RESEARCH ARTICLE

Growth in the Larval Zebrafish Pectoral Fin and Trunk Musculature

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After initial patterning, muscle in the trunk and fins of teleosts grows extensively. Here, we describe muscle growth in zebrafish, with emphasis on the pectoral fin musculature. In the trunk, slow muscle fibers differentiate first. In contrast, slow muscle does not appear in the pectoral fin until the beginning of the juvenile period. Mosaic hyperplasia contributes to trunk muscle growth, and new fibers are apparent within the muscle as early as 6 mm standard length. In the pectoral fin muscle, mosaic hyperplasia is not evident at any examined stage. Instead, the predominant mode of hyperplasia is stratified. In larval pectoral fin muscle new fibers appear subjacent to the skin, and this correlates with the expression of myogenic genes such as muscle regulatory factors and Pax7. Our results suggest that regulation of fiber type development and muscle growth may differ in the pectoral fin and trunk. *Developmental Dynamics 237: 307–315, 2008.* © 2007 Wiley-Liss, Inc.

Key words: myogenesis; limb; zebrafish; *pax7*; fast muscle; slow muscle

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INTRODUCTION

The form and function of adult muscle is dependent not only on its initial patterning during embryogenesis, but also on its growth during the postembryonic period. Postembryonic growth of muscle in vertebrates is supported by myogenic precursors that continue to proliferate after the initial embryonic muscle pattern is established. Myogenic precursors involved in this muscle growth undergo a molecular developmental program similar to myogenic precursors during embryonic development: expression of transcription factors such as Pax7 or Pax3, followed by expression of muscle regulatory factors (MRFs), expression of sarcomeric proteins, and finally elongation into fully differentiated cells (Parker et al., 2003). The primary distinction between embryonic muscle formation and subsequent muscle growth is that, while embryonic myogenesis is regulated primarily by cell-intrinsic signals and by growth factors from the immediately surrounding environment, many aspects of myogenesis during growth are regulated by long-range signals such as innervation and circulating hormones (Shavlakadze and Grounds, 2006).

Fin muscle in teleosts, like limb muscle in amniotes, is derived from the somites—segmentally repeated blocks of paraxial mesoderm in the trunk (Hollway and Currie, 2003; Holley, 2007). In amniotes, the dorsal portion of the somite gives rise to the dermomyotome (Buckingham et al., 2003). The dorsomedial dermomyotome then provides cells to the primaxial myotome, while the ventrolateral region provides cells to the abaxial musculature, including the limb and body wall muscles (Burke and Nowicki, 2003; Winslow et al., 2007). In zebrafish, the most external portion of the somites also has properties of a dermomyotome, and provides cells to the growing myotome (Devoto et al., 2006; Stellabotte and Devoto, 2007). It is not yet clear whether the zebrafish dermomyotome is regionalized in its contribution to different populations of skeletal muscle. However, in both zebrafish and amniotes, fin/limb muscle precursors migrate from the ventral dermomyotome into the fin/limb bud, and differentiate only after they have reached their target.

The early development of teleost

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pectoral fin muscle shares many features with tetrapod limb muscle development: precursors expressing *lbx1* and *c-met*, but not MRFs, migrate from the somite to the fin bud and commence myogenic differentiation there (Neyt et al., 2000). As in amniotes, myogenic precursors in the fin form two distinct masses and differentiate into muscle fibers (Grandel and Schulte-Merker, 1998). In zebrafish, these two muscle masses grow and then split into three dorsal and three ventral muscles (Thorsen and Hale, 2005). Growth of muscle within limbs and fins occurs long after the end of the migration of cells into the limb bud. Therefore, to ensure that myogenic precursors necessary for growth remain, a mechanism must be in place to maintain a balance between the proliferation and differentiation of these cells (Amthor et al., 1999).

The pattern of growth in the trunk musculature of teleost fishes has been studied extensively (Rowlerson and Veggetti, 2001). Hyperplasia, a process in which new muscle fibers are added, begins in many fishes as the embryonic period ends, just before hatching. In the first phase of hyperplasia, known as stratified hyperplasia, new muscle fibers are added at distinct regions called myogenic germinal zones. Myogenic germinal zones are a source of both new slow and fast fibers, and are found between the superficially located slow fibers and the deeper fast fibers, as well as at the dorsal and ventral extremes of the myotome and surrounding the lateral line nerve (Rowlerson and Veggetti, 2001). A second phase of hyperplasia is known as mosaic hyperplasia, because new fibers are added throughout the muscle, creating a mosaic of smaller, new muscle fibers, and larger, older muscle fibers. While these phases of growth are known to occur in several teleosts, such as sea bream (*Sparus aurata*; Mascarello et al., 1995) and brown trout (*Salmo trutta lacustris*; Steinbacher et al., 2007), the pattern of hyperplastic growth in the zebrafish trunk and pectoral fin has not been documented. Muscle growth also occurs by a separate process called hypertrophy, in which muscle fibers increase in size. It is likely that, as in amniotes, the growing fibers incorporate new nuclei from nearby myogenic precursors to maintain approximately the same sized myonuclear domain (Allen et al., 1999). Understanding the patterns of muscle growth during later stages of development will provide the framework for understanding the mechanisms underlying these processes.

Zebrafish provide an excellent model to study the process of limb muscle growth. Because the early development of the musculature is similar in both zebrafish and amniote models, it is reasonable to predict that the mechanisms underlying later growth are also similar. However, unlike in amniote models, the zebrafish pectoral fin musculature remains as two masses for a significant period of time during its larval and juvenile development. During this period, the pectoral fin is readily accessible, and the simplicity of the masses during growth periods allows for easier study.

Here, we describe the growth of the zebrafish pectoral fin musculature, with particular emphasis on the addition of new muscle fibers. We first describe the pattern of growth in the trunk of zebrafish during the late larval period as a basis for comparison to the growth of fin musculature during the same period. We find that, while axial muscle during the late larval period has begun to grow by mosaic hyperplasia, the main mechanism of hyperplastic growth in the pectoral fin musculature is stratified. We also characterize changes in fiber type diversity within the fin musculature during the larval and juvenile stages.

RESULTS

Hyperplasia in Larval Trunk Musculature

Two main types of hyperplastic growth have been identified in fish species: stratified hyperplasia, in which new fibers are added at discrete growth zones outside of the muscle mass; and mosaic hyperplasia, in which new fibers are added throughout the myotome. Zebrafish hatch at a total length (TL) of 3.3–3.5 mm (Kimmel et al., 1995), reach the juvenile stage at approximately 7 mm, and grow continuously throughout their lives to a typical size of approximately 30 mm, but can attain sizes of 50 mm (Schilling, 2002). To determine the pattern of hyperplastic growth in zebrafish, we have examined the trunk musculature of larval zebrafish between 4.5 and 7.0 mm (standard length [SL]). At 4.5 mm, the majority of hyperplastic growth occurs in a stratified manner (Fig. 1A), with new small diameter fibers found mainly on the surface of the fast musculature. By 6.0 mm, mosaic hyperplasia has begun, evident by the presence of new, small diameter fibers within the fast muscle, adjacent to older fibers (Fig. 1B), creating a mosaic appearance. Cells expressing *myogenin* and other MRFs are distributed throughout the myotome (Fig. 1C, data not shown), suggesting that myogenic differentiation is widespread.

Pectoral Fin Muscle Morphology During Late Larval Development

During larval development, there are two distinct pectoral fin muscles on either side of an endoskeletal disk, one functioning as an adductor, and the other an abductor (Fig. 2; Grandel and Schulte-Merker, 1998; Thorsen and Hale, 2005). The musculature remains in this configuration until approximately 8 mm, which corresponds to the period of transition of the pectoral fin into its adult position (Fig. 2A,B; Grandel and Schulte-Merker, 1998). The two muscle masses then begin to split into the adult complement of muscles (Fig. 2C,D). Until at least the time at which the muscles divide, individual fin muscle fibers run the entire length of the muscle mass, originating at the base of the fin and ending near the tip (Thorsen and Hale, 2005). To best understand the process of muscle growth in the pectoral fin, we used the period of growth during late larval development between 4.0 and 7.0 mm as our developmental window. Because the fin musculature remains as two muscle masses during this period, we can easily assess the patterns of growth within the fin and make comparisons to patterns of growth within the trunk musculature. Because amniote muscle also begins as two muscle masses, examination of muscle growth during this period enables comparison be-

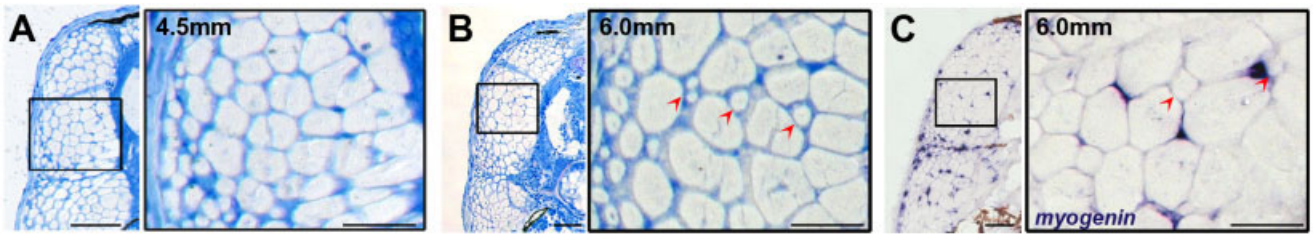

Fig. 1. Hyperplastic growth of trunk muscle in zebrafish larvae. **A:** Methylene blue–stained section through the trunk of a 4.5 mm larva. Hyperplastic growth at this stage is predominantly by stratified hyperplasia. Note the abundance of small diameter fibers at the lateral surface of the fast muscle, and absence of small diameter fibers within the fast muscle. **B:** Methylene blue–stained section through the trunk of a 6.0 mm larva. Small fibers are apparent within the fast muscle, indicating mosaic hyperplasia (red arrowheads) **C:** *Myogenin* labeling in a 6.0 mm larval trunk. Note the mosaic appearance of myogenin labeling in the trunk, suggesting mosaic differentiation of muscle. Mosaic hyperplasia is also evident by the appearance of small diameter fibers within the fast muscle (red arrowheads). Scale bars = 50 μm in A–C, 25 μm in insets.

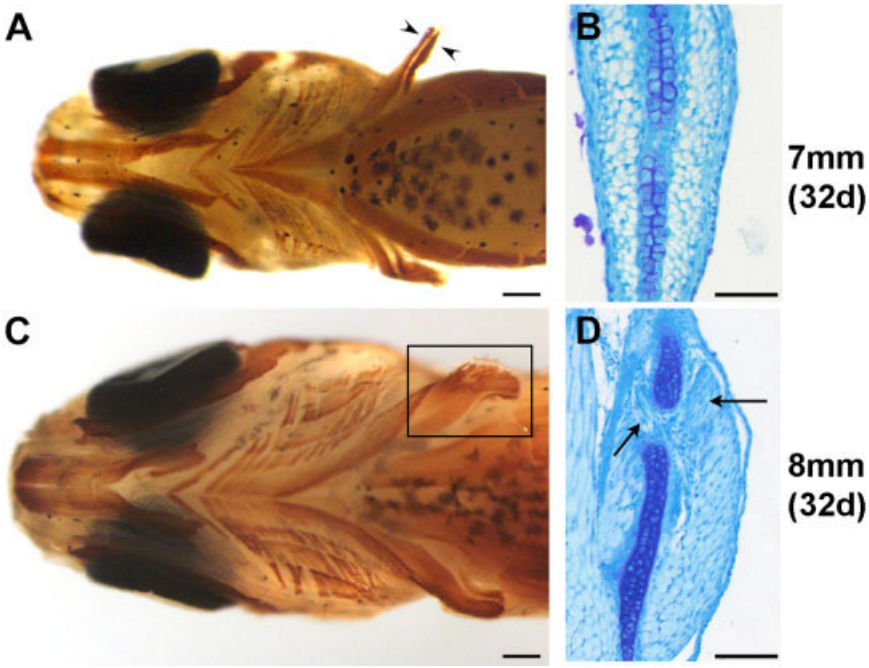

Fig. 2.

Fig. 2. Changes in pectoral fin musculature between 7.0 mm standard length (SL; A,B) and 8.0 mm SL (C,D), **A:** Ventral view of the musculature of the 7.0 mm larva. Musculature has been labeled in whole-mount with MF20 antibody (brown). The pectoral fin musculature consists of two distinct masses on either side of the endoskeletal disk (arrowheads). **B:** Transverse section of the pectoral fin of a 7.0 mm larva stained with methylene blue, showing muscle masses on either side of the central endoskeletal disk. **C:** Ventral view of the musculature of the 8.0 mm larva labeled with MF20 (brown); the fin muscle is boxed. **D:** Transverse section of a pectoral fin of an 8.0 mm larva demonstrating the beginning of splitting of the muscle masses (arrows). Scale bars 250 μm in A,C, 50 μm in B,D.

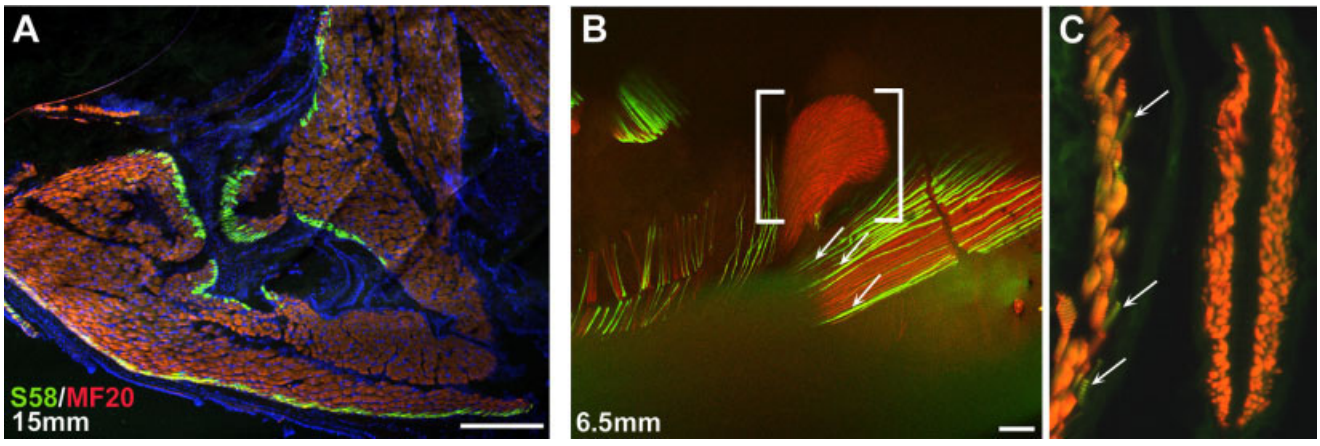

Fig. 3. The pectoral fin musculature during the late larval period consists of only fast muscle fibers. **A:** Transverse section of juvenile pectoral fin (15 mm SL). There are small populations of S58-positive slow fibers at the periphery of the individual muscles. **B:** Lateral view of the pectoral fin of a 6.5 mm larva stained in whole-mount for slow fibers (S58, green) and all fibers (MF20, red). There are no slow fibers stained within the pectoral fin (bracket), but slow fibers are apparent within the trunk (arrows). **C:** Transverse section of a 6.5 mm larva demonstrating that the pectoral fin musculature consists of only fast fibers. Slow fibers within the trunk are labeled by S58 at this stage (arrows). Scale bars = 100 μm in A,B, 25 μm in C.

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tween amniote limb muscle and zebrafish pectoral fin musculature.

Fiber Type Diversity in the Larval Pectoral Fin

Juvenile and adult zebrafish fin muscles consist of at least two different muscle fiber types. Slow muscle fibers, labeled by the S58 antibody, are found on the surface of the muscles as early as 7 mm and can be seen on the surface of all muscles in juvenile zebrafish (Fig. 3A, data not shown). These slow muscle fibers develop during later development, and the pectoral fin musculature consists of only fast, and possibly intermediate, muscle fibers until at least 6.5 mm (Fig. 3B,C). These fibers are unreactive for S58, and label with MF20, which labels all muscle fibers in zebrafish, and zm4, which is specific to fast muscle fibers in zebrafish (Fig. 3C, data not shown). Using currently available antibodies, intermediate muscle fibers cannot be distinguished from fast muscle fibers. Intermediate muscle fibers are thought to be a subset of fast muscle fibers (Bone, 1978), and for the purpose of this study, we are considering all zm4-positive cells as fast muscle. The absence of the typically smaller slow fibers within the fin before the juvenile period is an advantage, as it simplifies analysis of muscle growth.

Characterization of Pectoral Fin Muscle Growth

To determine the extent of hyperplastic growth in the larval pectoral fin, we determined the total number of muscle fibers in cross-sections of pectoral fins of larvae between 4.0 and 6.5 mm. Between 4.0 and 6.5 mm, the total cross-sectional area of the pectoral fin musculature dramatically increases (Fig. 4A–C). There was also a significant increase in the number of fibers per pectoral fin between these developmental time points. Between 4.5 and 6.5 mm, the number of muscle fibers nearly doubled, suggesting that hyperplasia is contributing significantly to growth of the pectoral fin musculature (Fig. 4D; 4.5 mm, n = 3; 5.5 mm, n = 4; 6.5 mm, n = 4).

We also examined the contribution of hypertrophy to the growth of the pectoral fin musculature during this period. To reduce the possibility of including new fibers in our analysis of hypertrophy, we restricted our analysis to the 20 largest fibers, which were likely to be older fibers. The mean cross-sectional area (CSA) of these fibers at 6.5 mm is 150% that of the 20 largest fibers at 4.5 mm (Fig. 4E; all groups n = 3). During this same period, the total CSA of the muscle increases by over 250%, indicating that both hypertrophy and hyperplasia play major roles in the growth of the pectoral fin muscle.

Within one muscle fiber type, new fibers have a smaller diameter than older fibers. The majority of new, small diameter fibers in larval pectoral fins were subjacent to the skin (Fig. 5A), and few small fibers were within the muscle mass itself. We have also looked at hyperplasia in pectoral fin in juveniles (15 mm) and adults (32 mm) to address whether the mode of hyperplasia changes later, as it does in the trunk. We have not identified any small diameter muscle fibers within the fast muscle of the pectoral fin in the juvenile or adult, but small diameter fibers are easily identified on the surface of the muscles (Fig. 5B, data not shown). Therefore, during the development and growth of the adult pectoral fin muscles, hyperplasia remains stratified in the pectoral fin. To quantitatively map the spatial distribution of small fibers during larval stages, we charted the relationship between the CSA of each muscle fiber and its distance from the skin. Using this analysis, we determined that small fibers are found closest to the skin (Fig. 5C). To simplify presentation of these data, we binned muscle fibers into groups by CSA: small (less than 10 μm^2), medium (10–20 μm^2), and large (greater than 20 μm^2). The small muscle fibers were localized closest to the skin in all sizes examined. For example, in the 6.5 mm specimens small fibers were on average 7.2 μm from the skin, while large fibers were on average 20.9 μm from the skin (Fig. 5D; 4.5 mm, n = 3; 5.5 mm, n = 3; 6.5 mm, n = 4). Thus, hyperplasia in the pectoral fin, as in the larval trunk, occurs in a stratified manner.

Molecular Characterization of Muscle Growth in the Fin

To further characterize the growth of the pectoral fin, we examined the expression of genes involved in muscle development. Pax7, a transcription factor involved in myogenesis, was expressed in cells on the surface of the existing muscle masses at all stages examined (Fig. 6A, data not shown). As in the trunk (Devoto et al., 2006), a subset of the Pax7-positive nuclei co-express Myogenin, indicating that the Pax7-positive cell population includes myogenic precursors (Fig. 6A, insets). The muscle regulatory factors *myoD*, *myogenin*, and *myf5* were also expressed on the surface of the muscle (Fig. 6B,C; data not shown). The location of these Pax7 and *myoD*, *myogenin*, and *myf5* positive cells correlates with the location of the small diameter fibers, suggesting that these cells contribute to muscle growth in the pectoral fin. There was, however, some expression of MRFs more centrally, within the fin muscle. This may reflect muscle precursors being incorporated during hypertrophy.

DISCUSSION

Fiber Type

We have found that the larval pectoral fin musculature contains no slow fibers until approximately 7 mm (SL), when a few slow fibers can be found at the surface of the muscle masses (data not shown). By 15 mm, slow fibers are evident on the periphery of all of the pectoral fin muscles (Fig. 3A). The late development of slow fibers in the zebrafish fin contrasts with the development of fiber type in tetrapod limb muscles, in which slow and fast fiber types are present during the embryonic patterning stages (Crow and Stockdale, 1986). By 15 mm, the zebrafish pectoral fin has largely developed into its adult form; the musculature has split into the adult complement of six muscles, and the girdle has rotated into its adult position horizontal to the body axis (Thorsen and Hale, 2005). The function of the pectoral fin has also shifted by this time, from heavy involvement in swimming in larvae, to functioning only in initiation of swimming and maneuvering during juvenile stages

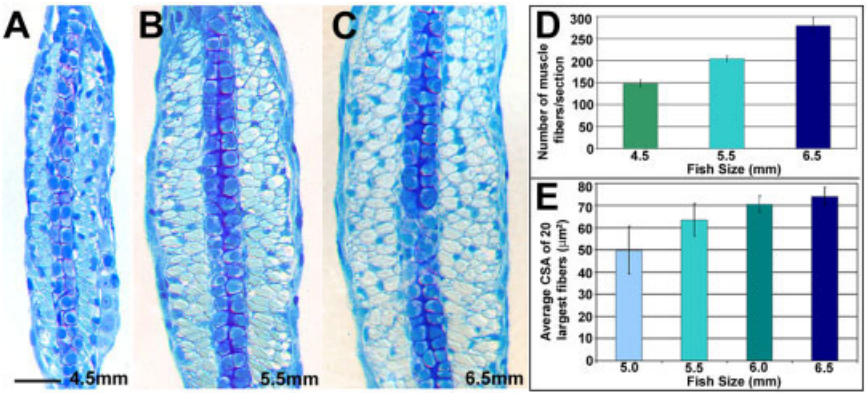

Fig. 4.

Fig. 4. Growth of muscle in the late larval pectoral fin occurs by hyperplasia and hypertrophy. **A–C:** Transverse sections of methylene blue–stained pectoral fins during the late larval period. There is a large increase in overall size of the pectoral fin musculature from 4.5 mm (A) to 6.5 mm (C). **D:** Quantification of total muscle fibers/section shows an increase in the number of muscle fibers during the late larval period. Between 4.5 and 6.5 mm, the number of fibers almost doubles, increasing from a mean of 148 fibers to a mean of 279 fibers (4.5 mm, n = 3; 5.5 mm, n = 4; 6.5 mm, n = 4). **D:** Increase in the mean cross-sectional area of the 20 largest fibers suggests that hypertrophy is contributing to muscle growth during this period (all sizes, n = 3). Scale bar = 25 μm in A–C.

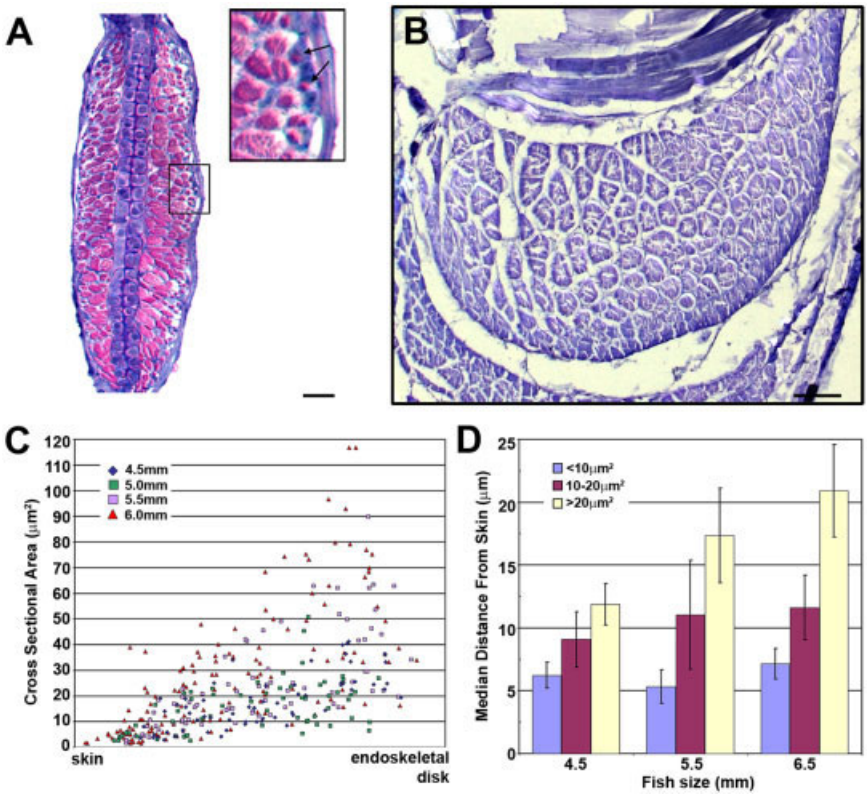

Fig. 5.

Fig. 5. Addition of new muscle fibers occurs at the periphery of the existing muscle masses. **A:** Transverse section of the pectoral fin of a 6.5 mm larva stained with methylene blue and basic fuchsin. Fibers with a small cross-sectional area (CSA) are closest to the skin. Inset shows small fibers near the skin. **B:** Methylene blue–stained transverse section through an adductor muscle in the pectoral fin of an adult (32 mm). No small, new fibers are within the mass of the muscle. Ventral is to the bottom. **C:** Scatter plot showing the relationship between the CSA of a muscle fiber and its position relative to the skin and endoskeletal disk. Each point represents one muscle fiber. Distances were determined using the GIS program ArcGIS by mapping the relationship between the center of each fiber and the closest point on the skin or endoskeletal disk. **D:** Chart showing the relationship between bins of fiber size (CSA) and the median distance from the skin. Muscle fibers that are less than 10 μm^2 in CSA have a smaller median distance from the skin in all larval specimen sizes (4.5 mm, n = 3; 5.5 mm, n = 3; 6.5 mm, n = 4). Scale bar = 25 μm in A, 100μm in B.

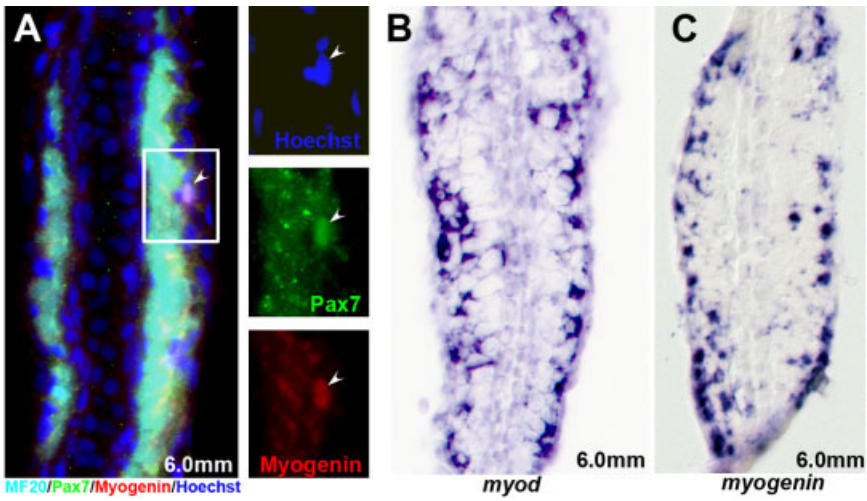

Fig. 6.

Fig. 6. Molecular characterization of myogenesis corresponds with the location of new, small diameter fibers. A–C: Expression of molecular markers of myogenesis in 6.0 mm larval pectoral fins. **A:** Pax7 (Green) is expressed in cells superficial to the existing muscle masses (MF20, Cyan). A subset of Pax7 expressing cells coexpress Myogenin (Red), signifying myogenic differentiation. Insets signify boxed region and show a nucleus that expresses both Pax7 and Myogenin (arrowheads). **B:** The muscle regulatory factor *myod* is expressed by cells at the periphery of the muscle masses. **C:** *Myogenin* is expressed by cells at the periphery of the muscle masses. Scale bar = 25 μm.

(Thorsen and Hale, 2005). The appearance of slow fibers in the zebrafish pectoral fin at 7 mm correlates with the beginning of the juvenile stage and directly precedes the transition of the pectoral fin from its larval to adult form. The development of slow fibers at this stage, as well as the distribution of slow fibers in pectoral fin muscles at 15 mm, may reflect the change in function of the pectoral fin during the larval to juvenile transition.

The segregation of fiber types is a common feature of zebrafish muscles. For example, trunk slow fibers develop in a layer at the surface of the myotome during embryonic development, overlying the differentiating fast muscle. This separation of slow and fast fibers is maintained in the adult, where slow muscle fibers are located in a wedge lateral to the fast fibers. The separation of fiber type within the trunk is important for muscle function during swimming, as the laterally located slow muscle fibers provide the most efficient locomotion (Bone, 1978). Larval and adult cranial muscles also show segregation of slow and fast fiber types, with slow fibers on the outside surface of a larger population of fast muscle fibers (Hernandez et al., 2005). Slow fibers develop very early in the trunk, from adaxial cells, and likely underlie movement of the trunk even before hatching (Devoto et al., 1996). Later-developing myogenic precursors in the dorsal and ventral myotome contribute to growth of the embryonic slow muscle fiber layer after its initial development (Barresi et al., 2001). Thus, even though trunk slow fibers derive from multiple sources, they attain the same superficial position. The much later development of slow muscle in the fin, as compared to the trunk, likely reflects differences in requirements for slow fibers for movement of the pectoral fin and trunk. The precursors to these late developing slow muscle fibers in the fin are likely to arrive in the fin during the embryonic period, when dermomyotome cells migrate into the fin bud. It remains unclear whether the precursors responsible for the growth of trunk slow muscle precursors, fin fast muscle fibers, and/or the initial fin slow muscle fibers share a lineage relationship. The factors regulating slow and fast muscle fiber type specification from dermomyotome-derived myogenic precursors are unknown.

Patterns of Growth

Prior work in several teleost species has indicated that mosaic hyperplasia is predominantly used by fish that reach a large adult size (Rowlerson and Veggetti, 2001). This mode of growth, in which new fibers are added within the muscle mass in a "mosaic" manner, is thought to be the primary mechanism which allows muscle mass in large species to attain large size, and has been studied extensively in commercial species for this reason (Rowlerson and Veggetti, 2001). During early larval development in zebrafish, the primary mode of hyperplasia in the trunk is stratified. However, by 6.0 mm, small fibers are evident within the muscle mass of the trunk—a hallmark of mosaic hyperplasia. This finding indicates that zebrafish undergo two phases of myogenic hyperplasia, with mosaic hyperplasia beginning during later larval growth and overlapping temporally with stratified hyperplasia in the trunk during the examined stages. This secondary phase of myogenesis has not been previously documented in zebrafish, and is an unexpected finding, as mosaic hyperplasia is not typically thought to contribute to hyperplastic growth in fish of small ultimate size. For example, in the guppy (*Poecilia reticulata*) hyperplastic growth of trunk muscle has been reported to occur only in a stratified manner (Veggetti et al., 1993). This is also true of other small species, such as members of the families Channichthydiae and Harpagiferidae, which only grow to a few centimeters in length (Johnston et al., 2003).

During the same developmental period as mosaic hyperplasia is occurring in the zebrafish trunk, we have found that new, small diameter fibers are distributed at the surface of the pectoral fin muscle masses. We have not identified any new, small diameter fibers within the fast muscle of the pectoral fin of any examined stages (up to 32 mm), indicating that mosaic hyperplasia is strongly reduced or absent in the pectoral fin musculature. Therefore, it appears that stratified hyperplasia is the predominant mode of hyperplasia in the fin, and occurs concurrently with mosaic hyperplasia in the trunk. It is also worth nothing that, while MRF-positive muscle precursors are concentrated on the surface of the pectoral fin muscle masses during the late larval period, indicative of stratified hyperplasia, there are MRF-positive cells deep as well. Because of the apparent lack of new muscle fibers in the medial portions of the fin musculature, it is likely that these deep MRF-positive cells contribute to hypertrophic growth of existing fibers.

Muscle growth also occurs by hypertrophy, as individual muscle fibers increase in size. In the zebrafish pectoral fin, the largest fibers individually grew in CSA by approximately 50%, while the overall muscle grew by 250%, thus both hypertrophy and hyperplasia are very important in the growth of the fin muscle. Hyperplasia is mostly completed before or shortly after birth in amniotes, and hypertrophy is the major mechanism of growth of skeletal muscle (Rayne and Crawford, 1975; Ontell and Dunn, 1978). Addition of new fibers, as well as repair of muscle, is mainly the result of differentiation of satellite cells (Cossu and Biressi, 2005). In contrast, in zebrafish, as well as in other teleosts, hyperplasia and hypertrophy play major roles in muscle growth at least into the juvenile stages.

Myogenic Precursors

Pax7 is expressed by myogenic precursors on the surface of the zebrafish trunk musculature during embryonic and larval stages, and these cells have been shown to contribute to myogenic growth (Devoto et al., 2006; Feng et al., 2006; Stellabotte et al., 2007). In the embryonic zebrafish, Pax7 is also highly expressed by cells in the dorsal neural tube as well as migratory neural crest cells. In the trunk, these can easily be distinguished from myogenic Pax7 cells by level of Pax7 expression, morphology, as well as the lack of expression of myogenic markers. Pax7-positive cells on the surface of the fin muscle masses in the zebrafish are adjacent to small fibers and MRF expression. These cells have a similar morphology, position, and level of Pax7 labeling to the Pax7-positive cells on

the surface of the myotome. As in the myotome, some of these Pax7-positive cells also express Myogenin, indicating that these cells are differentiating into muscle cells and, therefore, contributing to muscle growth in the fin musculature. The pattern of $Pax7^+$ myogenic precursors external to differentiating fin muscle fibers is reminiscent of the pattern of myogenic precursors seen in the developing limb musculature of amniotes such as chick and mouse (Amthor et al., 1998; Swartz et al., 2001; Relaix et al., 2004). Pax7 is also expressed by muscle precursors in anamniotes such as *Xenopus* (Satoh et al., 2005). In chick, muscle precursors express Pax7 before their migration into the limb, and continue to express Pax7 after they have populated the limb (Swartz et al., 2001). In mouse and zebrafish, migratory limb muscle precursors express only Pax3 and not Pax7 during their migration into the limb and upon reaching the limb Pax7 expression is turned on by these cells (Relaix et al., 2004; Hollway et al., 2007; data not shown).

The spatially restricted stratified hyperplasia in the pectoral fin during late larval growth contrasts with the distributed, mosaic hyperplasia in the trunk. This finding suggests that the mechanisms regulating trunk muscle growth are separate from those regulating pectoral fin muscle growth, and that it is not systemic hormonal influences that determine the onset of mosaic hyperplasia. It is possible that the precursors that support mosaic hyperplasia in the trunk are not present in the pectoral fin. This explanation could reflect differences between the dermomyotome cells that migrate into the fin and the dermomyotome cells that enter into trunk myotome during its growth. Alternatively, it may reflect differences in the muscle environment between the fin and the trunk. These could be differences in the size of the muscle, differences in the distribution of growth factors, and/or differences in innervation between the trunk and the fin muscles. Because of the localization of new muscle fiber formation to the region of the pectoral fin closest to the skin, we suggest that localized signals from this region may be regulating hyperplasia in the pectoral fin.

EXPERIMENTAL PROCEDURES

Fish Strains and Staging

Wild-type zebrafish (Oregon AB strain) housed at Wesleyan University since 1997 were used in this study. Fish were reared using standard techniques. Zebrafish are exothermic, and larvae of the same age can vary greatly in size. Therefore, staging of larvae was done based on length; we used the standard length (SL), which is the distance from the tip of the nose to the base of the tail.

Antibodies and Immunohistochemistry

Antibodies used were MF20, a mouse monoclonal antibody obtained from the Developmental Studies Hybridoma Bank (DSHB) that labels differentiated skeletal muscle cells in all species examined (Bader et al., 1982); S58, a mouse monoclonal antibody obtained from DSHB raised against chicken myosin (Crow and Stockdale, 1986) that labels slow muscle fibers in zebrafish (Devoto et al., 1996); zm4, an antibody that specifically labels fast fibers in zebrafish (Barresi et al., 2001) and Pax7, a mouse monoclonal antibody obtained from DSHB that specifically recognizes Pax7 protein in chicken (Kawakami et al., 1997). zm4 supernatant was purchased from the Zebrafish International Resource Center, which is supported by grant P40 RR12546 from the NIH-NCRR. All primary antibodies were used at 5 μg/ml. Secondary antibodies used were IgG1-Alexa546 (Invitrogen) for Pax7 and zm4, IgG2b-Alexa546 (Invitrogen) and IgG2b-Alexa488 (Invitrogen) for MF20, and IgA-fluorescein isothiocyanate (Sigma) for S58.

Whole-mount antibody labeling of larvae was carried out essentially as in Hernandez et al. (2005). Briefly, larvae were fixed overnight in Dent's Fixative and Dent's bleach was used to reduce pigment to better visualize staining and to quench endogenous peroxidases. Antibody labeling was done using standard techniques. Secondary antibody against MF20 was IgG-horseradish peroxidase (Sigma, 1:200). Following the diaminobenzidine reaction, specimens were washed with PBT and cleared and stored in 80% glycerol. Larvae were photographed in glycerol, using an Axiocam MR camera on a Zeiss AxioPlan microscope. Images were adjusted in Photoshop, and all processing was carried out on the entire image. Immunohistochemistry on sections was done by standard procedures (Barresi et al., 2000; Hernandez et al., 2005; Feng et al., 2006).

Plastic Sectioning and Staining

Larvae were fixed overnight in 4% paraformaldehyde. Fixative was washed out, and specimens were dehydrated to 100% ethanol for embedding in JB-4 (Polysciences). Infiltration and embedding into JB-4 plastic resin was performed per the manufacturer's instructions. Blocks were sectioned using an ultramicrotome, and sections were stained with methylene blue or Lee's Methylene Blue/Basic Fuchsin, dried, and cover-slipped in Permount.

Analysis of Distance Between Muscle Fibers and Skin or Endoskeletal Disk Using ArcGIS (ESRI)

Digital images of methylene blue or Lee's methylene blue/basic fuchsin stained plastic sections of larval pectoral fins were taken using an AxioCam MR camera attached to a Zeiss Axioplan microscope. Sections were selected based on proximal–distal position within the fin, and only sections taken through the mid-region of the fin muscle were analyzed. Muscle fibers in the dorsal muscle mass were circled digitally, and the *x,y* coordinates of the center of each muscle fiber were exported into database format (.dbf) files. Points were drawn along the skin overlying the dorsal muscle mass and along the dorsal surface of the endoskeletal disk, and the *x,y* coordinates of each point were exported into database format files. These files were imported into ArcMap. Polylines corresponding to the skin and endoskeletal disk were drawn connecting the imported points. To determine the distance of each muscle fiber center to the skin and endoskeletal disk, the lines corresponding to the skin and cartilage were joined to the points

corresponding to the center of each muscle fiber. Joining was done using the "Joins and Relates" function based on spatial location. Distances were exported into Microsoft Excel and analyzed. This process was repeated for the ventral muscle, using the ventral skin and ventral edge of the endoskeletal disk as reference lines. The number of animals used is as follows: n = 3 for 4.5 mm specimens, n = 3 for 5.5 mm specimens, and n = 4 for 6.5 mm specimens. Both dorsal and ventral muscle masses were used for each specimen.

In Situ Hybridization on Sections

Specimens for in situ hybridization were fixed in 4% paraformaldehyde and sections were obtained as for immunohistochemistry. Section in situ hybridization was carried out as in Myat et al. (1996). Digoxigenin-labeled antisense RNA probes for *myogenin*, *myoD*, or *myf5* were diluted in hybridization buffer and applied to rehydrated sections overnight at temperatures appropriate for hybridization for each probe (62°C for *myogenin* and *myf5*, and 64°C for *myoD*). After color reaction, sections were washed with PBS and dehydrated and coverslipped in Permount.

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