exons to prevent amplification of any contaminating genomic DNA. Each tube contained 12.5 µl of the SYBR green supermix, (1 µl of both positive and negative primers from 10 µM stocks, 9.5 µl DEPC-treated H₂O, 1 or 2 µl of cDNA for each tube and 1 or 2 µl of H₂O for the primer control). Three different RNA preparations were used and each sample was run in triplicate and averaged to correct for any errors in pipetting. Relative expression was calculated according to the method of Pfaffl (2001) where Calibrator @ Day 5 and Test @ Day 5 yields Relative expression equal to one. To verify the specificity of the primer pair,

PCR products were run on an agarose gel after real-time analysis. As control, we amplified chicken GAPDH using the primer pair

GAPDH.F (+) 5'-TCCTGTGACTTCAATGGTGA-3' and

GAPDH.R (-) 5'- CACAACACGGTTGCTGTATC-3'.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out with RNA from specific tissues as described previously (Denz et al., 2004; Wang et al., 2007). First strand cDNA was made with oligo dT as primer and was amplified subsequently with gene- and isoform-specific primer-pairs (listed in Table 1) following our published protocols (Denz et al., 2004; Zajdel et al., 2003). PCR-amplified DNA was subjected to agarose gel electrophoresis and southern hybridization was carried out with gene and isoform specific oligonucleotide probes (as shown in Table 1) end-labeled with ³²P by T4 kinase (Denz et al. 2004; Wang et al., 2007, 2008).

In situ hybridizations

Whole mount—HH [Hamburger and Hamilton, 1951] stage 15 embryos were removed from the yolk, rinsed, and fixed in 4% paraformaldehyde/PBS and processed for *in situ* hybridization. The riboprobes of myotilin used for *in situ* hybridization were prepared as follows: T3.Myot.(F): 5'- GAATTAACCCTCACTAAAGGATACAGCCAGACTCGAA-3'

T7.Myot(CR) : 5'-GTAATACGACTCACTATAGGGAATCAGGAGGCAGATC-3'

At the 5'-end of the plus primer, a sequence of the T3 RNA polymerase promoter (bold and underlined) was added. Similarly, the promoter sequence for the T7 RNA polymerase was fused to the 5'-end of the negative PCR primer. The PCR amplified products were gel purified separately and subjected to *in vitro* transcription. Upon transcription with T3 RNA polymerase, the template yielded sense probes (used as negative control), and yielded anti-sense probes when transcribed *in vitro* with T7 RNA polymerase. The *in vitro* transcription was carried out with DIG RNA Labeling kit (T3/T7) from Roche Diagnostic following manufacturer's specifications. The purity of the *in vitro* transcribed RNA probe, was checked by electrophoresis of aliquots of sense and antisense probes in a denatured agarose gel. An anti-digoxigenin antibody Fab alkaline phosphatase conjugated secondary antibody was used for the detection of the riboprobe (Boehringer Mannheim). For *in situ* hybridization, digoxigenin-labeled sense and anti-sense alpha-actin riboprobes 590 bp were used as negative and positive controls respectively. Detailed protocol for the *in situ* hybridization has been reported elsewhere (Linask et al., 1997).

Paraffin embedded tissue sections—Whole chicken embryos staged according to Hamburger and Hamilton (1951) were fixed at stages: HH9 (day 1.3), HH15 (days 2.3), HH20 (day 3), HH31 (day 7), and day 12, and prepared for *in situ* hybridization. The technique described by Jowett (1997) was used with the sense (negative control) and anti-sense RNA probes described above. The results are presented in **Figures 1 and 2**.

Western blot analysis

Western blot analysis was carried out with myofibrils isolated from embryonic (15-day-old embryos) and adult chicken (obtained from a local farm at Cornell University, Ithaca, NY) and with myofibrils isolated from mouse heart and skeletal muscles (kind gift from Dr. David F. Wieczorek, University of Cincinnati). The method described by Rajan et al. (2010) was used for isolation of myofibrils. Western blotting was performed with enhanced chemiluminescence detection as described in our published protocols (Thomas et al., 2010; Sanger et al., 2010). Polyclonal antibodies were raised against a 14-mer peptide “APKQLRVPTFSKY” in myotilin that is conserved both in humans and chickens (Wang et al., 2011). One cysteine residue was added at the N-terminus to aid in affinity purification (Covance Inc., Denver, PA). Sarcomeric alpha-actinin antibodies were obtained from Sigma (St. Louis, MO).

Cell Culture and Immunostaining

Skeletal myoblasts and cardiomyocytes were isolated respectively from the breast muscles and hearts of nine-day old quail embryos and plated on collagen-coated 35-mm MatTek (Ashland, MA) dishes at concentrations of 1×10^5 and 3×10^5 cells per dish according to procedures described in Dabiri et al. (1999). The cells were fixed with buffered 3% paraformaldehyde, and permeabilized as previously described (Dabiri et al., 1999). The cells were then stained with polyclonal anti-myotilin antibodies, Alexa 488 secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and rhodamine phalloidin (Molecular Probes, Eugene, OR).

RESULTS

Localization of myotilin transcripts in embryonic chicken heart and skeletal muscle by *in situ* hybridization

In situ hybridization was carried out on whole embryos (Figure 1 A-C), as well as on sections cut through the embryonic heart regions (Figure 2 A-D). During heart development the expression of myotilin mRNA was biphasic, showing higher levels in early embryonic stages (HH 9 and 15) (Figures 1 A and 2 A) than in the more differentiated cardiac tissue of later stages at Day 3 (HH 20) and Day 7 (HH 31) (Figures 1 C and 2 C). This was followed by increased expression in myocardium at Day 12 (HH 38) (Figure 2 D). In contrast to developing heart, throughout somite development there was a high expression of myotilin (Figures 1 A and B). Myotilin also was expressed in the developing neural tissue, including the eyes and otocysts (Figure 1 C).

Expression of myotilin transcripts during embryonic development of chicken skeletal and cardiac muscle—The expression of skeletal muscle myotilin measured by RT-PCR (Figure 3) was at a maximum on day 15 as seen both with ethidium bromide staining of amplified DNA (panel A, lane 3) and with ^{32}P -labeled oligonucleotide detector in Southern hybridization (panel B, lane 3). In adult skeletal muscle, the signal was barely detectable (panels A and B, lane 5). Analysis of the same RNA for TPM1 and TPM4alpha, whose stage- and tissue-specific expression is known (Zajdel et al., 2003) was done in parallel as confirmation of the integrity of the RNA. As expected, TPM1 transcript was

present in the adult stage (Figure 3, panel D, lane 5) and TPM4 α transcript was absent in both E20 and adult stages (Figure 3, panel E, lanes 4 and 5). These results are consistent with evidence that in adult chicken, TPM4 α is expressed exclusively in the heart, but in embryonic stages it is expressed also in low amount in skeletal muscle (Figure 3, panel E, lane 1-3) (Zajdel et al., 2003).

In comparison to myotilin expression in skeletal muscle, the expression levels in embryonic cardiac muscle were lower (Figure 4 panels A and B, lanes 1-3 vs. 5-7). However in 15-day post-hatch stages, expression levels were higher in chicken heart than in skeletal muscle (Figure 4 panels A and B, lanes 4 vs. 8). The RT-PCR data showing the increase in expression level of full-length myotilin transcript expression in embryonic hearts (Figure 4 B, lanes 1-3) are in good agreement with the *in situ* hybridization data (Figure 2 C, D).

Real-time qRT-PCR analysis of myotilin transcription in chicken cardiac and skeletal muscles (Figure 5) revealed that the relative expression of myotilin in the heart (H) increased during embryogenesis, and reached maximum level in the adult heart. In contrast, in skeletal muscle (S), the expression level of myotilin increased until embryonic day 15 after which it declined and was reduced significantly in adult skeletal muscles (Figure 5).

Western blot analysis of myotilin protein in chicken heart and skeletal muscle

To evaluate the relative amount of myotilin protein in developing chicken muscle we raised antibodies against a 14-mer peptide “APKQLRVRPTFSKY” that is conserved both in humans and chickens (Wang et al., 2011). As expected the myotilin antibodies stained the Z-bands of mature myofibrils (Figure 6; (Wang et al., 2011). Myotilin protein (MW = 57 kDa) was detected by western blot in myofibrils from adult chicken heart muscle (Figure 7 panel A, lane 2), but was barely detectable in myofibrils from embryonic heart (Figure 7 panel A, lane 1). In addition, a higher molecular weight signal was detected at ~72 kDa region in adult chicken cardiac myofibrils (Figure 7, panel A lane 2). A similar high molecular weight myotilin band was also detected in human heart (Moza et al., 2008), although there is no known messenger mRNA that might encode a 72 kDa myotilin protein or any logical posttranslational modification that could explain the increase in size (Moza et al., 2008).

In skeletal muscle myofibrils, a protein band of ~ 57 kDa was detected in both embryonic (Figure 7, lane 3), and, at a much lower amount, in adult myofibrils (Figure 7, lane 4). In addition, in the myofibrils in both embryonic and adult skeletal muscle (Figure 7, lanes 3 and 4), there were several prominent bands below ~57 kDa that could be breakdown products or alternatively spliced variants of myotilin. An alternatively spliced myotilin mRNA encoding a low molecular protein containing 314 amino acid residues is known to be expressed in humans (Accession # AK300088.1; GI: 184390257). Additionally, a 45 kDa myotilin protein could be generated from the myotilin mRNA due to an aberrant translational start point in the mRNA sequence [Salmikangas et al., 1998]. In the same gel, stripped and probed with anti- α -actinin, a single 97 kDa band characteristic of α -actinin is seen in all the lanes in panel B (Figure 7) and indicates that the extracts prepared from the adult and embryonic samples were of high quality.

DISCUSSION

A beating heart is formed in the chicken embryo at HH stage 10 (~33 hours or 9-to 10-somites). Our *in situ* hybridization results together with our RT-PCR and qRTPCR results reveal a biphasic transcriptional pattern for the *MYOT* gene in the developing heart. At HH stage 15 (23 somites), before beating begins, *in situ* hybridization results show a strong signal of myotilin transcripts in the myocardium of the embryos (Figure 2 A). An apparent second wave of *MYOT* transcription in the heart starts around embryonic day 10-12 (HH 36-38) (Figure 2 D; Figure 4 B, lanes 2 and 3) reaching maximum level in the adult stage (Figure 5; Figure 4 B, lane 4).

In situ hybridization data show the expression of myotilin transcription in a variety of mouse tissues (Mologni et al. 2001). During mouse embryogenesis, these authors reported a wider expression of myotilin in the embryo than in the adult mouse suggesting a role for myotilin in tissue remodeling during organogenesis. In contrast to the mouse system, myotilin gene expression in chicken appears to be down regulated in adult skeletal muscle, whereas it reaches maximum in adult cardiac tissues.

Mologni et al. (2005) isolated and characterized the upstream promoter region of the *MYOT* gene from mouse and found 13 E-boxes (CANNTG) and six putative MEF binding sites distributed along the promoter region. It is to be noted that myogenic bHLH family proteins like MyoD, myogenin, and Myf5 require the binding with E-box cis-elements for transcription in skeletal muscle. In addition, 15 GATA boxes, one Nkx2.5 consensus sequence, and one inverted SRF binding site were located in the immediate upstream regulatory region that may be involved in cardiac specific transcription. Additionally, a prominent GAGA box (at -843 bp) and a non-conventional TATA box (at -25 bp) were identified. These authors performed deletion and mutational analysis followed by expression analysis in transfected C2C12 cells in order to delineate the role of each cis-element present in the upstream regulatory region. They did not identify a cis-element that controls the *MYOT* gene negatively. In chicken, the upstream regulatory region of the *MYOT* gene may differ. In chicken, as in mouse, the *MYOT* gene is expressed in both heart and skeletal muscle. No transcriptional repression has been reported in mouse [Mologni et al. 2005]. Hence, it would be logical to speculate that the putative *cis*-elements in the upstream regulatory region of the *MYOT* gene and the corresponding trans-acting factor(s) in skeletal muscle may be different in the chicken/avian system. Furthermore, in chicken there may be a trans-acting factor that in conjunction with the *cis*-elements represses the *MYOT* gene in skeletal muscle but not in cardiac muscle.

Cardiac myosin light-chain 2 (*MLC-2*) is known to be expressed in both cardiac and skeletal muscles in early fetal cells (Saidepet et al. 1984). The expression of *MLC-2* undergoes transcriptional repression in adult non-cardiac muscle tissues. Ruoqian-Shen et al (1991) identified an 89-bp cis-elements in the upstream promoter region of the *MLC-2* gene, which in conjunction with a trans-acting protein may repress the *MLC-2* gene in adult skeletal muscle. Our observation of the reduced transcription of *MYOT* in adult chicken skeletal muscle may be explained with a similar argument that a transacting protein is expressed in adult skeletal muscle, which in conjunction with a cis-element present in the promoter

region of the chicken *MYOT* gene may significantly down regulate its expression in adult skeletal muscle.

Myotilin is expressed in the mammalian system in striated muscles, smooth muscle, and non-muscle tissues during embryogenesis (Frank et al., 2005; Mologni et al, 2001, Moza 2008). However, its transcription is restricted predominantly to the heart and skeletal muscles in adults (Mologni et al. 2001). Although the promoter analysis of mouse *MYOT* gene has addressed the expression of myotilin in striated muscle (Mologni et al. 2005), it does not explain how it is repressed in non-muscle and smooth muscle cells with the progression of development. In order to explain the differential level of myotilin transcription in heart and skeletal muscle, a detailed characterization of the promoter region is an essential prerequisite for future work.

In conclusion, *in situ* hybridization of embryonic stages shows myotilin gene expression in heart, somites, neural tissue, eyes and otocysts. RT-PCR and qRT-PCR data, together with *in situ* hybridization results point to a biphasic transcriptional pattern for *MYOT* gene during early heart development with maximum expression level in the adult heart. In skeletal muscle, the expression level starts decreasing after embryonic day 20 and declines in the adult skeletal muscles. Western blot assays of myotilin in adult skeletal muscle reveal a decrease in myotilin protein compared with levels in embryonic skeletal muscle. Our results suggest that *MYOT* gene may undergo transcriptional activation and repression that varies between tissues in developing chicken. These results strongly suggest that myotilin protein expression in cross striated muscles undergoes differential translational control in heart and skeletal muscle cells in a stage specific manner. There is precedence for such a translational control of various myofibrillar protein synthesis *in vivo*. For example, cardiac troponin-T (cTnT) protein undergoes translational control during chick embryogenesis (Antin et al. 2002). Although cTnT mRNA expression starts at HH stage 5 in lateral mesoderm in chick embryos and subsequently localizes to heart muscle cells by stage 14, no detectable cTnT protein is found until stage 9 of chick embryos (Antin et al., 2002). Precise translation of a variety of mRNAs in a restricted area in a cell may be controlled by a variety of repressors. These repressor molecules are specifically recruited to transport ribonucleoprotein particles and block translation at different steps (Besse and Ephrussi, 2008). Furthermore, there is the possibility of involvement of microRNA in translational control of myofibrillar protein(s) (van Rooij et al., 2009; Shieh et al., 2011; Wang et al. 2011).

Acknowledgments

The project was supported by Grant Number AR-57063 from NIAMS/NIH, and Grant Number HL080426 from NHLBI/NIH (JMS and JWS). JWS is also indebted to the Hendricks Fund for support of this work.

Grant Sponsor: NIH

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Figure 1.

Whole mount *in situ* hybridization of expression patterns of myotilin transcripts in chicken embryos during three stages of heart development.

A. In HH stage 9 (embryonic day 1.3) myotilin is present in the developing neural tissue, bilateral heart tissue as it fuses to form a single tubular structure (arrows), and in blocks of somite tissue.

B. In HH stage 16 (embryonic day 2.2) neural tissue, (including the eye region - upper arrow), the heart (lower arrow), and somites continue to express myotilin.

C. With increased backlighting of the embryo on embryonic day 3 (HH stage 20) a ring of myotilin expression is seen at the site of the otocyst (Ot) and the eye (Ey). The heart (lowest arrow) appears to show reduced expression as compared to early heart formation in A. The inset depicts the same ED stage 3 embryo at exposures used in A, B. Magnification bar =100 μ m for all panels (A,B,C).

Figure 2.

In situ hybridization patterns of myotilin expression in sections of different stages of chicken embryonic hearts. In all panels scale bar = 100 μm. Myo, myocardium; Endo, endocardium; AS, antisense RNA probe.

A. In an HH stage 15 embryo the myocardium (Myo) expresses a relatively high level of myotilin as compared with the endocardium (Endo). AS = anti-sense.

B. Section of an embryonic day 7 (HH stage 31) sense control.

C. Section of day 7 embryo hybridized with antisense probes (AS). As compared to the control section in panel B, only a low level of signal is detectable in the myocardium.

D. A slightly higher level of myotilin expression is apparent in the compact layer and trabeculae of the embryonic day 12 (HH 38) heart.

Figure 3.

RT-PCR analysis of myotilin gene expression in developing and adult chicken skeletal muscles.

M: Molecular marker; Lanes 1 - 5: Embryonic (E) days 5, 10, 15, 20, and Adult; Lane 6: primer control.

Panel A: Ethidium bromide (Ebr) staining of amplified myotilin cDNA (full length coding region).

Panel B: Southern hybridization of myotilin with ^{32}P -labeled probe

Panel C: Ebr staining of GAPDH-amplified DNA.

Panel D: Ebr staining of the TPM1 (total) cDNA

Panel E: Ebr staining of the total TPM4 cDNA. Note that TPM4 is down regulated in 20-day-old skeletal muscle and adult skeletal muscle (Lanes 4 and 5) as known from previous studies (Zajdel et al. 2003).

The nucleotide sequences of the primers used for amplification of various transcripts are presented in Table 1

Figure 4.

RT-PCR analysis of myotilin gene expression in heart and skeletal muscle in embryonic and 15-day post hatch chicken.

M: Molecular marker; Lanes 1-4 heart: Embryonic days (E) 7, 10, 15 and adult post-hatch (A) day 15. Lanes 5-8 skeletal: Embryonic days (E) 7, 10, 15 and post-hatch (A) day 15.

Lane 9: primer control.

Panel A. Ethidium bromide (Ebr) staining of amplified myotilin cDNA

Panel B. Southern hybridization of myotilin amplified DNA

Panel C. Ethidium bromide staining of PCR amplified DNA with GAPDH-specific primer-pair. The nucleotide sequences of the primers used for amplification of various transcripts are presented in **Table 1**.

Figure 5.

Real-time qRT-PCR of myotilin expression during development of heart and skeletal muscle in chicken. The relative change in myotilin expression in heart and skeletal muscle at embryonic days 5, 10, 15, and 20 and in adult muscle was calculated as:

$$\text{Relative Expression} = \frac{(E_{TARGET})^{DC_T \text{ TARGET}(=CALIBRATOR-TEST)}}{(E_{REF})^{DC_T \text{ REF}(=CALIBRATOR-TEST)}}$$

E = Efficiency; Calibrator is Day 5 data (which differs for Heart and Skeletal muscle); the ratio of Calibrator and Test at Day 5 yields Relative expression on Day 5 = 1. Note that although the relative expression of heart and skeletal muscle on Day 5 is 1, the actual expression levels are different for heart and skeletal muscle. Reference gene is chicken GAPDH.

Figure 6.

Immunohistochemical reactivity of Quail myotubes with anti-myotilin antibodies. The myotubes were fixed, permeabilized and stained with (A) myotilin antibody, and (B) phalloidin. The myotilin antibody stains the Z-bands of mature myofibrils and is present along premyofibrils in an aperiodic pattern in the myotube on the right side on the image. Phalloidin stains the Z-bands and the ends of overlapping thin filaments in the contracted sarcomeres of the myotube. (C) is the merge of (A) myotilin and (B) F-actin stainings. Scale = 10 microns.

Figure 7.

Western blot analysis of myotilin protein in chicken embryonic and adult heart and skeletal muscles. Samples for Western blot were prepared from isolated myofibrils with ~15 ug/lane of protein separated with SDS-PAGE. Gels were blotted and the blots stained with anti-myotilin antibodies raised in rabbits (**panel A**). Enhanced chemiluminescence was used to visualize the bands of proteins. Lane 1. Embryonic 15-day-old chick heart. Lane 2, Adult chicken hearts. Lane 3, Embryonic 15-day-old chick skeletal muscle. Lane 4, Adult chicken skeletal muscle. As a control, the blot in panel A was stripped of myotilin antibody and

stained with alpha-actinin antibody (**panel B**). Alpha-actinin is present in all lanes indicating that the myofibrillar extracts were of good quality. We conclude that the absence of myotilin in the embryonic heart is not due to the quality of the extracts. (**A**, lane 1). Molecular weight markers were run in lanes labeled M. The marker proteins do not react with the myotilin antibodies.

Table 1

Sequence of gene as well as isoform specific primer-pairs and detector oligonucleotides used in this study.

Gene	Amplification type	Primer/detector	Sequence
Motilin	Entire coding sequence	Forward primer (P1) Reverse primer (p2) Detector (D)	5'-GGATGTTTAACTACGAACGT-3' 5'-TTAAAGTTCATCGCIIICA-3' 5'-TGGCTCGCAGGCTGCTCGGA-3'
Myotilin	Real-time qRT-PCR	Forward primer Reverse primer	5'-ATACAGCCAGACTCGAATGC-3' 5'-GAATCAGGAGGCAGATCCTT-3
GAPDH	RT-PCR	Forward Primer Reverse primer	5'-GTCTCCTGTGACTTCAAT-3' 5'-ACAGATCAGTTTCTATCA-3'
GAPDH	Real-time qRT-PCR	Forward primer Reverse primer	5'-TCCTGTGAC TTCAATGGTGA-3' 5'-CACAAACACGGTTGCTGTATC-3'
TPM1	RT-PCR for total TPM1 transcripts	Forward primer Reverse primer Detector	5'-GCTGAGAGTGAAGTAGCTTCC-3' 5'-TAGATCATCAATGCTCTTC-3' 5'-AAATGTGCTGAGCTTGAAGAG-3'
TPM4 α	RT-PCR for entire coding sequence	Forward primer Reverse primer Detector	5'-ATGGATGCCATCAAGAAAAAGATG-3' 5'-AGACTACAGGGAGGTCATATATCATT-3' 5'-GCAGAAGGTGAGGTGGCGGCG-3'