Effect of Shz-1, a cardiogenic small molecule, on expression of tropomyosin in axolotl heart

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Abstract:*A family of sulfonyl-hydrazone (Shz) small molecules including shz-1 was found to induce Nkx2.5 gene and subsequently augmented the expression of sarcomeric □-tropomyosin in P19CL6 cells. In order to understand the mechanism by which shz-1 induced sarcomeric □ tropomyosin , we employed Mexican axolotl (Ambystoma mexicanum) as the animal model, which we use in our laboratory for studying the structural/functional relationship of tropomyosin in relation to cardiogenesis and cardiac myofibrillogenesis. Tropomyosins are a family of actin binding proteins that show cell specific diversity by a combination of multiple genes and alternative RNA splicing. Of the 4 tropomyosin genes, both TPM1 and TPM4 genes play pivotal roles in myofibrillogenesis as well as cardiac contractility in axolotl. Two isoforms known as TPM1□ and TPM1□ of the TPM1 gene as well as the sarcomeric isoform designated as TPM4□ of the TPM4 gene are expressed in axolotl heart. Upon injection intraperitoneally into juvenile axolotl, shz-1 augmented the expression of transcripts of TPM1□, TPM1□□ and TPM4a in heart but not in skeletal muscle. Interestingly, the expression of TPM2 transcript was also increased. HOWEVER, TPM3 and cardiac TnT expression remained unchanged. In contrast to transcript expression, our western blot analysis with sarcomeric tropomyosin-specific antibodies did not show any significant increase in tropomyosin expression in shz-1 treated striated muscles. Similarly, western blot analysis with extracts of whole embryos failed to record any increases in tropomyosin expression in embryos maintained for four days in the presence or absence of 5□M shz-1. The contradictory results of transcript analysis by RT-PCR and protein analysis by western blotting strongly suggest that sarcomeric tropomyosin transcripts in axolotl heart may undergo translational control similar to that had been proposed in transgenic mice over expressing TPM1□ in a cardiac specific manner and also in hearts of TPM1-ablated mice.*

Key Words: *Ambystoma mexicanum*, Shz-1, Nkx2.5, sarcomeric tropomyosin, gene expression

1. INTRODUCTION

A family of sulfonyl-hydrazone (Shz) molecules can trigger cardiac specific mRNA and protein expression in a variety of embryonic and adult stem/progenitor cells. Shz molecules including Shz-1 are potent inducers of Nkx2.5 and a subset of other cardiac markers, including myocardin, troponin-I, myogenin, etc. (Sadek et al., 2008). The homeobox gene Nkx2.5, an ortholog of the *Drosophila* gene *tinman* is the earliest known marker in cardiac lineage in vertebrate embryos. The expression of Nkx2.5 starts during early development and persists into adulthood in vertebrates (Lien et al 1999). In knockdown studies, embryonic mice homozygous (-/-) for Nkx2.5 resulted in death ~9 days postcoitum (Lyons et al 1995). The knockout mice formed normal heart tubes, however, rightward looping of the ventricle failed. The results suggest an essential role of this gene in cardiac looping during morphogenesis (Lyons et al 1995). Development of the cardiac conduction system has also been linked to Nkx2.5 expression (Moskowwitz et al 2007). Besides its effect on induction of Nkx2.5 and other cardiac specific genes, shz molecules were also found to induce sarcomeric □-tropomyosin (S□TM or TPM1□) in stem cells. The rapid and robust induction of S□TM in mouse H9c2 cells by Shz molecules was evaluated by RT-PCR and western blot analyses (Sadek et al., 2008).

Tropomyosin is one of the six proteins comprising the thin filaments of sarcomeres in vertebrates. Four different TM genes (*TPM1, TPM2, TPM3,* and *TPM4*) has been identified in vertebrates (Lees-Miller and Helfman 1991; Schevzov et al 2011; Pittenger et al 1994; Narshi et al 2005). This gene duplication and alternative RNA processing have resulted in a diverse group of TM isoforms. Mexican axolotl, *Ambystoma mexicanum*, is an intriguing animal model for studying cardiogenesis and cardiac myofibrillogenesis because some of these animals carry a mutation in gene c (for cardiac lethal) (Humphrey 1972; Lemanski 1973; Lemanski 1979; Zajdel et al 1999). The hearts of homozygous recessive mutant embryos (c/c) are non-contractile and lack organized myofibrils (Lemanski 1973; Lemanski 1979; Zajdel et al 1999). As a result, the mutant embryos die after hatching due to lack of circulation. Even though mRNA expression of the sarcomeric tropomyosin isoforms is comparable in normal and mutant hearts and most of the myofibrillar proteins are at near normal quantities, the mutant hearts are deficient in sarcomeric tropomyosin protein (Zhang et al 2009; Spinner et al 2002).

We have identified and characterized three striated muscle isoforms of TM in axolotl hearts (TPM1□, TPM1□, and TPM4□) (Luque et al 1997; Thomas et al 2010; Denz et al 2004; Rajan et al 2010). TPM1□ and TPM1□ are the two alternatively spliced TM expressed in axolotl and other vertebrate hearts (Narshi et al 2005; Zajdel et al 1999; Zhang et al 2009; Spinner et al 2002). We have already reported the in vivo expression of TPM1□ mRNA in chicken (Zajdel et al 2003) and humans (Denz et al 2004). In chicken and humans, the expression of TPM1□ appears to be cardiac specific; it is not expressed in skeletal muscles. TPM1□, however, is expressed in both cardiac and skeletal muscles in axolotl (Thomas et al 2010).

Our objective of the present study is to examine whether or not Shz-1 can augment the expression of various myofibrillar proteins, especially tropomyosin in normal axolotl hearts. Although Sadek et al reported the effect of Shz molecules on sarcomeric tropomyosin (TPM1□) in various cell lines (Sadek et al 2008), the authors did not address the effect of this cardiogenic molecule on other sarcomeric tropomyosin isoforms e.g. TPM1□, TPM2□, and TPM4□. TPM1□, TPM1□, and TPM2□ are expressed in mammalian hearts (Luque et al 1997; Thomas et al 2010; Denz et al 2004). TPM4□ is expressed in amphibian (Spinner et al 2002; Hardy et al 1995) and avian hearts (Fleenor et al 1992). We have studied the effect of shz-1 on the expression of various known sarcomeric tropomyosin isoforms, cardiac troponin-T, and other sarcomeric proteins in heart and skeletal muscles of juvenile axolotl treated with this cardiogenic molecule. We also compared the expression of p53 transcripts in controls and Shz-1 treated animals. The tumor-suppressor protein, p53, is a transcriptional activator of muscle-specific phosphoglycerate mutase (M-PGAM) that in turn may contribute to various cardiac gene expression *in vivo* (Ruiz-Lozano et al 1999).

2. MATERIALS AND METHODS

(a) Shz-1 treatment of juvenile axolotl

Axolotl (1-2 cm long) were obtained from the Ambystoma Genetic Stock Center, at the University of Kentucky (Lexington, KY). Shz-1 was procured from Enzo Life Science (product # ALX-270-514) and solution was prepared (1-2 mg/ml) in DMSO. The master solution(s) was kept in brown colored tube to protect it from light at -20^0 C. Shz-1 was administered by intraperitoneal injection (10 □g/animal). Control axolotl were injected with an equal volume of DMSO. The animals were placed in separate fish bowl in Holtfreters solution (3.46 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $NaHCO_3$, 0.2 g $MgSO_4$, [pH 7.4] per liter of distilled H_20). Second injection was performed on day 2. Axolotl were monitored over a period of 4 days; both control and shz-1-treated axolotl were sacrificed for their tissue. MS-222 (Tricaine methanesulfonate) was used to anesthetize the axolotl prior to decapitation (Thurston et al 2009). Axolotl hearts and skeletal muscle were then dissected out using a scalpel and watchmaker forceps. The dissected tissues were quickly frozen in culture tubes placed in absolute ethanol containing dry ice. Frozen tissues were used for RNA isolation and protein extraction.

(b) Shz-1 treatment in axolotl embryos (35-37 stages [29])

Wild type embryos were obtained from the University of Kentucky axolotl colony. The embryos were kept in an incubator at 17°C until the required stages for study were reached. At the required stages (35 to 37 stages), the embryos were dejellied, placed in modified Steinberg's solution in small Petri dishes (60 X 15 mm; Falcon Plastics) (Zajdel et al 1999). To the experimental group(s) shz-1 (final concentration 5 mM) or to the control group equal volume of DMSO was added. After 4 days at 17^0 C, embryos were used for making homogenate for western blot analysis with tissue extraction buffer (Invitrogen) supplemented with protease inhibitors as mentioned under the method for western blot analysis.

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(c) RNA isolation from juvenile axolotl hearts and skeletal muscle

RNA was isolated from axolotl tissues using the RiboPureTM kit (Ambion), following the manufacturers protocol. Frozen tissues were homogenized using a mortar and pestle followed by passage through an 18-gauge needle using a syringe. The optical density was measured using a UV spectrophotometer to determine the yield of total RNA.

(d) RT-PCR for gene expression in Shz-1 and control axolotl tissues

cDNA was synthesized from total isolated RNA using iScriptTM cDNA synthesis kit (Bio-Rad). 0.1mg of total RNA was used to make 20 □l of cDNA, from which 1□l was utilized in each Polymerase Chain Reaction. Each PCR was run for 35 cycles of the protocol: denature 1 min at 95°C, anneal 1 min at 55°C, and elongate 1 min at 72°C. Agarose gel electrophoresis of the amplicons and subsequent southern hybridization was carried out following our published procedure (Thomas et al 2010)]. T4 polynucleotide kinase (Invitrogen) was used to label detector oligonucleotides with [□-^{32}P]ATP (6000Ci/mmol, Perkin Elmer) for southern hybridization. Nucleotide sequences of primer-pairs for amplification and detector oligonucleotides used in subsequent southern hybridization for various genes are presented in Table 1.

(e) Gel extraction and sequencing of TPM2 and TPM3 amplified DNA

PCR amplified DNA bands of TPM2 and TPM3 were isolated using Qiagen gel extraction kit and subsequently determined the nucleotide sequences from both sides at the DNA-sequencing facilities of Cornell University, Ithaca, NY.

(f) Western blot analysis

Protein was extracted from control and shz-1 injected axolotl using a Tissue extraction buffer buffer (50 mM Tris, pH 7.4; 250 mM NaCl; 5 mM EDTA; 2 mM Na_3VO_4; 1 mM NaF; 20 mM $Na_4P_2O_7$; 0.02% NaN_3) (Invitrogen), supplemeted with 1 mM PFSF and Complete Protease Inhibitor (Roche). The frozen samples were homogenized first and subsequently sonicated as described before [23]. Cell homogenates were centrifuged at 10,000 RPM for 5 minutes to pellet the tissue debris. Protein quantitation was done using BioRad protein reagent following the manufacturer's protocol. LDS sample buffer (Invitrogen) and sample reducing agent (Invitrogen) were added to the protein extract. The protein samples (10-15 □g protein per lane) and SDS-PAGE was carried out using Novex NuPAGE 4-12% Bis-Tris gels in MOPS running buffer using the direction supplied by the manufacturer. After transferring the proteins onto nitrocellulose filter(s) (Invitrogen), Ponceau reversible staining was carried out to test loading consistency and transfer efficiency. The blots were blocked with 5% dry fat-free milk powder (Carnation) in TBST (1 x TBS, 0.05% Tween 20) for subsequent treating with primary antibodies for overnight at 4^0 C or for 2 hours at room temperature. All secondary antibody incubation (with appropriate dilution) was done for 1 hour at room temperature. Chemiluminescence was performed by using ECL detection reagents (GE Heath Care or Invitrogen) and exposing the blot to x-ray film, following the manufacture's protocol. CH1 monoclonal antibody was procured from Hybridoma bank (University of Iowa); anti-sarcomeric □-actinin (Sigma-Aldrich) and TM311 monoclonal anti-tropomyosin antibodies were purchased from Sigma; Affinity purified anti-TPM1□ antibody a kind gift from Dr. David Wieczorek, University of Cincinnati (Rajan et al 2010).

3. RESULTS

(a) Gene expression in shz-1-treated and control axolotl tissues

We chose to examine the expression of several genes in shz-1-treated and control axolotl striated muscles by RT-PCR followed by Southern hybridization (Figures 1-5). Nkx2.5 was selected because shz-1 is known to induce Nkx2.5 in embryonic P19 cells (Sadek et al 2008) and also because of its importance in embryonic heart development in various other species from Drosophila to human (Tanaka et al 1998). The tumor-suppressor gene, p53, was chosen because it is known to signal the induction of apoptosis in cells (Nam et al 2006). TPM1□ and TPM1□ are alternately spliced sarcomeric isoforms of the *TPM1* gene. Both isoforms are thought to play important roles during myofibrilogenesis (Zajdel et al 1998). TPM4□, the striated muscle isoform of the *TPM4* gene, is essential for cardiac myofibrillogenesis and cardiac contractility in amphibians including axolotl (Spinner et al 2002; Hardy et al 1995; Zajdel et al 2005). In addition, we chose TPM2 and TPM3, which are expressed in vertebrate striated muscle. Nucleotide sequences for axolotl TPM2 (GenBank Accession #BI817951.1) and TPM3 (Accession # BL015_BH03) were obtained from GenBank. GAPDH was also amplified as a housekeeping gene. In addition, we have chosen cardiac troponin-T (TNNT2), which is expressed in axolotl hearts (Sferrazza et al 2007). Our aim is to evaluate whether shz-1 treatment can affect the expression of various genes in axolotl hearts.

Our RT-PCR results revealed that the expression of Nkx2.5 was increased significantly in hearts in juvenile axolotl injected with Shz-1 (panel a, Figure 1). However, Nkx2.5 is not expressed in skeletal muscle of both control and shz-1 treated animals (lanes 3 & 4, panel a; Figure 1). The absence of expression of Nkx2.5 in axolotl skeletal muscle is consistent with our previously reported finding (Thurstob et al 2009). We performed RT-PCR amplification of Nkx2.5 with 3 different cDNA preparations. The amplified DNA was subsequently hybridized (panel b; Figure 2) with Nkx2.5 – specific [^{32}P]-labeled oligonucleotide probe (nucleotide sequences presented in Table 1). Both ehidium staining (panel a) and southern hybridization (panel b) data showed an increased level of Nkx2.5 expression in all three cDNA preparations from shz-1 -treated axolotl hearts (compare lanes 1 & 2; lanes 3 & 4; lanes 5 & 6).

Besides Nkx2.5, we compared the expression of transcripts of various tropomyosin isoforms in heart and skeletal muscle from normal and shz-1-treated axolotls. Results in Figure 3 show the amplification of TPM1□ and

TPM1 using isoform specific primer pairs. Expression of both TPM1 (panel a & b) and TPM1 (panel c & d) were significantly higher in shz-1 treated hearts (lane 1) compared to control (lane 2). Although no such increase was observed in the expression of TPM1 in shz-1 treated skeletal muscle (lanes 3 & 4; panels a & b), our results did show an increase in TPM1 expression in skeletal muscle treated with shz-1 (lanes 3 & 4; panels c & d). Again, GAPDH expression remained unchanged in treated and untreated tissues (panel e). The total TPM2 transcript level in shz-1 treated and control axolotl striated muscle showed an interesting pattern. TPM2 expression was significantly augmented in shz-1 treated hearts (compare lane 1 with lane 2 in panels a & b, Figure 4) whereas it some how was down regulated in shz-1 treated skeletal muscle (compare lane 3 with lane 4 in panels a & b). We do not know the complete nucleotide sequence of TPM2, thus we are not sure whether specifically TPM2 a was upregulated. Since we performed RT-PCR of TPM2 for the first time, we isolated the amplified DNA from heart and skeletal muscle (both treated and control) from agarose gel (using gel extraction kit from Qiagen). We determined the nucleotide sequences of the amplified DNA and subsequently compared with the axolotl TPM2 sequences available from the databases. The *TPM2* gene in vertebrates generates more than one isoforms [5-8]. Unlike TPM2, TPM3 transcript level remained unaltered in treated and control hearts (lanes 1 & 2; panels c & d, Figure 4) and skeletal muscle (lanes 3 & 4; panels c & d, Figure 4). We isolated the amplified total TPM3 DNA and determined the nucleotide sequences.

Interestingly, Shz-1 upregulated the TPM4a expression significantly in axolotl heart (lane 1 & 2; panel e, Figure 4). TPM4 is known to play critical role in axolotl heart and is not expressed in skeletal muscle. Our results showed no expression of TPM4 in both shz-1 treated and control juvenile axolotl skeletal muscle (lanes 3 & 4, panel c in Figure 4). The results led us to conclude that shz-1 did not induce TPM4, the cardiac specific tropomyosin isoform, in skeletal muscle. As previously mentioned, we included p53 and cardiac TNNT2 in this gene expression study in heart and skeletal muscle from shz-1-treated and untreated juvenile axolotl.

Results depicted in Figure 5 suggest that p53 expression level remained unaltered in heart (lanes 1 & 2) and skeletal muscle (lanes 3 & 4) in shz-1 treated animals (panel a - ethidium bromide staining and panel b- southern hybridization with specific probe). The nucleotide sequences of the primer-pairs and oligonucleotde probe are presented in Table 1. The expression level of TNNT2, which is known to be express in axolotl hearts, remained unchanged in shz-1-treated heart (lanes 1 & 2; panel d, ethidium staining of the TNNT2 amplified DNA and panel e, southern hybridization with TNNT2 specific probe). No TNNT2 expression was detected in shz-1 treated and control skeletal muscle. Again, shz-1 did not induce the expression of TNNT2 in skeletal muscle. Ethidium bromide staining and subsequent southern hybridization with a gene specific primer pair (panel e) and subsequent southern hybridization with an oligonucleotide probe (sequences of the oligonucleotides given in Table 1) showed that the expression remained unaltered in control (lane 2 for heart & lane 4 for skeletal muscle) and shz-1-treated (lane 1 for treated heart and lane 3 for treated skeletal muscle) axolotl tissues (panels e & f in Figure 5).

(b) Effect of shz-1 on the expression of sarcomeric tropomyosin proteins in heart and skeletal muscle

Shz-1 injection led to the augmentation of mRNA expression of various sarcomeric tropomyosin isoforms in axolotl heart. Five sarcomeric isoforms of the four TPM genes designated as TPM1, TPM1, TPM2, TPM3, and TPM4 are all recognized by CH1 monoclonal antibody (Schevzov et al 2011; Spinner et al 2002; Rajan et al 2010). To the best of our knowledge, there is no gene specific antibody that recognizes the gene specific sarcomeric isoform. For example, no specific antibody exists that recognizes only TPM1 but not TPM2, or TPM3, or TPM4, except for TPM1 (Rajan et al 2010). For TM protein analysis, we chose CH1 antibody that recognizes all five sarcomeric isoforms as mentioned above. Panel a (Figure 6) represents the blot stained with Panceau dye, which showed the loading consistency. Panel b (Figure 6) shows the signal with CH1 monoclonal antibody that recognizes the total sarmoeric tropomyosin expression. We failed to detect any significant increase in sarcomeric tropomyosin protein either in heart or in skeletal muscle due to shz-1 injection. Next, we used TM311 monoclonal anti-tropomyosin antibody that recognizes all tropomyosin isoforms containing exon 1a generated by any of the four TPM genes in vertebrates (Schevzov et al 2011). Again, we did not find any significant changes in the tropomyosin protein concentration in heart and skeletal muscle after shz-1 injection. We also performed western blot analysis with anti-TPM1 that recognizes only TPM1 and no other sarcomeric tropomyosin (panel c, Figure 6). No significant changes in TPM1 expression were recorded due to shz-1 treatment.

Our western blot analysis with CH1 and TM311 antibodies using extracts of axolotl embryos (stage 35 -37) maintained in presence or absence of 10 M Shz-1 did not show any changes in sarcomeric or total high molecular weight tropomyosin expression (Figure 7).

The combined results with the three different antibodies viz. CH1, TM311, and TPM1 argued against the upregulation of sarcomeric tropomyosin expression in shz-1 heart. Results of our western blot analysis are in contrast with our RT-PCR results. Thus we believe, tropomyosin expression in axolotl may undergo some kind of translational control, which conceptually is not new.

4. DISCUSSION

Nkx2.5, in conjunction with other transcription factors such as GATA-4, regulates the expression of various cardiac specific-genes during heart development (Chen and Schwartz 1996; Biben and Harvey 1997). Sadek et al (2008) reported that the Shz-1 molecule activates myocardin gene in P19CL6 cells. Myocardin is a cardiac- and smooth muscle-specific cofactor for the ubiquitous transcription factor serum response factor (SRF) (Small et al 2005). Shz-1 has also been reported to induce other cardiac-specific genes like cardiac troponin-I (cTnI) in human cells (M-PBMC). Most importantly, the Shz-1 molecule has been shown to induce the expression of sarcomeric □-tropomyosin protein. This was demonstrated by western blot analysis using CH1 monoclonal antibody, and by RT-PCR using a gene specific primer pairs (Sadek et al 2008) . Sarcomeric□□□□tropomyosin is indeed a highly specific marker of striated muscle cells and a very early marker of stem-cell cardiogenesis (Muthuchamy et al 1995). Various vertebrate cardiac tissues are also known to express TPM1□, an alternatively spliced tropomyosin isoform of the *TPM1* gene (Luque et al 1997; Thomas et al 2010; Denz et al 2004). CH1, a widely used monoclonal antibody, recognizes all sarcomeric tropomyosin isoforms including TPM1□, TPM1□, TPM2□, TPM3□, and TPM4□□. Sadek et al (2008) however, did not address whether or not Shz-1 affects the expression of other sarcomeric tropomyosin isoforms in cells during cardiogenic transformation. Of the four TPM genes, *TPM1* is the most versatile. It generates at least 10 different isoforms by alternate splicing and/or by using different promoters (Lees-Miller and Helfman 1991; Schevzov et al 2011; Narshi et al 2005). Two of the ten known isoforms are striated muscle specific, which are designated as TPM1□ and TPM1□ (Narshi et al 2005). The *TPM1* gene is expressed in most, if not all tissues, but the isoform expression is tissue-specific.

Sadek et al. (2008) did not explore the mechanism by which shz-1 induces the expression of sarcomeric -□ tropomyosin (TPM1□) in various non-cardiac cells. Shz-1 treatment may induce some of the cardiac specific genes in p19 cells and one or several of these genes, in turn, may change the splicing pattern of the *TPM1* gene. Or, shz-1 may induce the transcription of the promoter upstream to exon 1a of the TPM1 gene that leads to the expression of high molecular weight tropomyosin containing 284 amino acid residues including sarcomeric trpomyosin. In that case, one has to assume that the transcription of various tropomyosin isoforms in p19 cells initiates from the internal promoter of the *TPM1* gene that resides between exon 2b and exon 1b. In other words, only low molecular weight tropomysin isoforms containing 248 amino acids are expressed in p19 cells; Shz-1 specifically activates the extreme upstream promoter and initiates the transcription of high molecular weight TPM proteins including sarcomeric-□-TM (TPM1□).

Mexican axolotl is a unique animal model for studying the isoform diversity of tropomyosin (Zajdel et 1999). Although most of the myofibrillar proteins are present at near normal quantities, the cardiac mutant axolotl hearts are deficient in sarcomeric tropomyosin protein. However, mRNA expression of various sarcomeric tropomyosin isoforms is comparable in normal and mutant hearts (Zhang et al 2009; Spinner et al 2002). We cloned and sequenced Nkx2.5 cDNA from normal and mutant axolotl hearts, and we found no differences in nucleotide sequence between these two phenotypes. Our results strongly suggest that the mutant phenotype of cardiac mutant is not due to a mutation in the *Nkx2.5* gene. Moreover, our qRT-PCR results ruled out the hypothesis that the cardiac mutant phenotype in axolotl is due to an insufficiency of Nkx2.5 expression (Thurston et al 2009). The hearts in mutant axolotl embryos are developed, but the mutant hearts do not beat because they do not form organized myofibrils due to a lack of sarcomeric tropomyosin protein(s). Ectopic expression of tropomyosin protein in mutant hearts form organized myofibrils, which results in ventricular contraction in situ (Zajdel et al 1998). To the best of our knowledge, the conduction system in mutant hearts is not affected by the mutation in gene c. Furthermore, p53 expression is not inhibited in mutant hearts (Thurston et al 2009). Hence, we believe mutant hearts are not the best system for studying the effect of Shz molecules on sarcomeric proteins in cardiac tissues. The results on the effect of such chemical agents on sarcomeric protein expression in mutant hearts, we believe, will be difficult to explain.

Our results reveal that the expression of Nkx2.5 is augmented in hearts in juvenile axolotl injected with Shz-1 (Figure 1). However, Nkx2.5 is not expressed in skeletal muscle in control and shz-1 treated animals (Figure 1). The absence of expression of Nkx2.5 in axolotl skeletal muscle is consistent with our previously published observation (Thurston et 2009). Interestingly, RT-PCR results suggest an increasing level of transcripts for both TPM1□ and TPM1□ in cardiac tissues from shz-1 treated animals. In skeletal muscle, the transcript level of TPM1□ is also increased (Figure 3 panels c and d). We found a significant increase in TPM4 transcripts in shz-1 treated axolotl hearts. Sarcomeric TPM4□ has been shown to play an essential role for cardiac myofibril formation and cardiac contractility in axolotl hearts (Zajdel et al 2005) and is not expressed in skeletal muscle (Figure 4). Our results also revealed that the transcript level of the total TPM2 expression in treated heart was increased. As we do not have yet the complete nucleotide sequence of TPM2□, we are not in a position to conclude whether the transcript level of sarcomeric isoform of the *TPM2* gene designated as TPM2□ (Narshi et al 2005) is increased specifically. This is, however, the first demonstration that TPM2 is expressed in axolotl hearts and skeletal muscles. TPM2a is known to be expressed in amphibian system like xenopus. Currently, we are in the process of characterizing various isoforms of the *TPM2* gene expressed in axolotl. Unlike TPM2, the total TPM3 expression in heart and skeletal muscle in shz-1 treated animals remained unaltered (Figure 4). It is known that the

sarcomeric isoform of the *TPM3* gene designated as TPM3 is expressed in vertebrate skeletal muscles but not in cardiac tissues (Lees-Miller and Helfman 1991; Schevzov et al 2011).

In contrast to the expression of transcripts of various TM isoforms, we were unable to detect any increase in sarcomeric tropomyosin protein expression by western blot analysis either with TM311 or with CH1(Figure 6), or with TPM1 (Figure 6) specific antibodies in shz-1 injected animals. We also performed western blot analysis with total extracts of control and shz-1 treated axolotl embryos (stages 36-38) with CH-1 (Panel a, Figure 7)and TM311 (Panel b) antibodies. We were unable to detect any increase either in sarcomeric tropomyosin expression (as judged by the signal detected with CH1 antibody) or the total high molecular weight tropomyosin protein (signal obtained with TM311 antibody) (Figure 7). TM311 recognizes tropomyosin isoforms with exon 1a (Schevzov et al 2011).

5. SUMMARY AND CONCLUSION

Shz-1 induces the expression of transcripts of TPM1, TPM1, and TPM2 in axolotl hearts. However, we failed to detect any significant increase in sarcomeric tropomyosin protein in heart or skeletal muscle. This is most likely due to a translational control of various tropomyosin transcripts. Muthuchamy et al (1995) reported such translational control in hearts of transgenic mice over-expressing TPM2 in a cardiac- specific manner. An over-expression of TPM2 in hearts in several transgenic mice led to a significant decrease in TPM1 protein. As a result the level of expression of total sarcomeric tropomyosin remained the same. Rethinasamy et al (1998) reported that mice that are heterozygous for *TPM1* in *TPM1* ablated mice live well and reproduce normally. mRNA expression of TPM1 was reduced by 50% in hearts of transgenic mice. However, the level of TPM1 protein in hearts of TPM1 ablated mice was the same as in non-transgenic mice. Also, there was no increase in TPM2 protein. The authors concluded that translational regulation might control tropomyosin expression. Similar translational control of tropomysin expression may also be active in axolotl.

Although cardiac mutant axolotl hearts express the transcripts of various tropomyosin isoforms, the mutant hearts are deficient in tropomyosin protein [11,12]. However, mutant hearts when transfected with a low molecular weight non-coding RNA known as "Myofibril Inducing RNA" (or MIR) produce tropomyosin protein and thereby promotes myofibril formation in mutant hearts in situ [13]. MIR may somehow promote the translation of various transcripts of sarcomeric tropomyosin in mutant axolotl hearts. The results showed a significant increase in transcription of TPM1, TPM1, and TPM4 in hearts of shz-1 -injected axolotl without affecting the synthesis of corresponding protein(s). Our findings point towards a translational control of sarcomeric tropomyosin protein expression in axolotl hearts.

ACKNOWLEDGEMENT

The work was supported by a grant from American Heart Association and a grant from
Golisano Children's Miracle Network, Syracuse, New York to DKD. We are thankful to Dr. Harold Thurston for his critical comments.

Conflict of Interest
The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. RT-PCR analysis of Nkx2.5 expression in heart and skeletal muscle from juvenile axolotl injected with shz-1.

Panel a. Ethidium Bromide staining of Nkx2.5 amplified DNA.
Panel b. Ethidium Bromide staining of GAPDH amplified DNA.

Lane 1: Heart from control axolotl; Lane 2: heart from shz-1 injected animals; Lane 3: skeletal muscle from control animals; Lane 4: skeletal muscle from shz-1 injected axolotl; Lane 5: primer control; Lane M: Molecular marker.

Figure 2. RT-PCR of Nkx 2.5 from 3 different cDNA prepared from total RNA isolated from hearts of control and shz-1 injected axolotl.

Panel a. Ethidium bromide staining of Nkx2.5 amplified DNA.
Panel b. Southern hybridization of Nkx2.5.

Lane 1: 1st cDNA from control animal; Lane 2: 1st cDNA from shz-1 injected animal; Lane 3: 2nd preparation from control injected animal; Lane 4: 2nd preparation from shz-1 injected animal; Lane 5: 3rd preparation from control axolotl; Lane 6: 3rd preparation from shz-1 treated axolotl; Lane 7: primer control.

Figure 3. RT-PCR and Southern hybridization of TPM1a and TPM1k expressed in heart and skeletal muscle in control and shz-1 injected axolotl.

Panel a: Ethidium Bromide staining of TPM1a amplified DNA.
Panel b: Southern hybridization with TPM1 probe

Panel c: Ethidium bromide staining of TPM1k amplified DNA.
Panel d: Souther hybridization with TPM1 probe.
Panel e: Ethidium bromide staining of GAPDH amplified DNA.

Lane 1: Shz-1 injected heart; Lane 2: Control heart; Lane 3: shz-1 injected skeletal muscle; Lane 4: Control skeletal muscle; lane 5: Primer control.

Figure 4. RT-PCR of various TPM genes expressed in hearts and skeletal muscle in control and shz-1 injected axolotl.

Panel a: Ethidium bromide staining of TPM2 amplified DNA.
Panel b: Southern hybridization with TPM2 probe.
Panel c: Ethidium bromide staining of TPM3 amplified DNA.
Panel d: Southern hybridization with TPM3 probe.
Panel e: Ehidium bromide staining TPM4a amplified DNA.
Panel f: Ehidium bromide staining of GAPDH amplified DNA.

Lane 1: Shz-1 injected heart; Lane 2: Control heart; Lane 3: Shz-1 injected skeletal muscle; Lane 4: Control skeletal muscle; Lane 5: Primer control.

Figure 5. RT-PCR and Southern hybridization of genes expressed in heart and skeletal muscle from control and Shz-1 injected axolotl.

Panel a: Ethidium bromide staining of p53 amplified DNA.
Panel b: Southern hybridization of p53.
Panel c: Ehidium bromide staining of troponin-T (TNNT2) amplified DNA.
Panel d: Southern hybridization of TNNT2.
Panel e: Ethidium staining of GAPDH amplified DNA.
Panel f: Southern hybridization of GAPDH.

Lane 1: Shz-1 injected heart; Lane 2: Control heart; Lane 3: Shiz-1 injected skeletal muscle; Lane 4: Control skeletal muscle; Lane 5: Primer control.

Figure 6. Western blot analysis of shz-1 injected and control axolotl hearts and skeletal muscle with various anti-tropomyosin antibodies.

Top panel: Blot stained with Ponceau red dye
Panel Kappa: Stained with TPM1k antibody
Panel CH1: Stained with CH1 antibody
Panel TM311: Stained with TM311 antibody

Lane TH: Shz-1 injected heart; Lane CH: Control heart; Lane TS: Shz-1 injected skeletal muscle; Lane CS: Control skeletal muscle; Lane M: Molecular weight marker.

Figure 7. Western blot analysis with whole body extracts of shz-1 treated and control axolotl embryos with CH1 and TM311 antibodies

Panel a. Stained with CH1 antibody
Panel b. Stained with TM311
Lane 1: Control embryos; Lane 2: Shz-1-treated embryos Lane M: Marker

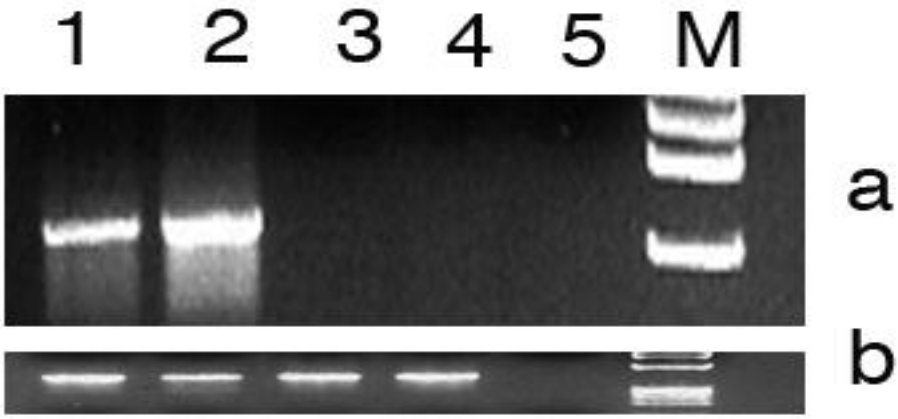
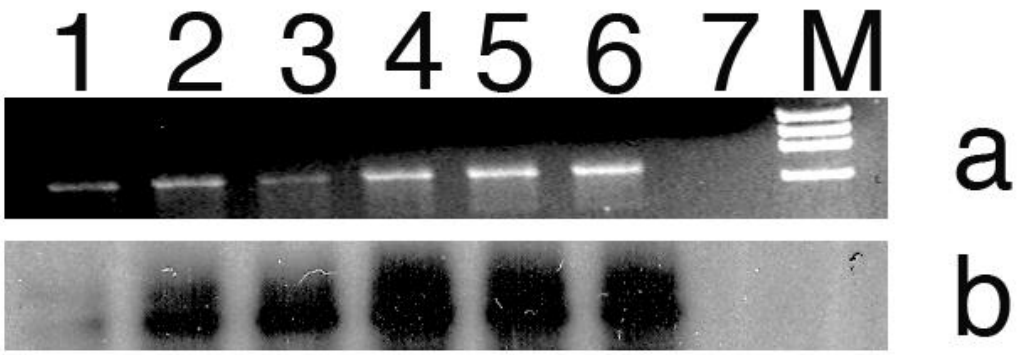


Figure 1.

Figure 2.



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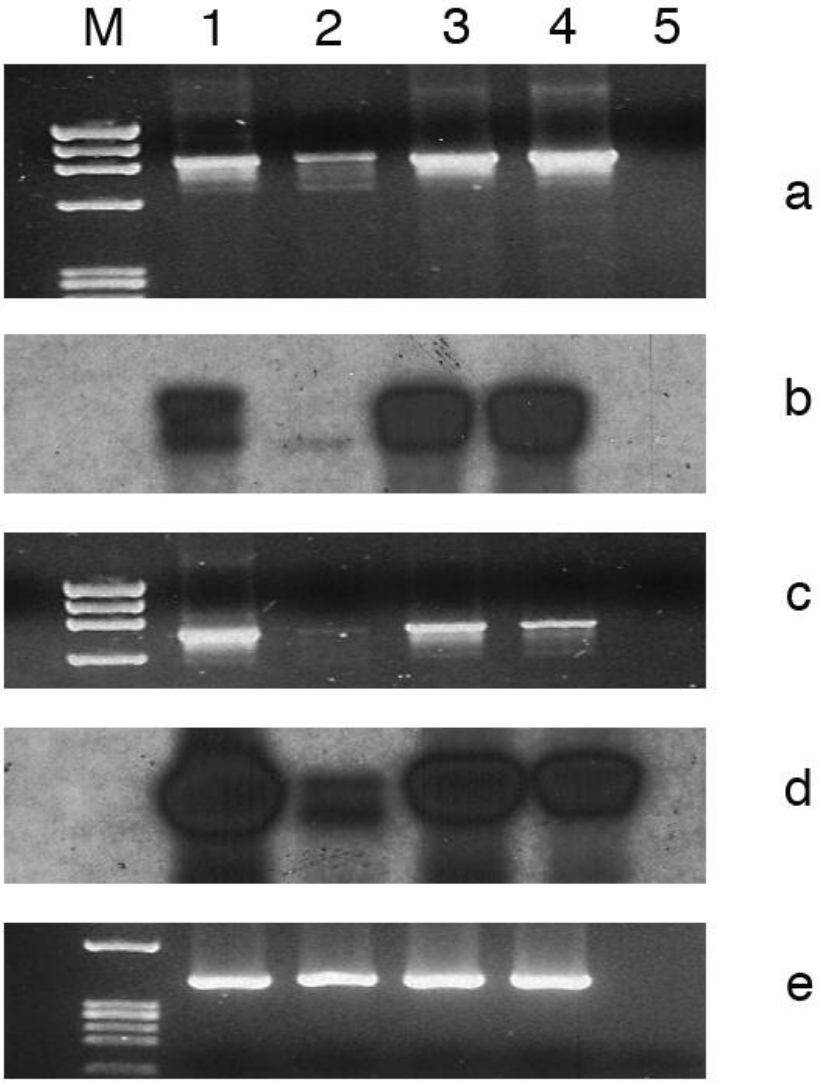
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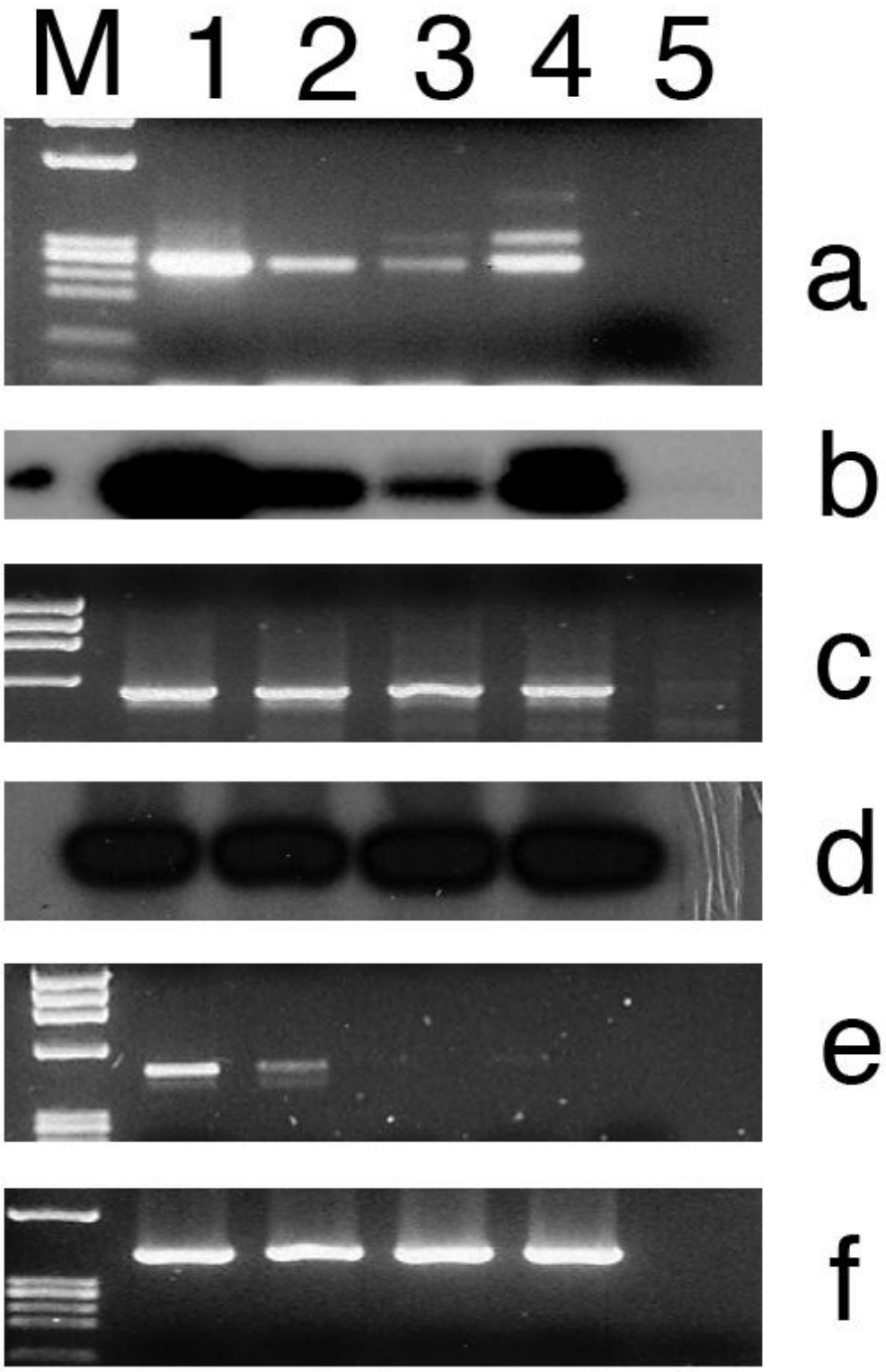
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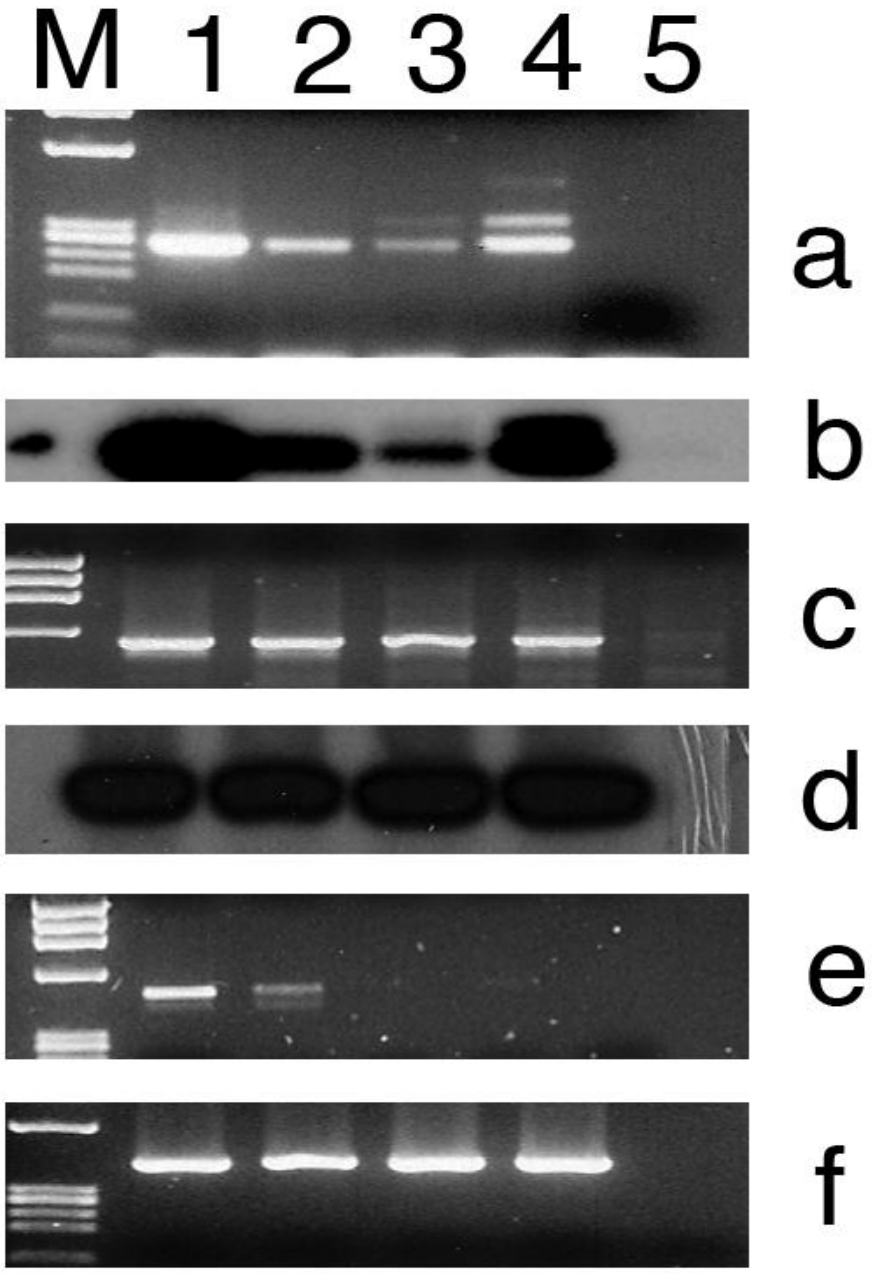

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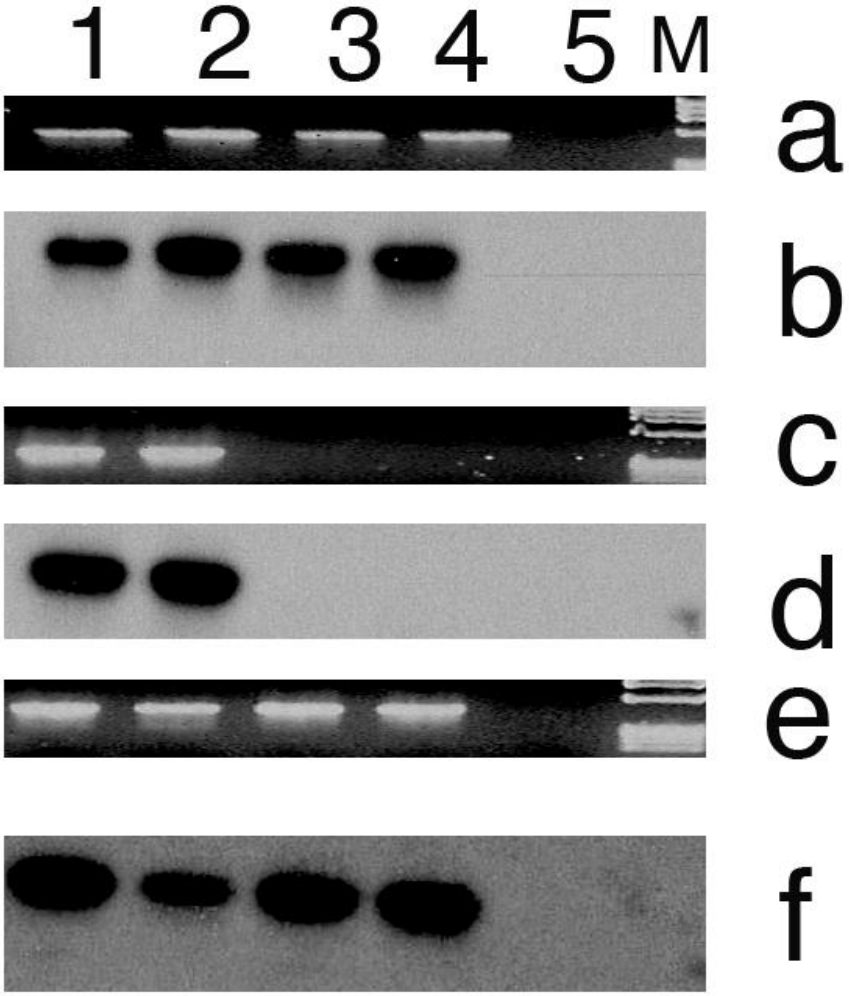

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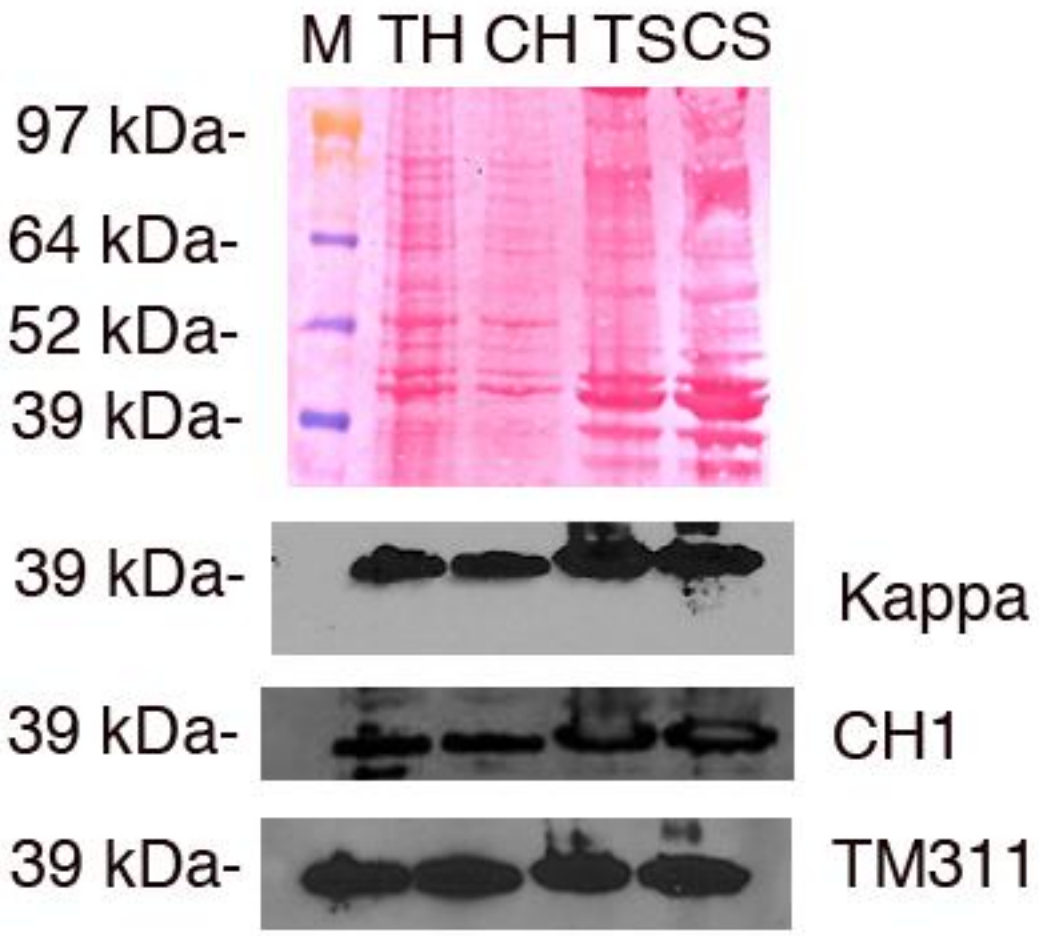
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Figure 7.

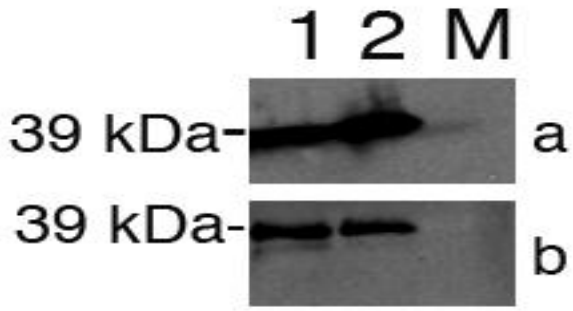

Table 1: Sequences of gene as well as isoform-specific primer-pairs and detector oligonucleotides used in this study

Gene	Amplification type	Primer/detector	Sequence
Nkx2.5	Coding sequence	Forward primer Reverse primer Probe	5'-TGGACCCAAAGGCGGACAA-3' 5'-AAAGGTCTACCAGGCTCTGAT-3' 5'-AAACCAGATCTTGACTTGCGT-3'
TPM1	Coding sequence for TPM1a	Forward primer Reverse primer Probe	5'-CTTGAGGACGAGCTAGTAGCCC-3' 5'-TACATTGAAGTCATATCGTTGTTGAG-3' 5'-GAAAGGGCAGAACTCCTCTGAAGG-3'
	Coding sequence for TPM1k	Forward primer Reverse primer Probe	5'-TAGAGGAGGATTGTGCA-3' 5'-TACATTGAAGTCATATCGTTGTTGAG-3' 5'-GAAAGGGCAGAACTCCTCTGAAGG-3'
TPM2	RT-PCR for total TPM2	Forward primer Reverse primer Probe	5'-AAGAGGCCAAACACATAGCA-3' 5'-GCCTCTTTCAGTTTGTCTC-3' 5'-AAGCCGAGTCCGGCAACTA-3'
TPM3	Coding sequence for total TPM3	Forward primer Reverse primer Probe	5'-CATCGAGGCCGTGAAGCGCA-3' 5'-TTGGGGAGTACTTTTCATC-3' 5'-TGAGGAGGCTGACCGGAAAT-3'
TPM4	Coding seuence for TPM4	Forward primer Reverse primer	5'-AGCCTTACAAAAACTGGAA-3' 5'-CAAGTTTGGCAACGGTCCTT-3'
Cardiac TnT	Coding sequence for high molecular wt cTNNT-1 & CTNNT2-2	Forward primer Reverse primer Detector/Probe ®	5'-AGGAGATGTGGCAATGGAT-3' 5'-TGGCAGAGGTGGAATGGATCAC-3' 5'-TGAATGCGGCGGTTCAAGGA-3'

P53		Forward primer Reverse primer Probe	5’-TTCCGGGCAGAGTACAAGAT-3’ 5’-TGAGGAATCATATCCTTCAGCT-3’ 5’-GTGCCTGTCCAGGTCGTGAC-3’