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ORIGINAL PAPER

Hsp27 is persistently expressed in zebrafish skeletal and cardiac muscle tissues but dispensable for their morphogenesis

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Abstract Constitutive expression of Hsp27 has been demonstrated in vertebrate embryos, especially in developing skeletal and cardiac muscle. Results of several previous studies have indicated that Hsp27 could play a role in the development of these tissues. For example, inhibition of Hsp27 expression has been reported to cause defective development of mammalian myoblasts in vitro and frog embryos in vivo. In contrast, transgenic mice lacking Hsp27 develop normally. Here, we examined the distribution of Hsp27 protein in developing and adult zebrafish and effects of suppressing Hsp27 expression using phosphorodiamidate morpholino oligonucleotides (PMO) on zebrafish development. Consistent with our previous analysis of *hsp27* messenger RNA expression, we detected the protein Hsp27 in cardiac, smooth, and skeletal muscle of both embryonic and adult zebrafish. However, embryos lacking detectable Hsp27 after injection of antisense *hsp27* PMO exhibited comparable heart beat rates to that of control embryos and cardiac morphology was indistinguishable in the presence or absence of Hsp27. Loss of Hsp27 also had no effect on the structure of the skeletal muscle myotomes in the developing embryo. Finally, embryos injected with antisense *hsp27* and scrambled control PMO displayed equal motility. We conclude that Hsp27 is dispensable for zebrafish morphogenesis but could play a role in long-term maintenance of heart and muscle tissues.

Keywords Hsp27 · Hsp70 · Muscle · Heart · Zebrafish

Introduction

Heat shock proteins play essential roles in all stages of an organism's life. Well-known functions of heat shock proteins include folding of nascent proteins and refolding of proteins damaged as a consequence of thermal injury and other stresses. Other vital functions include participation as cofactors in receptor-mediated signaling (Pearl and Prodromou 2006), scaffolding proteins for signaling elements (Rane et al. 2003), maintenance of cellular reduction/oxidation (redox) states (Arrigo 2001), and stabilization of cytoskeletal arrays (for representative review, see Mounier and Arrigo 2002). High-molecular-weight heat shock proteins use the energy of adenosine triphosphate (ATP) to modify or stabilize the conformation of other proteins, thereby preventing misfolding or restoring normal folding of target proteins. In contrast, small heat shock proteins (sHsps) lack ATPase activity but can undergo phosphorylation-dependent transition between high-molecular-weight oligomers and smaller subunits (Lambert et al. 1999).

Hsp27, also known as HspB1, is one of the most widely expressed and distributed small heat shock protein. Expression of Hsp27 is upregulated in response to sublethal injury by epithelial (Kiriyama et al. 2001; Bonham et al. 2003), neuronal (Costigan et al. 1998; Dodge et al. 2006), muscle (Knoll et al. 1994; van de Klundert et al. 1998), and other

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cell types, and orthologs of human Hsp27 are found in mammals, birds, fish, and amphibians (Gernold et al. 1993; Norris et al. 1997; Kawazoe et al. 1999; Mao et al. 2005; Tuttle et al. 2007). Altered expression and regulation of Hsp27 have been demonstrated in nerve and muscle tissues as a consequence of injury (Yoshida et al. 1999; Sakamoto et al. 2000), disease (Clemen et al. 2005), and aging (Chung and Ng 2006; Yamaguchi et al. 2007). A variety of functions for Hsp27 in injured cells have been proposed, including chaperone function (Jakob et al. 1993), modulation of apoptotic signaling cascades (Garrido et al. 1999; Charette et al. 2000; Pandey et al. 2000; Rane et al. 2003), and the regulation of cellular redox and glutathione levels (Arrigo 2001; Escobedo et al. 2004). In addition, Hsp27 can interact with cytoskeletal elements and stabilize cytoskeletal arrays in injured cells (Lavoie et al. 1993a, b). Many of these functions are also performed by other heat shock proteins, and there is evidence that small heat shock proteins may act, at least in part, as a cochaperone for high-molecular-weight heat shock proteins such as Hsp70 (for review, see Haslbeck et al. 2005).

In addition to stress-induced expression, *hsp27*, along with genes for a number of related small heat shock proteins, is expressed in some tissues in the absence of stress, including all types of muscle cells (Dillmann 1999; Benndorf and Welsh 2004). The role of constitutively expressed Hsp27 is not completely understood. Upregulation of *hsp27* expression is seen in differentiating myoblasts in vitro (Davidson and Morange 2000; Ito et al. 2001), as well as developing muscle cells of murine and amphibian embryos (Gernold et al. 1993; Tuttle et al. 2007). Similarly, we and others have reported muscle-cell-specific expression of Hsp27 in myotomes of developing zebrafish embryo (Mao et al. 2005; Marvin et al. 2008). Antisense *hsp27* messenger RNA (mRNA) expression also inhibits development of murine myoblasts (Davidson and Morange 2000) and induces cell death of embryonic stem cells (Mehlen et al. 1997) in vitro. Recently, knockdown of Hsp27 expression using phosphorodiamidate morpholino oligonucleotides (PMO) was reported to cause defects in heart formation and myofibril architecture in both skeletal and cardiac muscle of developing frog embryos (Brown et al. 2007). Together, these results suggest that Hsp27 may be required for differentiation of muscle cells. In contrast, *Drosophila* embryos lacking a homolog of human Hsp27 develop normally and, surprisingly, loss of Hsp27 does not alter the resistance of flies to heat shock or oxidative injury (Hao et al. 2007). The *Drosophila* homolog of Hsp27 lacks phosphorylated serines conserved among vertebrate homologs of Hsp27 (Ingolia and Craig 1982) and displays a constitutive localization to the nucleus of cells under control conditions (Marin and Tanguay 1996) not typical of vertebrate Hsp27. Therefore, it is not clear that direct comparisons between the roles of vertebrate and *Drosophila* Hsp27 are appropriate. However, normal development of mice lacking Hsp27 as a result of gene disruption has also been reported (Huang et al. 2007). Thus, there are currently conflicting data regarding the role of Hsp27 during embryogenesis.

In the present study, we have examined cellular and physiological consequences of transient Hsp27 knockdown in zebrafish embryos using injection of antisense *hsp27* PMO. We have also produced an antiserum suitable for immunolocalization and immunoblotting detection of zebrafish Hsp27. Data presented below demonstrate that skeletal muscle morphogenesis and function in zebrafish embryos lacking detectable Hsp27 are indistinguishable from that of embryos injected with a nonspecific control PMO. Assessment of cardiac beat frequency and morphology also failed to show an aberrant phenotype in the absence of Hsp27. Constitutive and stress-induced expression of Hsp70 was unaffected by the loss of Hsp27 in our experiments. However, Hsp27 was expressed in both embryonic and adult muscle tissues as well as a small number of other locations. These data reveal that Hsp27 plays a dispensable role in the morphogenesis of zebrafish and provides a new perspective on the functional significance of Hsp27 in vivo. Specifically, our findings are consistent with a role for Hsp27 in the maintenance of muscle tissue homeostasis.

Materials and methods

Zebrafish husbandry and experimental stress Adult zebrafish, *Danio rerio*, were reared and maintained as described previously (Mao et al. 2005). Embryos were collected from wild-type crosses and maintained at 26°C in embryo medium as described by Westerfield (1993). Embryos were heat-shocked at 36 h postfertilization (hpf) in a circulating water bath (IsoTemp 2150, Fisher Scientific, Pittsburgh, PA, USA) in parafilm-sealed 35-mm Petri dishes for 30 min at 39°C and allowed to recover at 26°C for 14 h before collection. Protocols for the use of animals in these experiments were approved by the Washington State University Animal Care and Use Committee and were in accord with National Institute of Health standards established by the Guidelines for the Care and Use of Experimental Animals.

Production of antiserum detecting zebrafish Hsp27 A complementary DNA coding for zebrafish Hsp27 (Mao et al. 2005) was cloned into the prokaryotic vector pET45b (Novagen, Madison, WI, USA) and expressed in *Escherichia coli*, strain BL21. The 6-His-tagged fusion protein was purified using Talon metal affinity resin (Clontech Laboratories, Palo Alto, CA, USA) and used to generate polyclonal Hsp27 antiserum (αHsp27) as described by Monteville et al. (2003). Pre-immune and αHsp27 sera were tested for

recognition of purified recombinant protein and zebrafish Hsp27 expressed in NIH3T3 fibroblasts (Mao et al. 2005) by Western blotting and indirect immunolocalization, respectively (data not shown).

SDS-polyacrylamide gel electrophoresis and Western blotting For expression analysis during development, pools of ten or more embryos were chilled and sonicated on ice in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris/HCl (pH 6.8), 2% SDS, 0.01% bromophenol blue, 10% glycerol) and samples were cleared by centrifugation at 10,000×*g* for 10 min at 4°C. For tissue analysis (Fig. 6), adult male zebrafish were euthanized in ice water and dissected to obtain select tissues. Tissue pieces were immersed in SDS-PAGE sample buffer containing 2.5 mM ethylenediaminetetraacetic acid (EDTA) and a 1:200 dilution of Protease Inhibitor Cocktail (SigmaAldrich P8340). Protein concentrations were measured using a Bio-Rad D_C Protein Assay Kit. After adding β-mercaptoethanol to a final concentration of 4%, proteins were separated using 4% acrylamide stacking and 12% running gels. Resolved proteins were transferred to membranes (NitroBind, 0.22 μm, GE Water & Process Technologies, Trevose, PA, USA) and stained with Ponceau S to confirm uniform loading and transfer of proteins. Immunoblotting was conducted using the αHsp27 serum described above, an anti-Hsp70 antibody (rabbit polyclonal SPA-812, Stressgen, Inc.), or an antisarcomeric actin antibody (SigmaAldrich, clone 5C5) and peroxidase-labeled secondary antibodies. Blots were developed and analyzed as described previously (Mao et al. 2005). Each experiment was performed at least three times.

Phosphorodiamidate morpholino oligonucleotide microinjections PMO were obtained from GeneTools, Inc. (Corvallis, OR, USA). PMO sequences were: Hsp27 antisense (*hsp27i*), 5'GTTTTGAAGAGTTGTT TTTCGGCTC3', and scrambled control (scr), 5'GGAGCTTAGTGA TGTTCTGTTCTTT3'. The *hsp27i* PMO used in our studies is complementary to the −11–36 bp of the 5'-untranslated region of the zebrafish *hsp27* mRNA sequence. The scrambled morpholino has the identical nucleotide composition in a random order. A BLASTn search of over 37 million annotated zebrafish nucleotide sequences identified only one sequence (*hsp27*/*hspB1*) with significant homology to *hsp27i*. PMO were dissolved in either sterile distilled water (see Figs. 1c and 3) or injection buffer (0.4 mM $MgSO_4$, 0.6 mM $CaCl_2$, 0.7 mM KCl, 58 mM NaCl, 25 mM HEPES pH 7.1) and aliquots were frozen at −80°C. Zebrafish embryos were injected as described by Liu et al. (2007) at the one- to four-cell stage using a Narishige coarse micromanipulator and MMPI-2 pneumatic pressure regulator (Applied Scientific Instruments, Inc., Eugene, OR, USA). Needles were pulled from borosilicate capillary tubes (1.00×0.78 mm×10 cm, Sutter Instruments Co., Novato, CA, USA) using a Sutter Instruments pipette puller (Model P-87). Injection volumes (assessed by injecting solutions into mineral oil and calculating the volume of droplets) were approximately 1.8 nl. In preliminary studies, injection of both *hsp27i* and scr PMO at a concentration of 1 mM caused equivalent embryo toxicity, manifested as reduced size at 24 hpf, abnormal appearance, heart and trunk muscle development, and white patches of dead cells, particularly in the developing brain (data not shown). Injection of the *hsp27i*

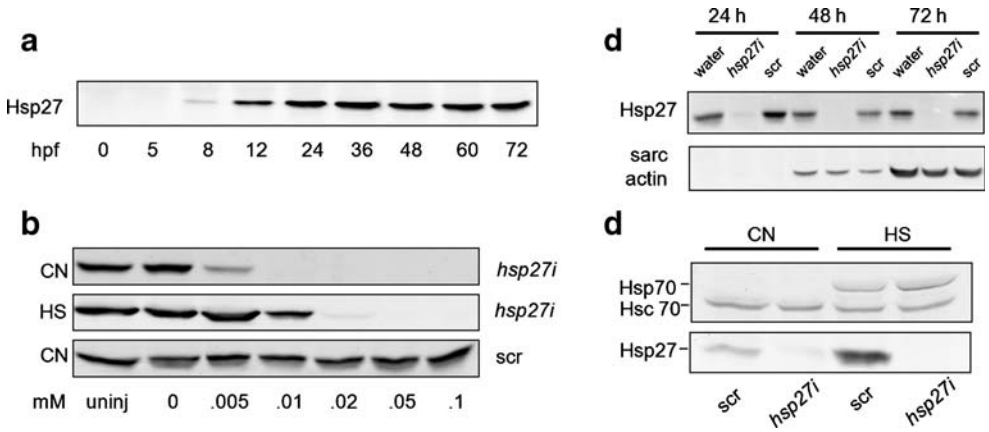

Fig. 1 Western blot detection of Hsp27 and Hsp70 in zebrafish embryos. **a** Developmental time course of Hsp27 expression. *Numbers* are time of sample harvest in hours postfertilization (*hpf*). **b** Concentration effects of scrambled (*scr*) and antisense *hsp27* (*hsp27i*) PMO injection on Hsp27 expression. *Numbers* represent injection concentration in millimolar. A sample from uninjected embryos (*uninj*) is also shown. **c** Effects of 0.15-mM *hsp27i* PMO injection on Hsp27 expression in embryos of varying ages. The expression of sarcomeric (*sarc*) actin is shown for comparison. **d** Effects of 0.1 mM *hsp27i* PMO injection on expression of constitutive Hsc70 and inducible Hsp70 in control (*CN*) and heat-shocked (*HS*) embryos. Samples were harvested at 36 hpf. All lanes were loaded with equal amounts (30 μg) of total embryo protein

PMO at a concentration of 0.02 mM or less had little or no effect on Hsp27 expression (Fig. 1b). Injection of *hsp27i* PMO at concentrations of 0.1 and 0.15 mM inhibited Hsp27 expression without affecting development or behavior of embryos, relative to solvent or scr-PMO-injected embryos (see "Results").

Fluorescent labeling of embryos To visualize myofibrils, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. Whole embryos were stained with rhodamine-labeled phalloidin (0.1 μg/ml) diluted in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 0.5% Triton X-100, pH 7.3) overnight at 4°C, then rinsed twice for 1 h in PBS containing 0.5% Tween 20 (PBST). Embryos were mounted on slides with Mowiol containing an antifade agent (DABCO, Sigma Chemical Co.). To visualize cardiac myofibrils, tails were removed from labeled embryos and the thorax was mounted in agarose. Images of embryos were obtained using a Zeiss LSM 510M confocal microscope (Carl Zeiss Inc., Thornwood, NY, USA) and a ×20 1.2 NA glycerol immersion objective. A through-focus series of images in the Z dimension was collected for each embryo at 3-μm intervals. For detection of Hsp27 in situ, whole embryos or dissected adult tissues were fixed for 30 min in 4% formaldehyde in PBST, permeabilized for >2 h in lysis buffer and then frozen in 100% OCT by immersion in liquid nitrogen. Twelve- to 15-μm-thick cryosections were cut using a Reichert-Jung cryotome (Cryocut model 1800) and placed on chrome-gelatin-subbed coverslips. Cryosections were fixed for an additional 10 min in 4% formaldehyde and then probed with αHsp27 serum and fluorescein-isothiocyanate-labeled secondary antibody. Fluorescent phalloidin was used as a counterstain. Images of cryosections were obtained using a Zeiss Axiovert 200M and an ORCA AG cooled charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ, USA). Different exposure times were used to image fluorescein- and rhodamine-labeled probes, but images were otherwise obtained using a single set of camera and illumination parameters for all embryos or tissue preparations. Postprocessing was conducted using Axiovision 4.1 (Carl Zeiss, Inc.) to set the background image intensity to black. This manipulation was applied simultaneously and equivalently to all images.

Motility assay and heart beat measurements Single batches of fertilized embryos were collected and injected with scr or *hsp27i* PMO at the one to two cell stages. Embryos were manually dechorionated at 50 hpf and placed in a 10-cm Petri dish containing embryo medium (Westerfield 1993) in a temperature-controlled chamber at 28.5°C. Movement was initiated by touching the embryo on the side and recorded using a digital camcorder (Panasonic Model PU-GS32). Videos were digitized using Window Movie Maker version 5.1 (Microsoft Corp.) at 30 frames per second and saved as AVI files, then converted to an image series using Quicktime Player Pro for Windows version 7.5.5 (Apple Inc.). Images were imported at six frames per second into ImageJ version 1.37 (Wayne Rasband, National Institute of Health, Bethesda, MD, USA) for further analysis. The X and Y coordinates of each embryo in each frame of videos were marked with a digital stylus and exported to Microsoft Excel. Excel was used to calculate the total distance between embryo positions in sequential frames. Average maximal rate of movement for each embryo was calculated by averaging the three highest velocity measurements from each swim.

Heart rates were measured by counting heartbeats during a 15-s interval for 50–52-hpf embryos. The heartbeats were counted by eye using a dissecting microscope (SMZ-2T, Nikon, USA). Data were obtained from a total of 30 embryos in each test group in three independent trials.

Myotome area measurements For analysis of myotome area, the myotome found dorsal to the caudal tip of the developing gut (the 12th myotome) was selected for analysis because it could be reliably identified in high-magnification confocal images of embryonic trunk regions. The area of the 12th myotome for each embryo was measured using ImageJ.

Statistical analysis Average values resulting from our quantitative analyses are presented with the calculated standard deviation or standard error of the mean as indicated. Average values measured for scr-PMO-injected embryos were compared to values measured from *hsp27i*-PMO-injected embryos, using Student's T test or Z test where indicated. Calculated p values of less than or equal to 0.05 were considered an indication of statistically significant differences in average values.

Results

Hsp27 expression during zebrafish embryogenesis

We previously demonstrated expression of zebrafish *hsp27* mRNA embryos as early as 8 hpf, with maximal expression observed at 24 hpf. Figure 1a shows a Western blot of equal amounts of total protein isolated from embryos at different ages. A single band at the predicted size of zebrafish Hsp27 (22 kDa) is detected on blots probed with αHsp27 serum in samples harvested as early as 8 hpf, consistent with our previous analysis of *hsp27* mRNA expression. Densitometry values (not shown) indicated that Hsp27 protein levels

increased approximately sevenfold between 9 and 24 hpf. Unlike *hsp27* mRNA expression levels that declined starting at 36 hpf, Hsp27 protein levels remained constant between 24 hpf and hatching at 72 hpf (Fig. 1a), although reduced expression, as a proportion of total protein, was seen by 1 week postfertilization (data not shown).

Morpholino knockdown of Hsp27 in zebrafish embryos

To investigate the potential roles for Hsp27 in morphogenesis, we injected zebrafish embryos at the one to two cell stages with antisense Hsp27 (*hsp27i*) or scrambled control (scr) PMO. Figure 1b shows effects of injecting PMO at varying initial concentrations on Hsp27 expression, evaluated by Western blotting. Injection of *hsp27i* PMO abrogated detectable expression of Hsp27 in embryos at concentrations >0.05 mM. In contrast, injection of scr PMO at all tested concentrations had no effect on Hsp27 expression (Fig. 1b).

Figure 1c demonstrates that injection of *hsp27i* PMO at a concentration of 0.15 mM inhibits expression of Hsp27 at all developmental time points examined in the present study. In contrast, no difference in Hsp27 protein expression is detected when comparing scr-PMO- and water-injected embryos at all time points. Figure 1c also shows immunoblotting to detect sarcomeric actin in samples obtained from embryos injected with *hsp27i* PMO, scr PMO, or water. There is no apparent difference in sarcomeric actin expression levels at any developmental time point between embryos expressing and lacking Hsp27, indicating that loss of Hsp27 does not affect muscle development at the level of sarcomeric actin expression.

Figure 1d examines if Hsp27 knockdown using 0.1 mM *hsp27i* PMO affects expression of the high-molecular-weight heat shock protein, Hsp70. Hsp70 has two isoforms in zebrafish, constitutively expressed Hsc70 and stress-upregulated Hsp70. Hsc70 expression (lower band) was unaltered after Hsp27 knockdown in both unstressed embryos and embryos recovering from heat shock. Additionally, Hsc70 protein expression is unaltered by heat shock, consistent with previous reports (Evans et al. 2005). As expected, inducible Hsp70 protein expression (upper band) is upregulated in embryos recovering from heat shock. The knockdown of Hsp27 had no effect on the expression of Hsp70 after heat shock.

Immunofluorescent detection of Hsp27 in 50-hpf embryos

Figure 2 shows cryosections of 50-hpf zebrafish embryos probed with αHsp27 serum and fluorescent secondary antibodies. Embryos were injected with equal concentrations (0.1 mM) and volumes of scrambled (scr; top and middle rows) or *hsp27i* PMO (bottom row) at the one to two cell stages. Consistent with published data on *hsp27* mRNA expression (Mao et al. 2005; Marvin et al. 2008), embryos injected with scr PMO exhibit ubiquitous low levels of Hsp27 expression and higher levels in the developing facial muscles (top left, arrows, and inset). Localization of Hsp27 within cryosections of the tail of scr-PMO-injected embryos (top center) also indicates ubiquitous low-level expression throughout developing myotomes and higher expression in a subset of myocytes on the outer edge of the myotome. This is most noticeable near the developing lateral line of the embryo where a subset of myocytes exhibits a high level of Hsp27 expression (top center, arrow). These cells were positively identified as myocytes by counterstaining with fluorescent phalloidin, a probe for F-actin filaments (data not shown). Finally, under control conditions, the highest level of Hsp27 fluorescence was observed in the heart (top right). Scrambled-PMO-injected embryos heat-shocked (30 min at 37°C) at 36 hpf and allowed to recover for 14 h showed global increases in Hsp27 expression in all tissues, including the trunk muscles, heart, brain, spinal cord, and eye (center row). In contrast, *hsp27i*-PMO-injected embryos processed for Hsp27 immunolocalization after an identical heat shock and recovery period display almost no fluorescence using the same imaging parameters (bottom row). This negative control experiment further demonstrates the effectiveness of the *hsp27i* PMO and the utility of our αHsp27 serum for immunofluorescence localization of Hsp27.

Zebrafish morphological development is normal in the absence of Hsp27

Previous studies have concluded that Hsp27 could play a role in regulating differentiation or survival of some vertebrate embryonic cell types. However, comparison of the overall morphology of zebrafish developed after injection with equal volumes of 0.15 mM scr and *hsp27i* PMO did not reveal changes in the appearance of resulting embryos (Fig. 3a). To provide a quantitative assessment of the morphology of these embryos, we measured the length of the tail (Fig. 3b), the diameter of the lens (Fig. 3c), and the two-dimensional (2-D) area of the yolk mass, an indirect measure of overall metabolism (Fig. 3d) of 24-hpf embryos. No significant differences in these measured values were detected ($p>05$).

Morphology and function of cardiac and skeletal muscles are unaltered by knockdown of Hsp27

Hsp27 is highly expressed in the skeletal and cardiac muscle tissues (Fig. 2) and previous reports have indicated that Hsp27 knockdown using phosphorodiamidate morpholino oligonucleotides can disrupt development of these tissues in frogs (Brown et al. 2007). Here, we have examined effects of Hsp27 knockdown on morphology and functional character-

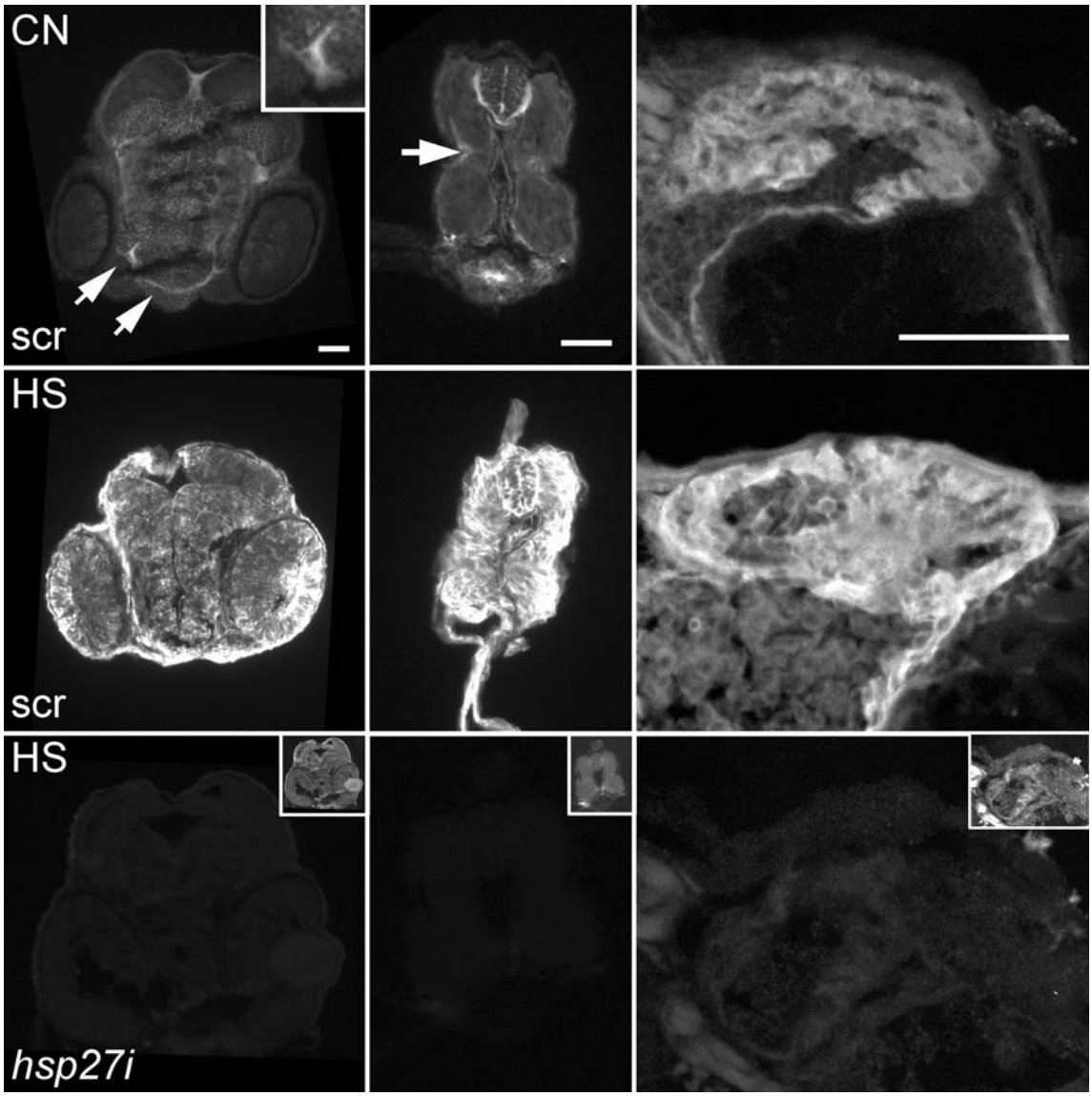

Fig. 2 Immunolocalization of Hsp27 in 50-hpf zebrafish embryos. All images were obtained with identical illumination and camera settings. *Top row*: Hsp27 expression is detected in the head (*left*), trunk (*middle*), and heart (*right*) of 0.1 mM scrambled (*scr*)-PMO-injected embryos under control conditions (*CN*). Hsp27 is most highly expressed in craniofacial muscles (*arrows*, *left*, and *inset*), in myocytes near the developing lateral line of the trunk (*center*, *arrow*), and in the heart (*right*). *Middle row*: Hsp27 expression increases in all tissues of 0.1 mM scr-PMO-injected embryos after heat shock (HS). Bottom row: Hsp27 immunostaining of embryos injected with 0.1 mM antisense *hsp27* (*hsp27i*) PMO and heat-shocked. The *inset* images in the *bottom row* (not to scale) were digitally enhanced to show the section morphology. Images show the dorsal surface *up* for head and trunk images and *down* for heart images. *Scale bars* are 50 μm

istics of these tissues in zebrafish embryos. Figure 4a shows representative images, centered on the 12th myotome, of skeletal muscle in 50-hpf embryos stained with fluorescent phalloidin. No overall morphological difference in skeletal muscle architecture is seen when comparing 0.1 mM *hsp27i* and scr-PMO-injected embryos. We also measured the 2-D area of the 12th myotome in 30 scr- and 30 *hsp27i*-PMO-injected embryos (Fig. 4b). Analysis of these values revealed no statistically significant difference ($p \geq 0.05$) between these groups of embryos.

Figure 4c shows confocal Z-series projections of phalloidin fluorescence in hearts of 50-hpf scr- and *hsp27i*-PMO-injected embryos. The thickened myocardium of the ventricle is the most obvious structure. These images are representative of four Z-series projections generated for embryos in each group. Although in this example the scr-PMO-injected embryo heart appears slightly larger and more brightly stained, this difference was not observed consistently. Importantly, these images are representative in showing no difference in overall morphology of the ventricle

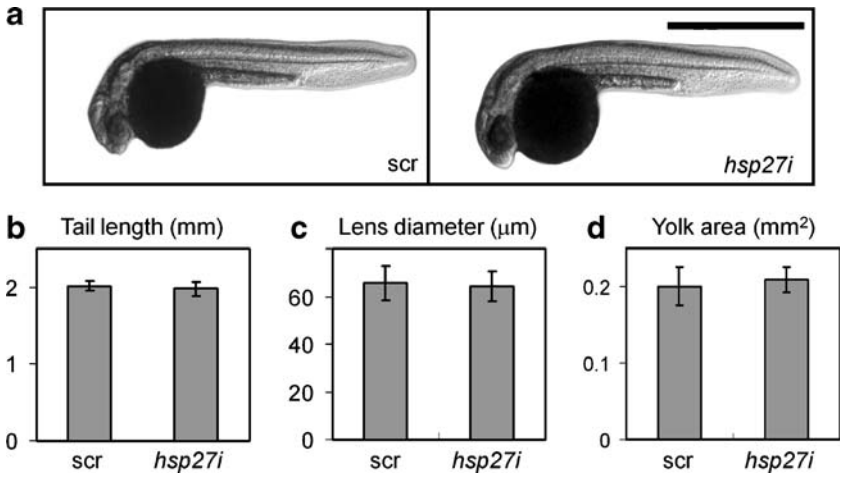

Fig. 3 Morphology of zebrafish embryos is not altered by Hsp27 knockdown. **a** Overall morphology of 24-hpf embryos after injection at the one to two cell stages with 0.15-mM scrambled (scr) or antisense *hsp27* (*hsp27i*) PMO. *Scale bar*=1 mm. **b** Tail length of embryos measured from the tip of the tail to the yolk mass (*arrows*, **a**). **c** Lens diameter. **d** Two-dimensional area of the yolk. Values were obtained from 20 or more embryos in each group collected in a total of three independent trials

or in myofilament prevalence, organization, or thickness. As an assay of function, we also analyzed heart beat rate in 40 scr and 40 *hsp27i*-PMO-injected embryos at 50 hpf in a total of three independent experiments (Fig. 4d). Analysis of these values revealed no significant difference in the heart beat rates between these groups of embryos ($p>05$).

Swimming velocity of zebrafish larvae is unaltered by Hsp27 knockdown

As an indirect measure of both skeletal muscle and motor neuron function, we assayed swimming motility during the startle response of dechorionated embryos at 50 hpf, the earliest developmental time point when robust swimming behavior was observed in uninjected controls (data not shown). Figure 5a shows representative paths of movement of a scr and an *hsp27i*-PMO-injected embryo. No overt changes in parameters such as distance or directional persistence are observed. Figure 5b shows a quantitative analysis of the average maximal swimming velocity displayed by 0.1 mM PMO-injected embryos ($n>30$ for each group). No difference in average value was detected ($p\geq0.05$) when comparing scr and *hsp27i*-PMO-injected embryos.

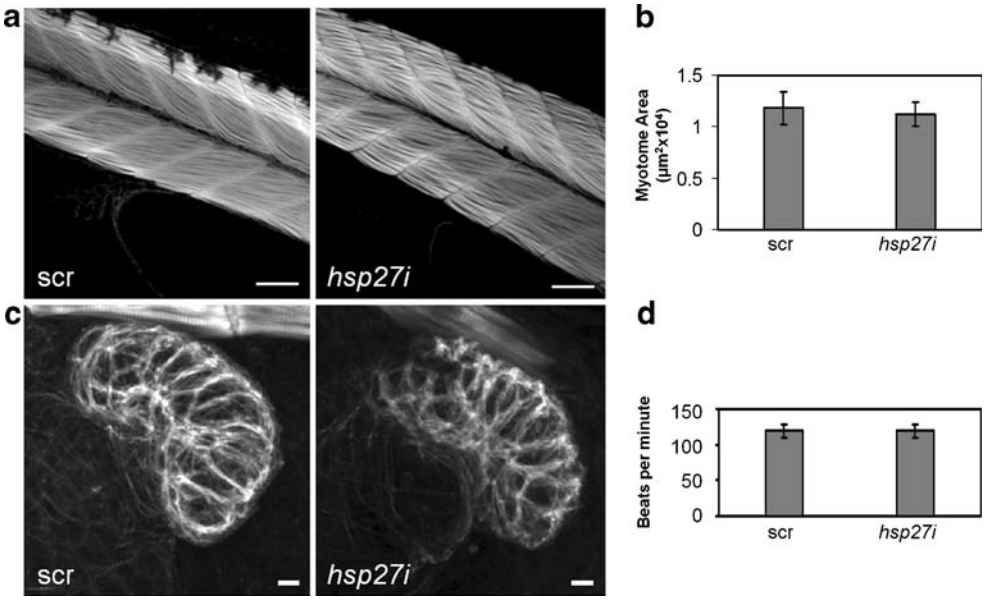

Fig. 4 Striated muscle architecture and heart rate in 50-hpf embryos are not altered by Hsp27 knockdown. **a** Phalloidin staining of skeletal muscle in embryos. These images are centered on the 12th myotome. **b** Area of the 12th myotome measured from 30 embryos injected with 0.1 mM scrambled (scr) or antisense *hsp27* (*hsp27i*) PMO in a total of three independent trials. **c** Z-series projections of F-actin in hearts of embryos injected with *hsp27i* or scr PMO. Images are representative of four projections in each group. **d** Heart beat frequency measured from 40 scr and 40 *hsp27i*-PMO-injected embryos. No significant differences in myotome area or heart rate ($p\geq0.05$) were detected. *Error bars* are standard deviations. *Scale bars* are 50 μm (myotomes) and 10 μm (hearts)

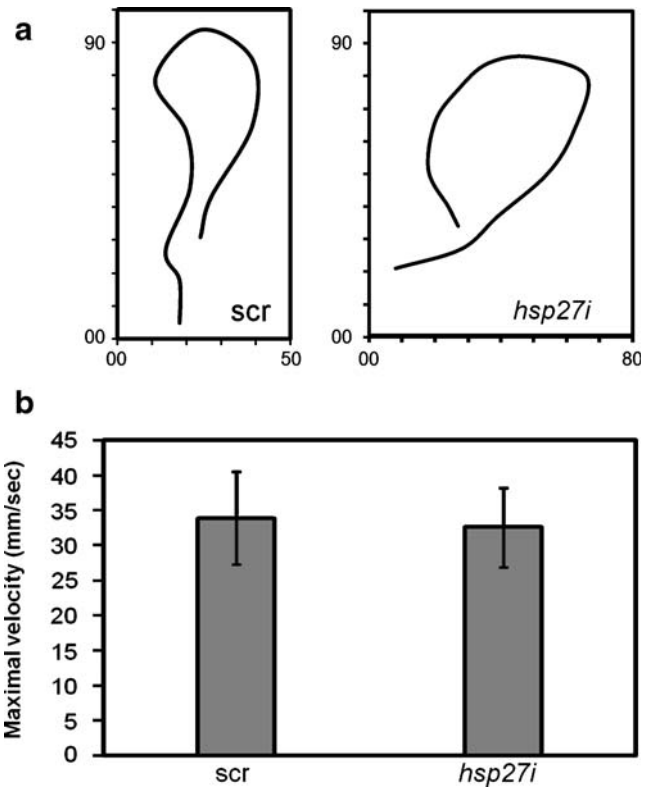

Fig. 5 Motility of 50-hpf embryos is not altered by Hsp27 knockdown. **a** Traces of motility by embryos injected with 0.1 mM scrambled (scr) or antisense *hsp27* (*hsp27i*) PMO. Traces are representative of at least 30 embryos per group measured in three independent trials. *Axes* show *x* (*horizontal*) and *y* (*vertical*) image coordinates. **b** Mean maximal velocity of *hsp27i* and scr-PMO-injected embryos in millimeter per second. No statistically significant ($p \geq 0.05$) difference was detected. *Error bars* indicate standard deviations

Hsp27 is persistently expressed in adult zebrafish tissues

Because Hsp27 appears to play no role in the morphogenesis of zebrafish tissues, we considered whether its expression might be involved in maintenance of zebrafish tissue homeostasis over longer periods. To begin to test this hypothesis, we conducted immunolocalization and immunoblotting studies of Hsp27 expression in tissues of unstressed adult zebrafish (Fig. 6). Immunolocalization of Hsp27 in tissue cryosections was most evident in ventricular myocardium (Hrt) and was somewhat more evident in peripheral muscle fibers than interior ones (Fig. 6, Hrt). Skeletal muscle cryosections also showed relatively high levels of Hsp27 (Fig. 6, Ske). The highest level of expression in this tissue was consistently observed in an outer lateral wedge of muscle previously shown in a number of fish species, including zebrafish, to consist of slow-twitch, or red, muscle (Greer-Walker and Pull 1975). Intriguingly, the greatest level of expression in skeletal muscle sections was detected in a subset of myocytes found at the border between the slow- and fast-twitch muscle tissues (Ske, arrow). Hsp27 is also detected, albeit at much lower levels, in a layer of tissue found at the outer lining of the intestinal tract (Int, arrow). Counterstaining with fluorescent phalloidin indicated that this layer contains smooth muscle tissue (data not shown). Internal structures of the adult zebrafish brain are largely devoid of Hsp27 expression. However, the outer epithelial lining of the adult brain displayed a thin layer of Hsp27-positive fluorescence (Br, arrow). Finally, Hsp27 was localized in cells surrounding seminiferous tubules in the testis (Fig. 6, Test). However, no Hsp27-expressing cells were observed within the tubule structures. To confirm these overall expression patterns within the adult zebrafish, we analyzed Hsp27 expression levels in adult zebrafish tissues using Western blots. Immunoblotting of Hsp70 was performed for comparison. Hsp27 expression was highest in heart tissues, skeletal muscle, and the lens but was also detected at lower levels in all tissues examined. Hsp27 expression was also consistently higher in red muscle than in white muscle. Interestingly, Hsp70 was also constitutively expressed in adult tissues, with the highest levels of expression detected in the brain, testis, and intestinal tissue, whereas little or no Hsp70 expression was detected in the heart, lens, or skeletal muscles.

Discussion

Hsp27 is not required for development of zebrafish under control conditions

Hsp27 expression is upregulated in muscle and some other tissues of the developing vertebrate embryo in the absence of stress (Gernold et al. 1993; Norris et al. 1997; Kawazoe et al. 1999; Mao et al. 2005; Tuttle et al. 2007). Results of previous in vitro studies have indicated that Hsp27 expression could be required for differentiation of myoblasts and other embryonic cell types (Mehlen et al. 1997; Davidson and Morange 2000); however, in vivo data addressing this hypothesis are limited and conflicting. Huang et al. (2007) showed no effects of *hsp27* gene deletion on mouse development, while Brown et al. (2007) have reported that transient knockdown of Hsp27 causes cardia bifida and myofibril defects in frogs. We have previously shown that zebrafish share with other vertebrates the expression of *hsp27* mRNA in cardiac and skeletal muscle early in development and the ability of zebrafish Hsp27 to protect cultured cells from thermal injury (Mao et al. 2005). In the present study, we have conducted functional studies of Hsp27 in zebrafish using an *hsp27i* PMO to inhibit expression during embryogenesis. Like results published previously for both mice and *Xenopus* (Brown et al. 2007; Huang et al. 2007), the global pattern of

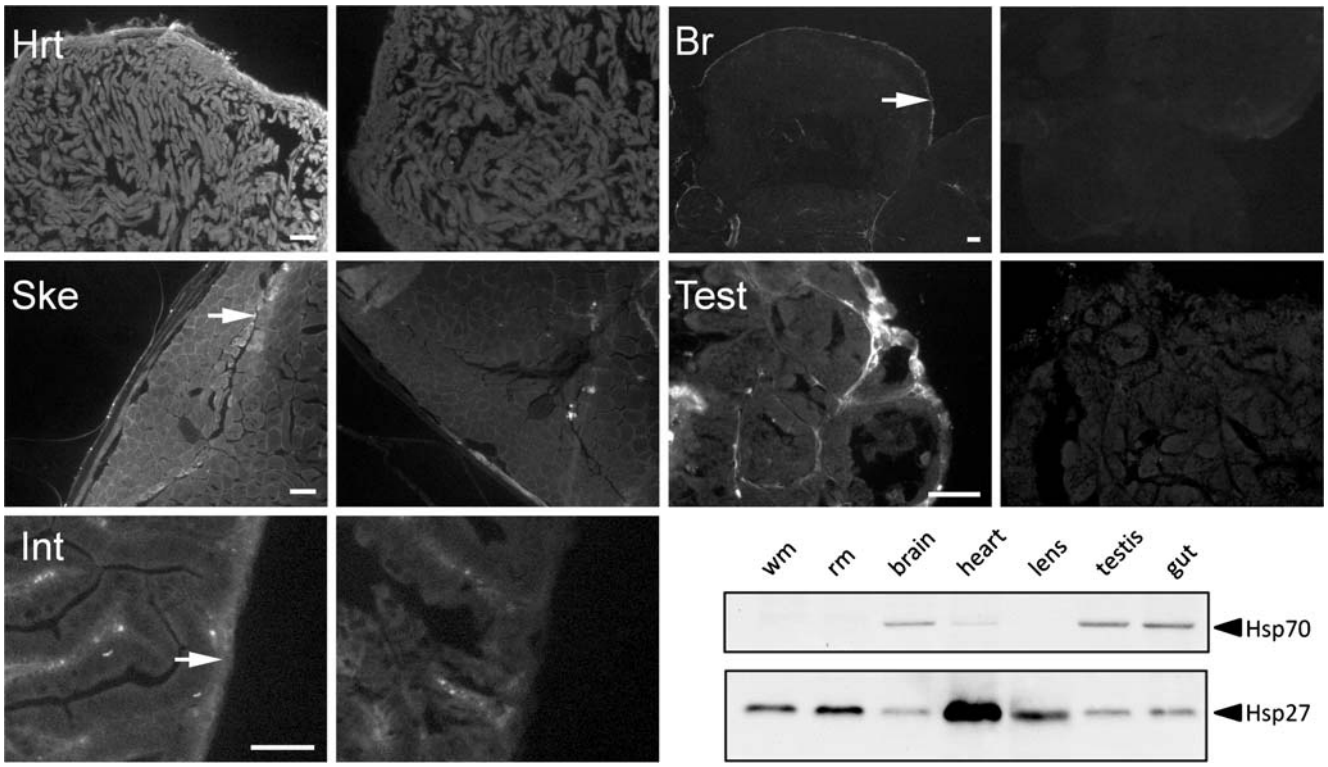

Fig. 6 Hsp27 immunolocalization in unstressed adult zebrafish tissues. Hsp27 was detected throughout the heart myocardium (*Hrt*) and skeletal muscle, especially in slow-twitch muscle and at the boundary between external slow and internal fast-twitch muscle (*arrow*, *Ske*). Hsp27 was detected within the smooth muscle surrounding the intestinal tract (*arrow*, *Int*) and in the lining of the brain tissues (*arrow*, *Br*). Hsp27 was also detected at the margin of seminiferous tubules in the testis (*Test*). *Right-hand panels* are secondary antibody-only controls of each tissue type. *Scale bars* are 50 μm. A Western blot probed for Hsp70 and Hsp27 expression in white, or fast-twitch, muscle (*wm*), red, or slow-twitch, muscle (*rm*), brain, heart, lens, testis, and gut tissues is shown for comparison

embryonic development was unaltered by the loss of Hsp27 in zebrafish. The present and two previous studies appear to provide a consensus that Hsp27 is dispensable for overall vertebrate developmental and thus that results obtained from in vitro studies of the role of stress proteins during development should be viewed with caution. One interpretation of available literature is that differentiation may be more difficult for cells in vitro than in vivo. In support of this view, oxidative stress in cultured cells has been well documented (Halliwell 2003). Expression of Hsps may promote survival and function of cells during differentiation in vitro, with implications for bioengineering of tissues and organs.

Cardia bifida and defects in myofibril organization were demonstrated in *Xenopus* embryos lacking Hsp27 as a result of morpholino depletion (Brown et al. 2007). Development of the zebrafish heart is regulated by a network of transcription factors that share both sequence and functional homology with those involved in the development of hearts in other vertebrates, including frogs, mice, and humans (Weinstein and Fishman 1996). Additionally, like hearts of other vertebrates, the zebrafish heart develops from bilateral heart fields that migrate toward and fuse at the embryonic midline to form the primitive heart tube, and defects in these morphogenic movements result in cardia bifida in zebrafish embryos (Chen et al. 1996; Yelon et al. 1999; Trinh and Stainier 2004; Wang et al. 2005; Matsui et al. 2007). Similar to studies of mice lacking Hsp27 as a result of gene deletion (Huang et al. 2007), our studies demonstrate that these events do not require the expression of Hsp27 in zebrafish embryos under control conditions. Additionally, unlike results from studies of frog embryos, myofibril architecture in heart and skeletal muscle were not detectably different between *hsp27i*- and scr-PMO-injected zebrafish embryos. Hsp27 has also been shown to play a role in the development and protection of the emerging motor neuron system in vitro (Williams et al. 2006). However, our results showed no difference in motility of zebrafish embryos lacking Hsp27 versus controls. Because swimming requires proper function of both the muscle and nervous systems, these results indicate that not only the skeletal muscle but also motor neurons were functionally intact. One possible interpretation of the available data is that mammals and zebrafish, but not frogs, express proteins that can compensate for the loss of Hsp27.

Humans and mice have genes for ten related small heat shock proteins (Fontaine et al. 2003), and zebrafish have genes for 15 such proteins (Elicker and Hutson 2007). In mammals, expression of the related αB crystallin in skeletal and cardiac muscle tissues has been well documented (see Inaguma et al. (1993), for example), and these proteins have some common functionality (Jakob et al. 1993). We and others have reported that αB crystallin is not detected in zebrafish muscle tissues (Posner et al. 1999; Mao and Shelden 2006). However, mRNAs for four other sHsps (Hspb7, Hspb8, Hspb9, Hspb11) are detected in somites of zebrafish embryos. In cardiac tissues, *hspb7* and *hspb12* mRNA are expressed through at least 48 h of development (Marvin et al. 2008). The number of potentially complementary proteins expressed in amphibians has not been determined, but it is conceivable that frogs and other amphibians exhibit greater reliance on Hsp27 during development than zebrafish and mice. Alternatively, interpretation of previous results may have been complicated by issues related to well-documented morpholino toxicity (Heasman 2002). To address these issues, the present study employed antiserum detecting zebrafish Hsp27 to determine the minimal PMO concentration effective in reducing Hsp27 expression in zebrafish. In addition, we compared results of our experiments using *hsp27i* PMO with a control PMO containing identical nucleotides in a scrambled order.

Our present study has several important limitations. Our studies may have lacked the sensitivity required for analysis of subtle changes in heart or skeletal muscle function, so we cannot dismiss the possibility of a unique role for Hsp27 in zebrafish embryogenesis. Our studies also did not address possible roles for Hsp27 during later stages of development, such as sexual maturation, and did directly address the performance of these tissues after stress- or injury-induced damage. These issues are under consideration in our laboratory. However, taken together, the expression of Hsp27 in metabolically active muscle tissues, coupled with the absence of morphological defects in embryos lacking Hsp27 during development most strongly support the view that Hsp27 has a role in protecting myocytes against mechanical or oxidative stress, rather than having a direct role in modulating morphogenesis or organization of the myofilament system.

The expression pattern of Hsp27 supports a role in maintenance of tissue homeostasis

Although Hsp27 expression patterns have been widely examined in several mammalian species, previous studies of Hsp27 expression in zebrafish and frogs have been limited to analysis of *hsp27* mRNA distribution patterns (Mao and Shelden 2006; Tuttle et al. 2007; Marvin et al. 2008) or gene reporter constructs in zebrafish embryos and larvae (Wu et al. 2008). For example, *hsp27* mRNA expression was detected in developing heart, craniofacial muscle and lens tissues of embryos (Marvin et al. 2008). In trunks of 48-hpf zebrafish embryos, *hsp27* mRNA was elevated in superficial skeletal muscles. This region contains developing slow-twitch muscle, leading Marvin et al. to suggest that Hsp27 expression might be specifically elevated in slow-twitch muscle fibers. Our results confirm that Hsp27 protein expression is elevated in zebrafish embryos, compared to surrounding tissues, in cardiac and craniofacial muscles and a subset of muscle fibers found at the lateral margin of embryonic trunk and tail muscle tissues. However, our antibody also detected ubiquitous lower-level Hsp27 expression, not seen in previous studies, in embryos raised under control conditions. The widespread expression of Hsp27 protein detected by immunofluorescence in zebrafish embryos in the present study, particularly in the developing brain and spinal cord, was unexpected and initially led us to consider whether this pattern could result from nonspecific binding of the αHsp27 serum used in our studies. However, our Western blotting studies corroborate the relative changes in Hsp27 expression seen by immunostaining during development, after heat shock, and in adult tissues. In addition, little or no staining is detected in embryos by immunofluorescence after knockdown of Hsp27 in our studies. These data indicate that there is significant constitutive expression of Hsp27 in most tissues of the zebrafish embryo.

Only a few studies have examined Hsp27 expression as a function of muscle fiber type. In mammals, Hsp27 and related αB crystallin protein or mRNA levels are generally higher in muscle tissues containing predominately slow-twitch muscle than in those containing mostly fast-twitch muscle (Inaguma et al. 1993; Huey et al. 2007; Ishihara et al. 2008). However, the opposite has also been reported (Kim et al. 2004). In addition, Hsp27 expression in predominately fast-twitch muscle has been shown to vary as a function of age, with declining expression correlating positively with increased age (Gupte et al. 2008). The present study demonstrates that Hsp27 protein expression is higher in lateral slow-twitch muscle fibers than more central fast-twitch muscles in both 50-hpf embryos and, for the first time, in adult zebrafish muscle tissues. These results suggest that Hsp27 expression and function in slow-twitch muscle fibers has been conserved between fish and mammals and that zebrafish may therefore be a useful model in which to address the functional significance of this expression. However, some differences can also be observed. For example, some studies have shown that Hsp70 is also highly expressed in mammalian slow-twitch muscle tissues (Tupling et al. 2007; Ishihara et al. 2008), but our present results are consistent with previous reports (Blechinger et al. 2002) showing that significant constitutive

expression of Hsp70 is not detected in zebrafish muscle tissues. In addition, both slow- and fast-twitch muscle tissues in mammals express significant levels of the related αB crystallin, but αB crystallin expression is detected only in the lens of zebrafish (Posner et al. 1999; Mao and Shelden 2006). Presently, it is unclear whether Hsp27 plays a more significant role in muscle tissues of zebrafish than mammals or if other heat shock proteins found in fish species perform the roles played by Hsp70 and αB crystallin in mammals.

Expression patterns of Hsp27 and Hsp70 are distinct in zebrafish under control conditions

Finally, recent attention has been focused on the potential role of Hsp27 as cochaperone for high-molecular-weight Hsp70. For example, several studies have shown that Hsp27 and other small heat shock proteins can prevent irreversible unfolding of damaged proteins in vitro (Haslbeck et al. 2005; Bryantsev et al. 2007) and in cultured cells (Bryantsev et al. 2007) and that Hsp27 can synergistically promote recovery of cells when coexpressed with Hsp70 (Riordan et al. 2004). This function would presumably require the spatial and temporal coexpression and colocalization of both proteins. Consistent with this hypothesis, both Hsp70 and Hsp27 expression levels are ubiquitously induced in zebrafish embryos following sublethal thermal injury (Adam et al. 2000; Mao and Shelden 2006). However, Western blots presented here indicate that only the testis, intestinal tissues, and brain express both Hsp27 and Hsp70 at detectable levels in the absence of stress. In addition, Hsp27 expression is strikingly higher in tissues lacking detectable Hsp70 than in those in which Hsp70 is readily detected. Our data therefore have implications for understanding both regulation and function of Hsp27 and Hsp70 expression patterns in zebrafish. We suggest that the function of Hsp27 in zebrafish muscle tissues, at least under control conditions, does not require Hsp70 coexpression. These data also have some implication for understanding the regulation of Hsp27 expression. For example, the lack of protein coexpression under control conditions suggests differential regulation of gene expression in the absence of stress. Therefore, transcription of *hsp27* under control conditions may reflect activity of tissue-specific factors as opposed to general stress-responsive regulation. These conclusions also suggest that Hsp27 performs a function in muscle tissues that may be distinct from that involved in stress protection.

Summary

Our studies indicate that Hsp27 is not required for the differentiation of muscle cells or the establishment of normal cell architecture, including that of muscle cells, during development. However, our results confirm earlier studies of *hsp27* mRNA distribution suggesting that Hsp27 is preferentially expressed in skeletal and cardiac muscle cells in the zebrafish and demonstrate that expression in these tissues persists throughout the life of the animal. These data seem to support the view that Hsp27 promotes the integrity of muscle cell architecture or survival under conditions of mechanical strain or oxidative stress. The similarities in expression pattern and functional significance of Hsp27 observed in zebrafish in the present study and in mammalian systems indicate that zebrafish may be a useful model with which to establish the interrelationships between members of the small heat shock protein family.

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