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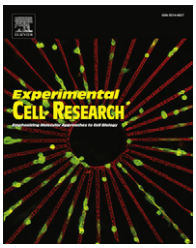

Research Article

Small heat shock protein HSPB1 regulates growth of embryonic zebrafish craniofacial muscles

Ryan C. Middleton[a], *Eric A. Shelden*[a,b,c,*]

[a]*School of Molecular Biosciences, College of Veterinary Medicine, Washington State University, Pullman, WA, USA*
[b]*Center for Reproductive Biology, Washington State University, Pullman, WA, USA*
[c]*Washington Center for Muscle Biology, Washington State University, Pullman, WA, USA*

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ABSTRACT

The small heat shock protein HspB1 (Hsp27) is abundantly expressed in embryonic muscle tissues of a wide variety of vertebrate species. However, the functional significance of this expression pattern is not well established. In the present study, we observed specific, high level expression of HspB1 protein and an HspB1 gene reporter in developing craniofacial muscles of the zebrafish, *Danio rerio*, and examined the consequences of reducing HspB1 expression to the development and growth of these muscles. Quantitative morphometric analyses revealed a reduction in the cross-sectional area of myofibers in embryos expressing reduced HspB1 levels by as much as 47% compared to controls. In contrast, we detected no differences in the number of myofibrils or associated nuclei, nor the number, size or development of chondrocytes in surrounding tissues. We also did not detect changes to the overall organization of sarcomeres or myofibrils in embryos expressing reduced levels of HspB1. Together our results reveal a critical role for HspB1 in the growth of myofibrils and provide new insight into the mechanism underlying its developmental function.

Introduction

The embryonic development of muscle cells is a complex but well characterized process involving coordinated changes in gene expression, cell movement and cytoskeletal organization [1–3]. In brief, most myogenic precursor cells arise from embryonic mesenchymal tissues [4]. Commitment to myogenic cell lineages leads to generation of proliferating myoblasts marked by expression of MyoD and Myf5 [5]. Terminal differentiation of myoblasts into myocytes involves a halt to cell cycle progression, cell fusion (in skeletal muscle), and expression of muscle-specific structural proteins under the control of regulatory factors such as Mrf4 and myogenin [6,7]. The maturation of myocytes, regulated by myocyte enhancers such as Mef2c/d, leads to the formation of contractile myofibrils. Myofibrillogenesis also requires both the high-level expression and precise assembly of filamentous cytoskeletal proteins such as actin, myosin and titin [8]. Individual muscle cell lineages, or fiber types, express unique isoforms of these proteins, and arise at different stages of development in a muscle- and organism-specific manner. For example, in the body muscle of chick embryos, a first wave of myogenesis produces cells expressing slow-twitch myosin, while later waves express fast-twitch myosin [9,10]. In body muscle of fish, transient expression of the transcription factor Blimp-1/ubo/Prdm1

*Correspondence to: 341 Biotechnology Life Science Building, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-7520, USA. Fax: +1 509 335 1907.
E-mail address: eshelden@vetmed.wsu.edu (E.A. Shelden).

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suppresses default development of fast-twitch muscle, yielding slow-twitch fibers [11,12]. Mechanisms regulating the mass attained by muscles after their initial development are not fully understood. However, growth factors such as insulin-like growth factor1 (IGF-1) and fibroblast growth factor (FGF) are known to regulate overall growth of muscle fibers in part by activating the p38 and Akt/mTor, and inhibiting the myostatin signaling pathways [13–17]. Interestingly, physiological conditions resulting in muscle hypertrophy have also been correlated with enhanced expression of heat shock proteins [18]. However, functional studies demonstrating a role for heat shock proteins in the growth of myocytes are lacking.

Hspb1 (also known as Hsp27), a member of the small heat shock family of proteins, is highly expressed both in response to a wide variety of stresses in almost all cells as well as in the absence of apparent stress in some cell types [19–23]. In adult vertebrate muscle tissues, Hspb1 is highly expressed in slow-twitch and cardiac muscle, with lower but still significant expression in fast-twitch skeletal muscle [24–26]. Small heat shock proteins play important roles in adult muscle cells. Overexpression of Hspb1 in transgenic mice protects heart function after ischemia [27,28], and mutation of the related Hspb5/alpha-B crystallin results in cardiac and skeletal muscle myopathies [29–31]. Regulation of smooth muscle contraction by HspB1 has also been demonstrated [32,33]. These functions may be linked to interaction of small heat shock proteins with cytoskeletal proteins, especially actin filaments [34–36]. In adult cardiomyocytes, Hspb5/alpha-B crystallin in mammals and Hspb1 in fish also interact with titin filaments [37–39], and interaction of Hspb1 with desmin has been reported in cardiomyocytes [40]. Finally, small heat shock proteins may play roles in adult muscle cells in ways that do not directly involve interaction with the cytoskeleton. For example, Hspb1 has been shown to play a role in regulating both the p38 and Akt signaling pathways [41–43].

Hspb1 is expressed in embryos of vertebrates including mice, frogs and fish. Hspb1 mRNA expression is ubiquitous in gastrulae but found in an increasingly restricted pattern as development proceeds [22,44–48]. Similarly, Hspb1 expression is also seen in undifferentiated murine embryonic stem cells in vitro and this expression declines rapidly once differentiation is initiated [49,50]. Hspb1 expression is seen primarily in somites after neural tube formation [44,47,48] followed by expression in differentiating embryonic skeletal and cardiac muscle tissues [44,46–48,51]. In the zebrafish embryo, ubiquitous expression of Hspb1 mRNA in body muscle declines during maturation of most muscle cells, but expression remains high in the heart as well as regions of muscle tissue associated with the presence of slow-twitch muscle fibers [46,47,52]. The functional significance of Hspb1 expression in embryonic development is still not clear. Results of in vitro studies have suggested that Hspb1 is required for differentiation but not proliferation of cardiomyocytes [53], while gene knockdown of Hspb1 in frog embryos produced defects in heart tube formation and actin filament organization in both somites and cardiomyocytes [44]. However, comparable studies in zebrafish did not reveal overt changes to muscle morphology in zebrafish [52], and no defects in development of cardiac or skeletal musculature were reported in a study of transgenic Hspb1 knockout mice [54].

Recently, we (see Results) and others [55] developed a transgenic zebrafish line expressing an enhanced green fluorescent fusion protein under the control of the zebrafish HspB1 promoter. Intriguingly, the reporter is expressed most highly in developing zebrafish embryos in heart and muscles of the eye, jaw and face, collectively referred to as craniofacial muscles [56]. We hypothesized that these muscles might display responses to manipulation of HspB1 expression levels not seen in previous studies of trunk muscle tissues [52]. In the present study, we characterized the growth of representative zebrafish craniofacial muscles and examined the developmental expression and function of Hspb1 protein in them using quantitative microscopy and molecular approaches. Zebrafish craniofacial muscles develop rapidly from both mesenchymal precursors and neural crest cell derivatives [57,58], leading to the formation of functional muscles within three days of fertilization [59]. Individual craniofacial muscles contain fewer than fifty myofibers and display characteristic and unique distributions of slow- and fast-twitch myosin positive cells [60]. These muscles are therefore a useful system in which to examine factors affecting muscle differentiation, growth and fiber-type specification. Expression of Hspb1 mRNA has been reported in zebrafish craniofacial muscles along with weaker and more restricted expression of mRNA for another small heat shock protein, Hspb8/Hsp22 [47]. Here, we report that embryos expressing reduced levels of HspB1 displayed a muscle specific reduction in the measured cross-sectional area (CSA) of both slow- and fast-twitch myosin positive myofibers compared to controls. The magnitude of this change, up to 47% over three days of development, agrees well with that reported by other authors investigating effects of more well-established factor regulating muscle growth or hypertrophy [61–64]. In contrast, the number of myofibers and nuclei within individual muscles, and the number of slow- and fast-twitch myosin expressing fibers within muscles were not altered by reduction in HspB1. Our results indicate that Hspb1 is not required for determination or proliferation of myogenic precursors, but is essential for optimal growth of craniofacial myocytes.

Materials and methods

Zebrafish husbandry

Zebrafish were raised and maintained as described [65,66]. The transgenic strain, TM1, expressing GFP under the control of a carp β-actin promoter [67] was a generous gift of Dr. Barrie Robison, University of Idaho. Protocols for the use of animals were approved by the Washington State University Animal Care and Use Committee in accord with National Institute of Health standards established by the Guidelines for the Care and Use of Experimental Animals. To create the zebrafish HspB1 promoter reporter line, approximately 2.5 Kb of zebrafish genomic DNA were PCR amplified with Platinum PfX (Invitrogen Life Technologies, Grand Island, NY) and primers (5′- ATGCCAGCAGCGGCCGCACAGGTGTGATTG-3′ (forward) and 5′- TCGACCGGTCTGCTGCCCTGATACCAGTCCC-3′ (reverse)). Amplification was performed in the presence of 100 mM tetramethylammonium chloride (Sigma-Aldrich, St. Louis, MO; see Ref. [68]) using an annealing temperature of 55 °C and a 4 min extension at a temperature of 63 °C. The PCR product was digested with NotI and AgeI (New England Biolabs, Ipswich, MA) and cloned into corresponding restriction sites in the vector pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA) modified as