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Distinct modes of vertebrate hypaxial muscle formation contribute to the teleost body wall musculature

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Abstract

The formation of the body wall musculature in vertebrates is assumed to be initiated by direct ventral extension of the somites/myotomes. This contrasts to the formation of limb muscles and muscles involved in feeding or respiration/ventilation, which are founded by migratory muscle precursors (MMPs) distant to the somites. Here, we present evidence from morphology and expression of molecular markers proposing that the formation of the two muscle layers of the teleost body wall involves both of the above mechanisms: (i) MMPs from somites 5 and 6 found an independent muscle primordium (posterior hypaxial muscle, PHM) which subsequently gives rise to the most anterior two segments of the medial obliquus inferioris (OI) muscle. (ii) Direct epithelial extension of the hypaxial myotomes generates the OI segments from somite 7 caudalward and the entire lateral obliquus superioris (OS) muscle. The findings are discussed in relation to the evolution of hypaxial myogenic patterning including functional considerations. We hypothesize that the potential of the most anterior somites to generate migratory muscle precursors is a general vertebrate feature that has been differently utilised in the evolution in vertebrate groups.

Keywords

fish muscle; myogenesis; *lbx1*; *Pax7*; migratory muscle precursors; evolution

Introduction

The segmental organization of the skeletal trunk musculature is a universal characteristic of all vertebrate embryos and reflects its developmental origin from the somites. The trunk muscles of vertebrates with jaws (gnathostomes) are further subdivided into epaxial (dorsal) and hypaxial (ventral) domains. This classification is based on the positional relationship of somite derivatives to the horizontal septum (most obvious in fish) and the innervation of the resident muscles by dorsal and ventral rami of the spinal nerves, respectively. In amniotes, the muscles of the back are classified as epaxial muscles, while diaphragm, tongue, abdominal, intercostal and limb muscles are classified as hypaxial. In adult teleost fish, the epaxial myotomes are frequently described as constituting one single muscle (*M. epaxialis*) as muscle fibers aligning to helical trajectories run through several myotomes. By contrast, the hypaxial myotomes within the region covering the body cavity are divided into two muscles which span the myotomes in different directions: the *M. obliquus superioris* (OS)

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running from anterodorsal to posteroventral in a superficial sheet, and the *M. obl. inferioris* (OI) running from anteroventral to posterodorsal in a deep sheet (Winterbottom 1974). In contrast to the epaxial musculature, which appears to arise directly within the somites without further dislocation, hypaxial muscle formation involves complex cell migrations.

Hypaxial muscle formation in vertebrates

All hypaxial muscles in amniotes derive from precursor cells in the ventro-lateral dermomyotome of the somites. These cells are specified by Bmp4 and Wnt signals from lateral plate mesoderm and dorsal ectoderm and are characterized by the expression of *Pax3* (review Vasyutina and Birchmeier 2006). Depending upon a somite's axial position, the hypaxial muscle precursors are further specified into a migratory and a non-migratory subpopulation which directly account for the two distinct mechanisms by which all amniote hypaxial muscles are formed: long-range migration and epithelial myotome extension (review Evans et al. 2006). (i) Migratory muscle precursors (MMPs) delaminate from the dermomyotome after epithelial-to-mesenchymal transition (EMT) and undergo long-range migration while retaining proliferative capacity. In amniotes, these MMPs arise in occipital, cervical, fore limb and hind limb level somites and contribute to fore and hind limb, tongue and laryngeal muscle formation, and to the mammalian diaphragm and cutaneus maximus muscles. They express downstream targets of *Pax3* such as *c-met* and *Ibx1*, the latter being exclusive for these cells (Dietrich et al. 1998). (ii) Non-migratory precursors arise in inter-limb level somites and characteristically do not express *Ibx1*. They enter the ventral part of the myotome directly from the ventral dermomyotome lip and are progressively shifted further ventrally to generate hypaxial body wall and intercostal muscles. This mechanism is commonly referred to as the epithelial extension of the myotome (Christ et al. 1983).

Fate mapping studies in chick (Nowicki et al. 2003) and mouse (Durland et al. 2008) have shown that migratory and non-migratory muscle precursors differentiate in two distinct mesodermal environments. This has inspired a new terminology defining musculoskeletal domains based on developmental criteria (Burke and Nowicki 2003). Thus, non-migratory myoblasts differentiate in an environment composed completely of somitic cells and contribute to the so-called 'primaxial domain'. MMPs, however, leave the somite and differentiate within and mix with the lateral plate mesoderm. They form the 'abaxial domain', which is composed of lateral plate connective tissue and somitic myoblasts. These domains are separated by a boundary called the lateral somitic frontier (Nowicki et al. 2003). The terminology is aimed to describe dynamic cell behavior during development. It differs from – but does not contradict – the classical epaxial/hypaxial subdivision, which is based on adult positional and functional criteria.

Our present understanding of the cellular mechanisms of hypaxial muscle formation in teleost fish is more limited. Only recently, it has been shown in zebrafish that pectoral fin muscles are generated by long-range migration of *Ibx1*⁺ MMPs which arise from a small number of pre-determined somites (2-4) (Neyt et al. 2000), similar to amniote limb development. Lineage tracing has indicated that zebrafish pectoral fin muscle precursors derive from the somitic anterior border cells which also give rise to the teleost dermomyotome (Hollway et al. 2007). Zebrafish studies also provide information on a migratory origin of a second muscle posterior to the pectoral fin which attaches to the cleithrum, the first-formed element of the teleost pectoral girdle. The founder cells of this muscle arise from somites 5 and 6 and express the same marker genes of cell migration (*Ibx1* and *met*) as pectoral fin muscle precursors (Haines et al. 2004; Neyt et al. 2000). This additional muscle has been named 'posterior hypaxial muscle' (PHM; Haines et al. 2004; term also used in present study). Other studies investigating early trunk myogenesis and cranial muscle development and function referred to the PHM as 'ventral-most hypaxial muscle' (Barresi et al. 2001), 'inferior obliquus muscle', (Hernández et al. 2002), or

‘anterior hypaxial muscle’ (Hernández et al. 2005). Hernández et al. (2002) suggested a transient importance of the PHM during early larval suction feeding (in co-action with the sternohyoideus muscle). However, little is known about the further development of the PHM, its functional integration into the trunk musculature and its relation to the double-layered ventral body wall musculature of adult teleosts.

The present study provides new information on hypaxial muscle formation in teleost fish and relates embryonic patterns of myogenesis to the muscles found in the adult body wall. We show that the MMP-founded PHM initiates the formation of the medial body wall muscle (OI) posterior to the pectoral fin. At the same axis level, direct extension of the myotomes leads to the formation of the lateral body wall muscle (OS). Posterior expansion of both muscle layers proceeds by direct extension of the adjoining myotomes, most probably by aligning medial muscle fibers with those of the PHM and lateral muscle fibers with those of the anterior hypaxial myotomes. We discuss the implications of our data with respect to the evolution of hypaxial muscles in vertebrates.

Materials and Methods

Investigations were carried out on early life stages of the European pearlfish *Rutilus meidingeri*, a large-sized cyprinid known to provide optimum definition of cellular patterning (Steinbacher et al. 2006). Fish were laboratory reared at 16°C from artificially inseminated eggs. Fish were over-anaesthetised with MS-222 (Sigma).

Specimens intended for whole mount in situ hybridisation (ISH) were fixed in PBS-buffered 4% PFA (8h, 4°C), rinsed in PBS, subjected to a graded transfer into methanol and stored at -20°C. Plasmids with cDNA encoding for zebrafish *lhx1* (PD Currie, University of New South Wales), *sim1* (FC Serluca, Harvard Medical School), *tbx2a* and *tbx3b* (JC Izpisua Belmonte, Salk Institute, La Jolla), *mef2d* and *mef2a* (BS Ticho, University of California) were employed. Standard protocols were used for plasmid linearisation and synthesis of digoxigenin-labelled antisense and sense riboprobes. ISH procedures followed those of Steinbacher et al. (2006).

Whole mount immunostaining was performed on specimens fixed in 4% PFA (as above), or in Carnoy's fixative using the protocols of Steinbacher et al. (2006). Anti-chicken Pax7 IgG1 (1:20; DSHB), anti-human Mef2 (1:100; Santa Cruz), anti-chicken slow myosin heavy chain (MyHC) S58 (undiluted; DSHB), anti-teleost slow MyHC 4/96-3c (1:500; A Rowleson, University of London), and anti-chicken light meromyosin IgG2b MF20 (1:10; DSHB) were used as primary antisera. HRP-conjugated anti-rabbit IgG (1:100; DAKO) was applied as secondary antibody and visualized with DAB.

For immunolabelling of cryostat sections, animals were cryo-fixed by plunging into liquid nitrogen-cooled 2-methylbutane and sectioned at 10 µm in a Leitz 1720 cryostat. Anti-Pax7, anti-Mef2 (see above) and anti-Phospho-Histone H3 (H3P, 1:100; Upstate) were used as primary antisera. Alexa 488-conjugated anti-rabbit IgG1 (1:800; Molecular Probes) and Alexa 546-conjugated anti-mouse IgG1 (1:800; Molecular Probes) served as secondary antibodies. Hoechst 33258 was used for nuclear counterstain.

For semithin section histology and TEM, animals were fixed in Karnovsky's fixative, postfixed in 1% OsO₄, embedded into epoxy resin (Glycid ether-100, Serva), and sectioned on a Reichert Ultracut microtome. Semithin sections (1.5 µm) were de-resinated and stained with azure II-methylene blue and basic fuchsin. Ultrathin sections for TEM were contrasted with uranyl acetate and lead citrate using standard facilities. A Leica Wild M10 stereomicroscope and a Reichert Polyvar microscope equipped for fluorescence microscopy and a Zeiss EM 910 TEM were used for digital photography of the results.

Results

The present study provides insight into the circumstances that give rise to teleost body wall muscle at the beginning of the larval period. A special focus is put on how this process is linked to the formation of MMP-derived (potentially abaxial) hypaxial muscles that arise in the embryonic period.

Prior to hatching, MMPs generate the PHM primordium posterior to the pectoral fin

During the segmentation period, pearlfish hypaxial myogenesis is confined to the somitic compartment. In order to determine the onset and axis levels of extra-somitic hypaxial muscle formation, we carried out ISH for the hypaxial-specific myoblast markers *lbx1*, *tbx2a*, *tbx3b* and *sim1*.

In embryos before the end of somitogenesis (40 out of 52 somites formed) *lbx1*, a marker specific to the migratory subset of hypaxial muscle precursors in mouse, *Xenopus* and zebrafish (Dietrich et al. 1998; Martin et al. 2006; Neyt et al. 2000), is expressed at high levels in the ventro-lateral domains of the somites 1-5 (Fig. 1A). In somite 1, *lbx1*⁺ tissue also bulges from the ventral border to extend in anteroventral direction. In fish after completion of somitogenesis, *lbx1* expression expands from somites 1-5, giving rise to three distinct anteroventrally oriented primordia (Fig. 1B). According to their location and in confirmation of previously published zebrafish results (Haines et al. 2004; Neyt et al. 2000; Schilling and Kimmel 1997) they can be identified (in direction from head to tail) as (i) sternohyoideus muscle (SHM), (ii) pectoral fin muscle (PFM), and (iii) posterior hypaxial muscle (PHM). In embryos shortly before hatching, the *lbx1* expression in the area of the PHM primordium is situated ventral to somite 4 and about triangular in shape. At its posterior tip, the primordium is in touch with the *lbx1*⁺ ventral edge of somite 5 (Fig. 1C,C'). Strong *lbx1* expression is now also present at the ventral edge of somite 6.

The hypaxial dermomyotome marker *sim1* is expressed in muscle progenitors which migrate into the limb buds of amniotes (Coumailleau and Duprez 2009) and to the ventral body wall of *Xenopus* (Martin et al. 2007). We found that *sim1* expression patterns largely mirrored those of *lbx1* temporally and spatially. Thus, *sim1* transcript is initially present in the ventral domains of the somites 1-5 and later also in cells at the location of the future SHM, PFM and PHM (Figs. 1D,E). From the end of somitogenesis onwards, the transcripts of *tbx2a* and *tbx3b*, two other hypaxial-specific markers in vertebrate fin/limb development (Gibson-Brown et al. 1998; Ruvinsky et al. 2000) and *Xenopus* body wall muscle formation (Martin et al. 2007), are expressed in the area of the PFM and PHM primordia, but are surprisingly absent from the somite edges and the more anteriorly situated area of SHM formation (Figs. 1F,G). Muscle precursor differentiation was assessed by staining for Mef2, a transcription factor expressed in the nuclei of nascent and maturing fish muscle fibers (Steinbacher et al. 2006). We found that cells at the position of the three extra-somitic muscle primordia (SHM, PFM, PHM) begin to react with *mef2d* probe when cells in the same areas still contain *lbx1*⁺ cells (Fig. 1H; for *lbx1* see Fig. 1C'). Anti-Mef2 immunostaining reveals that Mef2⁺ nuclei constitute just the same triangular-shaped structure (Fig. 1I) as also depicted by *lbx1* probe. However, the area of differentiating PHM cells is clearly separated from the somites while staining for *lbx1* – at the same time – demonstrates no gap between the PHM and somite 5 (cf. Fig. 1C). Staining for skeletal myosin with MF20 antibody confirms that the initial bunch of muscle fibers formed within the PHM of the prehatching fish is not linked to the nearby myotomes (Fig. 1J).

In agreement with the whole mount results, coss-sections through the PHM primordium show a loosely arranged cluster of mesenchymal cells 'bridging' the gap between the ventral dermomyotome edge and the primordium's dorsal edge (Fig. 1K), suggesting that muscle

precursor cells at the ventral edges of these somites undergo epithelial-to-mesenchymal transition - a characteristic feature of MMPs. Corresponding cryostat sections show that the muscle precursor marker Pax7 labels dermomyotome cells as well as mesenchymal cells between the somites and the PHM (Fig. 1L).

Before the onset of exogenous feeding, the PHM spans the yolk as a broad two-segmented muscle band

By the time of hatching, the PHM constitutes an obliquely oriented band extending from the area posterior to the pectoral fin to the ventro-lateral edge of the myotome of somite 5. Myogenic differentiation – as shown by ISH for *mef2a* (Fig. 2A) and immunolabelling for Mef2 and MF20 (Figs. 2B,C) – has extended the anterior rim of the PHM towards the pectoral fin, and has also spread into the narrower posterior part which contacts myotome 5. An additional cluster of muscle cells has emerged at the ventral edge of somite 6, separated from the posterior end of the PHM band (Fig. 2A-C) (cf. pattern of *lhx1* expression in Fig. 1C). On sections through the PHM anterior to its attachment site at myotome 5, loosely arranged mesenchymal cells populate the gap between the dermomyotome and the PHM (Fig. 2D, inset 1). The PHM itself by this time contains rounded cells with central nuclei in the core and a single layer of flattened cells at the lateral surface (Fig. 2D, inset 2). Immunolabelling demonstrates that most Mef2⁺ cells are located in the PHM core (Fig. 2E). Cells that stain for Pax7 are predominantly located in the surface layer, specifically in the distal part of the PHM (Fig. 2E). These cells are almost all Mef2⁻ but some co-stain with anti-H3P, indicating that they are mitotically active (Fig. 2F). Pax7⁺ cells are also found within the mesenchyme-filled gap between the PHM and the somites. Such cells sometimes also stain for Mef2 (Fig. 2E).

In posthatching fish (period until onset of free swimming), the PHM shows a continued substantial increase in both breadth and length (Figs. 3A-C). Immunostaining with anti-slow MyHC sera (S58 and 4/96-3c) was performed to visualize the orientation of individual muscle fibres (Figs. 3B,C). Labelling with the pan-muscle marker MF20 confirms that S58 and 4/96-3c are indeed able to define the true dimensions of the PHM and the myotomes (Fig. 3C). Results demonstrate that the anterior end of the PHM band now directly opposes the SHM just below the base of the pectoral fin. The posterior end meets the edge of myotome 5 as before, but - as a consequence of broadening - most of the muscle fibers now terminate at an elongation of the myoseptum between the myotomes 5 and 6 (Fig. 3B). Posterior to this myoseptum, newly formed muscle fibers provide a direct continuation of the band towards the edge of somite 6, thus adding a second segment to the PHM (Fig. 3A,B). The fibers of the two separately formed segments run oblique to those of the myotomes and show perfect myofibril alignment (Fig. 3B). Progressive ventral extension of both PHM segments subsequently leads to a nearly doubling of dorso-ventral breadth until onset of swimming (Fig. 3B, C).

ISH shows that up to this developmental stage, *lhx1* transcript is still present at low concentrations in both PHM segments, indicating that MMPs contribute to this extension process. *sim1* is still expressed at the posterior ends of the two PHM segments and along the ventral edge of somite 5 (Fig. 3E). The *tbx3b* probe generates a clear signal along the ventral edges of the somites 5 and 6, most times along with some less discrete staining laterally on the yolk sac (Fig. 3F). In addition, sections double immunolabelled for Pax7 and Mef2 or H3P demonstrate that Pax7⁺ cells occur at the ventral dermomyotome edge, and at the lateral surface and ventral edge of the PHM (Figs. 3G,H). PHM cells at the beginning of myogenic differentiation (Pax7⁺/Mef2⁺) are exclusively found at these sites (Fig. 3G). Mitotically active cells (Pax7⁺/H3P⁺) are restricted to positions at or close to the ventral edge (Fig 3H) thus indicating the formation of a precursor cell pool to sustain a growth zone

for the further ventral advancement of the edge, as visible in animals only one week older (Fig. 3I).

In early larvae, extension of the hypaxial myotomes promotes the formation of a two-layered body wall musculature

At onset of feeding (beginning of larval period) and a few days thereafter, two morphological changes become evident. First, the most ventral fibres of the myotomes 5 and 6 intersect with those in the most dorsal part of the PHM (Fig. 4A,B). Semithin sections illustrate that within this zone the PHM fibers run subjacent to the myotomal fibers (Fig. 4C). A concomitant expansion of the PHM itself (by addition of small, presumably new fibers) is indicated by a marked fiber size decrease towards the lateral surface and especially towards the ventral edge (Fig. 4D). Second, newly added muscle fibers at the ventral edge of myotome 7 align with those of the PHM instead of maintaining the orientation of the hypaxial slow fibers farther dorsally (Fig. 4B). In contrast to the situation at segments 5 and 6, there is no indication that the ventral extension of myotome 7 is promoted by MMPs (see lack of *Ibx1*, *sim1* and *tbx3b* expression in Figs. 3D-F).

Development after the onset of exogenous feeding is characterized by a substantial further longitudinal and ventral expansion of the body wall musculature. This occurs in increasingly complicated patterns. Whole mounts and semithin sections illustrate that the ventral body wall muscle in the area of the segments 5 and 6 is largely established by the PHM alone (Fig. 4C,D,F) while the ventral expansion of the hypaxial myotomes stagnates along a posteriorly slanting line in the lateral flank. This leaves the ventral body wall immediately posterior to the pectoral fin one-layered. In addition to its continued ventral expansion by recruitment of small fibers at the ventral edge, the PHM thickens by apposition of new fibers to the lateral surface (Fig. 4D). These growth sites are also preferred locations of Pax7⁺ and Mef2⁺ cells (Fig. 4E).

In contrast to the segments 5 and 6, the next following segments (from segment 7 to midbody) exhibit prominent ventral extensions of the hypaxial myotomes (Fig. 4F). These generate the body wall musculature of these segments which increasingly covers the body cavity while advancing towards the ventral midline. The ventral (most recently formed) area of this musculature shows no sign of a medial-lateral separation and its fibers adapt the oblique orientation of the anteriorly adjoining PHM fibers (Fig. 4F,J). By contrast, the hypaxial myotomes are two-layered at positions farther away from their ventral edges, whereby fiber trajectories of the medial and the lateral layer follow the templates set by the PHM fibers and the hypaxial fibers of the myotomes 5/6, respectively (Fig. 4F-H,J). We suggest that medial and lateral muscle layers separate gradually by muscle fiber re-orientation and correspond to the OI and OS muscles of the adults, respectively. The two body wall muscle layers are present up to the bases of the vertebrae (Fig. 4J). The ribs are interconnected by fibers that run roughly parallel to the body axis. Such fibers are found at the lateral boundary of the OI but strongly deviate in orientation from the deep OI fibers (Fig. 4H).

Immunostaining for Pax7 and Mef2 provides clear indication that the parting line between the two muscle layers is not a site of fiber recruitment. Thus, thickening growth of the body wall muscle is promoted by addition of small Mef2⁺ fibers to the lateral face of the lateral layer (just underneath the slow muscle fibers), but to the medial face of the medial layer (the latter being in contrast to the growth mode of the PHM) (Fig. 4I,J).

Discussion

The present work provides a comprehensive characterisation of the initial development and subsequent growth of the body wall musculature in the teleost pearlfish. The body wall of adult teleosts comprises two muscle layers, which correspond to those generally termed *Musculus obliquus superior* (OS) and *Musculus obliquus inferior* (OI) by Winterbottom (1974). We propose that the formation of the OI differs fundamentally between segments 5/6, and the more posteriorly positioned segments. At segments 5 and 6, the OI muscledescends from an independent MMP-derived muscle primordium (PHM). Direct epithelial extension of the hypaxial myotomes generates the OI segments from somite 7 caudalward. By contrast, the OS arises only by hypaxial myotome extension.

Body wall muscles at somites 5 and 6 are generated by two distinct developmental processes

Our data provide morphological evidence that the PHM represents the most anterior two segments of the OI. This conclusion is based on the fact that the PHM anticipates characteristic features of the adult body wall morphology (Winterbottom 1974): The dorsal part of the PHM lies underneath (i.e. medial to) the hypaxial myotome (Fig. 4C,G), just as the adult OI lies medial to the OS, and the PHM's muscle fiber trajectories are at anteroventral to posterodorsal orientation (Fig. 4B,F,G). The PHM is the first body wall muscle component established in the pearlfish finally providing the link necessary to insert the trunk OI to the cleithrum and coracoid as present in adult teleosts (Winterbottom 1974). The formation of the PHM most likely occurs by aggregation of predetermined MMPs from the dermomyotome of somites 5 and 6. This view is supported by (i) the expression of the MMP marker *lhx1* in the ventro-lateral edges of these somites and in the presumptive PHM primordium (Fig. 1A-C), (ii) the initial differentiation of PHM fibers distant to the somites (Fig. 1H-J) and (iii) the mesenchymal morphology of cells between the dermomyotome and the PHM primordium (Fig. 1K). All these features have previously been used to characterize the migratory mode of myogenesis in vertebrate development. The observed *lhx1* expression patterns are in close accordance with previous findings in the zebrafish (Haines et al. 2004; Neyt et al. 2000) and are also remarkably parallel to those in MMPs delaminating from amniote occipital/cervical somites to give rise to tongue, laryngeal and the mammalian diaphragm and cutaneous maximus muscles (Dietrich et al. 1998; review Vasyutina and Birchmeier 2006). A relation between PHM formation in teleost fish and MMP-dependent myogenesis in other vertebrates is further indicated by the finding that presumptive PHM precursors also express the hypaxial markers *sim1*, *tbx2a* and *tbx3b* (Fig. 1D-G). Just as *lhx1*, these factors are known to play a role in the migration of precursor cells from the ventral dermomyotome lip to promote hypaxial muscle development in amniotes at various sites including the limbs (Coumailleau and Duprez 2009; Gibson-Brown et al. 1998) and in the *Xenopus* trunk (Martin and Harland 2006).

After the formation of the PHM primordium, Pax7⁺ occasionally proliferative (H3P⁺) muscle precursors at the ventral edge of the muscle (Fig. 2E,F) indicate the formation of a resident progenitor pool to promote subsequent ventral growth (e.g. Fig. 4E). Prior to this stage, Pax7 is exclusively found in dermomyotome cells and in some of the mesenchymal cells in the gap between the hypaxial dermomyotome edge and the PHM (Fig. 1L). Whether the pool of resident Pax7⁺ precursors at the PHM edge originates by migration of Pax7⁺ from the dermomyotome lip via the mesenchyme, or whether this pool is established by turning on Pax7 in a previously Pax7⁻ sub-population of the PHM-founding MMPs remains to be determined. Evidence from mouse limb muscle formation argues for the latter conclusion, in that the a subset of *lhx1*⁺ MMPs turns on Pax7 while maintaining proliferative capacity and eventually gives rise to secondary limb muscle fibers and satellite cells (review Buckingham and Vincent 2009).

Collectively, our results strongly suggest that the pearlfish PHM is formed by the migratory mode of myogenesis, similar to what has been shown in zebrafish (Haines et al. 2004; Hollway et al. 2007). Based on these findings we assume that the most anterior two segments of the OI represents an abaxial domain within the body wall musculature. The formation of the lateral body wall muscle (OS) at the same axis level becomes evident when the ventrally expanding hypaxial myotomes of the segments 5 and 6 cover the most dorsal parts of the PHM. Morphological analysis indicates that this occurs by direct epithelial extension (i.e. stratified ventral outgrowth) of these myotomes (Fig. 4A-C), which is known to be promoted by Pax7⁺ dermomyotome cells (Steinbacher et al. 2006; Stellabotte et al. 2007). This enables us to conclude that in these segments, the OS belongs to the primaxial domain, just in contrast to the PHM/OI (see above). A somewhat surprising feature is that the ventral growth of the OS largely ceases after onset of exogenous feeding, thus leaving part of the body wall muscle directly posterior to the pectoral fin one-layered, deriving only from the PHM/OI (Fig. 4C,F). The reason for this remains unclear but may be associated with the subsequent formation of prominent pectoral fin muscles (particularly the abductor profundus and adductor superficialis) at this position (cf. Winterbottom 1974).

From somite 7 tailwards, myotomal extension alone accounts for body wall muscle formation

A striking result of the present investigation is that the two layers of the pearlfish body wall musculature (the OI and OS muscles) are initiated as separate structures (PHM and ventral myotome) in the segments 5 and 6 but appear to arise from a single common structure in all segments further caudalward. In the latter, the newly formed fibers of the ventrally extending myotomes align with the fibers of the PHM, thus stringing together the PHM (i.e. the most anterior part of the OI) and the prospective OI fiber trajectories of the trunk myotomes from segment 7 caudalward (Fig. 4F,G). We speculate that fiber orientation is influenced by the directional pull generated when the earlier formed PHM fibers contract during larval suction feeding (see also functional considerations below). The morphological separation of the hypaxial myotome extensions to create the two-layered OI/OS system with different fiber orientations appears to occur dorsal to the advancing edges, where the hypaxial muscle has already thickened (3 rows of fibers, Fig. 4J). The separation of a single muscle layer as observed in this study certainly requires further research but bears resemblance to hypaxial inter-limb body wall muscle formation in the chick. Also here, the body wall develops initially as a single muscle sheet from the ventral myotome before it becomes subdivided into 4 distinct muscles (obliquus abdominis externus, obliquus abdominis internus, transversus abdominis, rectus abdominis) (Christ et al. 1983). However, more recent work suggests that body wall muscle formation in amniotes involves both, migratory and non-migratory muscle precursors, rather than epithelial extension alone (review Evans et al. 2006).

Regarding the origin of the myogenic precursors accounting for teleost body wall formation from segment 7 caudalwards, the present results again suggest the dermomyotome as the most probable source. The observed myotomal extension is likely to occur by stratified hyperplasia (Fig. 4D,E,I) for which recent work in a variety of species has provided reliable evidence that it is driven by Pax7⁺ myogenic precursors from the dermomyotome but does not require 'classical' EMT (Hollway et al. 2007; Steinbacher et al. 2006; Stellabotte et al. 2007). Considering the primaxial/abaxial terminology, both muscle layers, the OI and OS, posterior to somite 6 most probably represent primaxial domains, thus leaving the most anterior part of the OI (PHM) as the only potentially abaxial domain within the body wall musculature. The conclusion that the OI is a hybrid muscle (partly primaxial and partly abaxial) is consistent with findings on trapezius and latissimus muscle composition in the Prx1Cre transgenic mouse (Durland et al 2008).

Functional and evolutionary considerations

Research on zebrafish has suggested that the PHM plays an important role in larval suction feeding by providing a functional connection between the SHM and the trunk muscle (Hernández et al. 2002). Consistent with this assumption, the present study demonstrates that the SHM is apposed to the PHM at the pectoral girdle prior to hatching and a massive broadening/strengthening of the PHM takes place prior to the onset of exogenous feeding (Fig. 3B,C). We further expand the previous understanding of this mechanism by showing that the body wall muscle involvement in larval feeding kinetics is not confined to the PHM but instantly strengthened by inclusion of myotomal fiber trajectories (Fig. 4A,B). Considering the early functional requirement of the PHM, a rapid establishment of the primordium by MMPs (instead of 'slow' myotomal extension) seems reasonable and may be a pre-requisite to achieve this. From this point of view, cellular mechanisms (migration, extension) of hypaxial muscle formation are variably employed depending upon factors such as distance of precursor cell translocation and available developmental time.

The view that the PHM originates from dermomyotome-derived MMPs is also of particular interest in a broader vertebrate/craniate context. The potential of specific somites to generate MMPs is generally regarded as a derived feature of vertebrate hypaxial myogenesis, while direct extension of the ventral myotomes is regarded as the more ancient/primitive mode of vertebrate hypaxial muscle formation (Haines and Currie 2001). Evidence has been obtained from most vertebrate groups that the ventral edges of the most anterior somites have the potential to generate MMPs with similar molecular, morphogenetic and functional features. The same has been shown for the limbless agnathan lampreys, demonstrating that regulatory cascades accounting for MMP specification have already emerged before the agnathan/gnathostome divergence (Kusakabe and Kuratani 2005). Thus, all MMPs concerned are characterised by the expression of *lhx1*, *tbx3* and/or *pax3* and migrate as mesenchymal cells to generate muscles that are, although in some cases only transiently, involved in feeding or respiration/ventilation. This holds for the teleost SHM and PHM just as for agnathan hypobranchial muscle, tetrapod laryngeal and tongue muscles, and mammalian diaphragm muscle (Dietrich et al. 1998; Haines et al. 2004; Kusakabe and Kuratani 2005; Neyt et al. 2000; this study). The obvious correspondence in origin, position, function and gene expression may point towards a shared structural and developmental ancestry of all these muscles. The MMP-generating potential of the anterior somites would then have been differently utilized in the evolution of vertebrate groups (see Fig. 5). It is in this sense that Kusakabe and Kuratani (2005) have speculated that the hypobranchial muscle of lampreys is homologous to the amniote tongue.

However, a challenging difference between the teleosts and the other vertebrate clades exists relating to the contribution of anterior somites to forelimb formation. Only in teleost fish but not in amniotes, MMPs from the most anterior somites (somites 2-4) also migrate to the forelimb (pectoral fin) anlage to give rise to fin muscles (Neyt et al. 2000; see also Figs. 1B-E, Fig. 5). In this context, positional considerations may be relevant. It is as yet unclear whether the forelimb originally evolved at the level of the MMP-producing anterior somites or spatially separated from these somites. Considering the fossil record it appears clear that the earliest known paired fins (those of the agnathan osteostracans) arose at a position similar to that of the pectoral fins of extant teleosts (review Johanson 2010). Thus it is tempting to speculate that the available potential of the anterior somites to release MMPs could have also been utilised to supply muscles to the newly evolved forelimb/fin. Amniote limb development, however, would be a derived feature requiring an extension of the MMP-generating potential towards somites at positions farther posteriorly (eg. somites 15-20 in chick, somites 9-12 in mouse). Cumulative evidence on gene expression further corroborates that fin/limb-generating muscle precursors bear a striking resemblance to those that account for the formation of lamprey hypobranchial muscle, amniote tongue muscle, and teleost

PHM and SHM (all of which are positive for *tbx3*, *lhx1*, *pax3/7*, and *mox2*) (eg. Candia et al. 1992; Gross et al. 2000; Huang et al. 1999; Kusakabe and Kuratani 2005; Neyt et al. 2000; Ruvinsky et al. 2000; and results of this study).

Despite the importance that is attached to the MMP-dependent mode of hypaxial myogenesis as discussed above, it only contributes to the very initial part of the teleost body wall musculature. All further muscle formation to complete the wall is achieved via direct epithelial somite/myotome extension. This ‘primitive’ mode of growth, is accomplished by ‘simple’ stratified hyperplasia, the probably earliest and still most important mechanism of vertebrate myotome expansion in both embryogenesis and evolution. Beside its essential role for myotome growth in teleost embryos and larvae, the mechanism accounts for body wall myogenesis in amniotes at inter-limb levels (Christ et al. 1983) and in chondrichthyans where it – in contrast to amniotes and teleosts – also generates fin muscle (Neyt et al. 2000).

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References

- Barresi MJ, D’Angelo JA, Hernández LP, Devoto SH. Distinct mechanisms regulate slow-muscle development. *Curr Biol*. 2001; 11:1432–1438. [PubMed: 11566102]
- Buckingham M, Vincent SD. Distinct and dynamic myogenic populations in the vertebrate embryo. *Curr Opin Genet Dev*. 2009; 19:444–453. [PubMed: 19762225]
- Burke AC, Nowicki JL. A new view of patterning domains in the vertebrate mesoderm. *Dev Cell*. 2003; 4:159–165. [PubMed: 12586060]
- Candia AF, Hu J, Crosby J, Lalley PA, Noden D, Nadeau JH, Wright CV. *Mox-1* and *Mox-2* define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. *Development*. 1992; 116:1123–1136. [PubMed: 1363541]
- Christ B, Jacob M, Jacob HJ. On the origin and development of the ventrolateral abdominal muscles in the avian embryo. An experimental and ultrastructural study. *Anat Embryol*. 1983; 166:87–101. [PubMed: 6220621]
- Coumalleau P, Duprez D. *Sim1* and *Sim2* expression during chick and mouse limb development. *Int J Dev Biol*. 2009; 53:149–157. [PubMed: 19123137]
- Dietrich S, Schubert FR, Healy C, Sharpe PT, Lumsden A. Specification of the hypaxial musculature. *Development*. 1998; 125:2235–2249. [PubMed: 9584123]
- Durland JL, Sferlazzo M, Logan M, Burke AC. Visualizing the lateral somitic frontier in the Prx1Cre transgenic mouse. *J Anat*. 2008; 212:590–602. [PubMed: 18430087]
- Evans DJ, Valasek P, Schmidt C, Patel K. Skeletal muscle translocation in vertebrates. *Anat Embryol*. 2006; 211:43–50. [PubMed: 17043770]
- Gibson-Brown JJ, I Agulnik S, Silver LM, Papaioannou VE. Expression of T-box genes *Tbx2-Tbx5* during chick organogenesis. *Mech Dev*. 1998; 74:165–169. [PubMed: 9651516]
- Gross MK, Moran-Rivard L, Velasquez T, Nakatsu MN, Jagla K, Goulding M. *Lbx1* is required for muscle precursor migration along a lateral pathway into the limb. *Development*. 2000; 127:413–424. [PubMed: 10603357]
- Haines L, Currie PD. Morphogenesis and evolution of vertebrate appendicular muscle. *J Anat*. 2001; 199:205–209. [PubMed: 11523824]
- Haines L, Neyt C, Gautier P, Keenan DG, Bryson-Richardson RJ, Hollway GE, Cole NJ, Currie PD. Met and Hgf signaling controls hypaxial muscle and lateral line development in the zebrafish. *Development*. 2004; 131:4857–4869. [PubMed: 15342468]

- Hernández LP, Barresi MJF, Devoto SH. Functional morphology and developmental biology of zebrafish: Reciprocal illumination from an unlikely couple. *Integr Comp Biol*. 2002; 42:222–231. [PubMed: 21708714]
- Hernández LP, Patterson SE, Devoto SH. The development of muscle fiber type identity in zebrafish cranial muscles. *Anat Embryol*. 2005; 209:323–334. [PubMed: 15761723]
- Hollway GE, Bryson-Richardson RJ, Berger S, Cole NJ, Hall TE, Currie PD. Whole-somite rotation generates muscle progenitor cell compartments in the developing zebrafish embryo. *Dev Cell*. 2007; 12:207–219. [PubMed: 17276339]
- Huang R, Zhi Q, Izpisua-Belmonte JC, Christ B, Patel K. Origin and development of the avian tongue muscles. *Anat Embryol*. 1999; 200:137–152. [PubMed: 10424872]
- Johanson Z. Evolution of paired fins and the lateral somitic frontier. *J Exp Zool*. 2010; 314B:347–352. [PubMed: 20535770]
- Kusakabe R, Kuratani S. Evolution and developmental patterning of the vertebrate skeletal muscles: perspectives from the lamprey. *Dev Dyn*. 2005; 234:824–834. [PubMed: 16252276]
- Martin BL, Harland RM. A novel role for *lhx1* in *Xenopus* hypaxial myogenesis. *Development*. 2006; 133:195–208. [PubMed: 16339190]
- Martin BL, Peyrot SM, Harland RM. Hedgehog signaling regulates the amount of hypaxial muscle development during *Xenopus* myogenesis. *Dev Biol*. 2007; 304:722–734. [PubMed: 17320852]
- Neyt C, Jagla K, Thisse C, Thisse B, Haines L, Currie PD. Evolutionary origins of vertebrate appendicular muscle. *Nature*. 2000; 408:82–86. [PubMed: 11081511]
- Nowicki JL, Takimoto R, Burke AC. The lateral somatic frontier: dorso-ventral aspects of antero-posterior regionalization in avian embryos. *Mech Dev*. 2003; 120:227–240. [PubMed: 12559495]
- Ruvinsky I, Oates AC, Silver LM, Ho RK. The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Dev Genes Evol*. 2000; 210:82–91. [PubMed: 10664151]
- Schilling TF, Kimmel CB. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development*. 1997; 125(15):2945–2960. [PubMed: 9247337]
- Steinbacher P, Haslett JR, Six M, Gollmann HP, Sängler AM, Stoiber W. Phases of myogenic cell activation and possible role of dermomyotome cells in teleost muscle formation. *Dev Dyn*. 2006; 235:3132–3143. [PubMed: 16960856]
- Stellabotte F, Dobbs-McAuliffe B, Fernandez DA, Feng X, Devoto SH. Dynamic somite cell rearrangements lead to distinct waves of myotome growth. *Development*. 2007; 134:1253–1257. [PubMed: 17314134]
- Vasyutina E, Birchmeier C. The development of migrating muscle precursor cells. *Anat Embryol*. 2006; 211:37–41. [PubMed: 16977478]
- Winterbottom R. A descriptive synonymy of the striated muscles of the Teleostei. *Proc Acad Nat Sci*. 1974; 125:225–317.

Fig. 1. MMPs generate the PHM primordium

During the segmentation period, the ventral edges of somites 1-5 express *lbx1* (A) and *sim1* (D). At the end of segmentation, the primordia of the SHM, PFM and PHM are defined by their expression of *lbx1* (B) and *sim1* (E). *lbx1* expressing cells also delineate the ventral edges of the anterior somites (now including somite 6). Shortly before hatching, the three primordia express *lbx1* (C, C') and *mef2d* (H), while *tbx2a* and *tbx3b* are confined to the PHM and PFM (F, G). Immunostaining with anti-Mef2 (I) and anti-myosin MF20 (J) defines the differentiating PHM as a triangular-shaped structure lying independently ventral to somite 4. The lens-shaped PHM profile is located distant to the myotome, mesenchymal cells (arrowheads) occur in the intermediate space (K). Double-immunolabeling demonstrates that Mef2⁺ nuclei populate the PHM (green arrowheads), Pax7⁺ nuclei (red arrows) occur at the proximal boundary and within the space towards the somite (L). Nuclei counterstained with Hoechst 33258 (blue). A-J: whole mounts (anterior trunk, anterior to the left); K, L: cross sections through somite 4 (dorsal to the top, midline to the left). Dotted lines indicate pectoral fin. dm dermomyotome, myo myotome, PF(M) pectoral fin (muscle), PHM posterior hypaxial muscle, SHM sternohyoideus muscle. Scale bars: A-H 250 μ m, I-J 100 μ m, K-L 25 μ m.

Fig. 2. The PHM contacts myotome 5

At hatching (12 dpf), the SHM, PFM and PHM express *mef2a* (A) and stain with anti-Mef2 (B) and anti-myosin MF20 (C). The band-shaped PHM (bracket) extends from the base of the pectoral fin to the edge of myotome 5. An additional cluster of differentiating muscle cells has emerged at the ventral edge of somite 6 (arrowheads in A-C). Mesenchymal cells create a chain-like link (arrows) between the PHM and the dermomyotome edge (D, inset 1). The PHM contains rounded cells in the core and is laterally covered by flattened cells (arrowheads in inset 2). Nuclei within the PHM stain for Mef2 and, in the distal part, for Pax7 (red arrowheads) (E); a Pax7⁺/Mef2⁺ cell (white arrowhead) is located in the space between the PHM and the somite/myotome. The distal part of the PHM contains mitotically active H3P⁺/Pax7⁺ cells (arrowheads) (F). A-C: whole mounts (dorso-lateral views, anterior to the left). D-F: cross sections through somite 4 (dorsal to the top, midline to the left). dm dermomyotome, myo myotome, PF(M) pectoral fin (muscle), PHM posterior hypaxial muscle, SHM sternohyoideus muscle. Scale bars: A-C 250 μ m, D-F 50 μ m.

Fig. 3. The PHM expands caudally and ventrally

Shortly after hatching, immunolabelling with anti-Mef2 (A) and anti-slow MyHC (4/96-3c) (B) shows that the PHM consists of two segments (brackets). Segment 1 extends from below the pectoral fin to the edge of myotome 5, segment 2 runs from an unstained ventral elongation of the myoseptum 5/6 to the edge myotome 6. In the time of onset of free swimming, anti-slow MyHC (S58) stained myofibrils of the two PHM segments insert to the myoseptal elongation 5/6 (arrowheads) (C); the pan-myosin marker MF20 confirms that S58 labels the entire PHM (inset in C). Low concentrations of *lbx1* transcript are present throughout both PHM segments (D). *sim1* and *tbx3b* are expressed at the ventro-lateral edges of the somites 5 and 6 (arrowheads) (E, F). Pax7⁺ nuclei are located at the lateral surface and at the ventral edge of the PHM (red arrowheads; insets highlight differentiating Pax7⁺/Mef2⁺ cell) (G). Mitotically active Pax7⁺/H3P⁺ cells (insets) reside preferentially close to the PHM's ventral edge (PHM delimited by dotted line) (H). Analysis under the TEM reveals that this site contains morphologically undifferentiated cells (arrowheads) and newly formed muscle fibers with small myofibrils (I). A-F: whole mounts (lateral views, anterior to the left). G-I: cross sections (dorsal to the top, midline to the left). ep epidermis, mf myofibrils, myo myotome, PF(M) pectoral fin (muscle), SHM sternohyoideus muscle. Scale bars: A-F 250 μ m; G,H 50 μ m, I 5 μ m.

Fig. 4. A double-layered body wall musculature arises

At onset of feeding, PHM myofibrils (arrowheads) stained with anti-slow MyHC (4/96-3c) cross those of the myotomes 5 and 6 (A) and 7 (B) to terminate at the posterior myosepta of these myotomes. Myofibrils of myotome 7 (arrow in B) adapt to the orientation of the PHM. Sections of early larvae confirm that PHM fibers pass underneath the ventral myotomes (C). This myotome/PHM arrangement obviously resembles the adult OS/OI muscle architecture. Small fibers (arrowheads in D) and Pax7⁺ and Mef2⁺ cells (E) assemble along the lateral PHM surface down to the ventral edge. In more advanced larvae, myofibrils fibrils of myotomes 7-10 align with the PHM (arrowheads) (F). OI fibers of myotome 7 insert at the ribs (arrow) and align with PHM (G). Lateral OI fibers interconnect the ribs (arrows), medial OI fibers run oblique to them (H). Pax7⁺ and Mef2⁺ cells are present at the medial and lateral surface of the OI and OS muscles, respectively (I). The OS/OI separation (arrow) is visible only at a distance away from the ventral myotome edge (J). Small fibers constitute the lateral OS surface and the medial OI surface (black/white arrowheads). A, B, F: whole mounts (lateral views, anterior to the left). C-E, I, J: cross sections through somites 6 (C-E) and 9 (I, J). G, H: horizontal sections through somites 6/7 (G) and 8/9 (H), anterior to the left. myo myotome, OI obliquus inferioris muscle, OS obliquus superioris muscle, PHM posterior hypaxial muscle. Scale bars: A, B, F 200 μ m, C-E, G-J 50 μ m.

Fig. 5.

Schematic representation of skeletal muscle patterning in craniotes/vertebrates via specification of migratory muscle precursors (MMPs), illustrating that the MMP-generating potential of the anterior somites is a common feature that emerged before the agnathan/gnathostome divergence. In lampreys (**A**), anterior somite MMPs migrate towards the head to form the hypobranchial muscle (hbm). In teleost fish (**B**), MMPs originating from somites 1-6 generate the sternohyoideus muscle (SHM, blue), pectoral fin muscle (PFM, orange) and posterior hypaxial muscle (PHM, blue). In amniotes (**C**), MMPs from anterior somites (occipital/cervical) give rise to tongue muscle (tm), laryngeal muscle (la), cucullaris or trapezius muscle (cu/tr) and the diaphragm (dp) (all in blue). Amniote limb muscles (lm, orange) arise from more posterior axis levels. Origin and destination of MMPs are highlighted in color; migration of muscle precursors is indicated by dashed lines. Ot, otic vesicle. Schematic modified after Kusakabe and Kuratani (2005).