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RESEARCH ARTICLE

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Anterior trunk muscle shows mix of axial and appendicular developmental patterns

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Abstract

Background: Skeletal muscle in the trunk derives from the somites, paired segments of paraxial mesoderm. Whereas axial musculature develops within the somite, appendicular muscle develops following migration of muscle precursors into lateral plate mesoderm. The development of muscles bridging axial and appendicular systems appears mixed.

Results: We examine development of three migratory muscle precursor-derived muscles in zebrafish: the sternohyoideus (SH), pectoral fin (PF), and posterior hypaxial (PHM) muscles. We show there is an anterior to posterior gradient to the developmental gene expression and maturation of these three muscles. SH muscle precursors exhibit a long delay between migration and differentiation, PF muscle precursors exhibit a moderate delay in differentiation, and PHM muscle precursors show virtually no delay between migration and differentiation. Using lineage tracing, we show that lateral plate contribution to the PHM muscle is minor, unlike its known extensive contribution to the PF muscle and absence in the ventral extension of axial musculature.

Conclusions: We propose that PHM development is intermediate between a migratory muscle mode and an axial muscle mode of development, wherein the PHM differentiates after a very short migration of its precursors and becomes more anterior primarily by elongation of differentiated muscle fibers.

KEYWORDS
lateral plate mesoderm, migratory muscle precursors, pectoral fin, posterior hypaxial muscle, sternohyoid

1 | INTRODUCTION

While axial and appendicular muscles are physiologically similar, and functionally and physically interconnected, they develop differently. Axial muscles, such as the deep muscles of the back, grow and expand within the somitic environment through the addition of dermomyotome-derived cells. This process is known as stratified hyperplasia in fish.[1-4] Therefore, axial muscles are primaxial, because the connective tissue cells within the muscle are derived from somitic cells.[5] In contrast, muscles associated with the pectoral appendage develop from cells which arise in the somites and then migrate to a distant site before differentiation.[6-9] The migratory muscle precursors (MMPs) that form these muscles move into the lateral plate mesoderm (LPM). Therefore, the appendicular muscles are abaxial, because the connective tissue cells within these muscles are derived from LPM.[5]

LPM and its persistent contact with ectoderm (together forming the somatopleure) provide incompletely understood signals for migration, differentiation, and patterning of the abaxial muscles.[4,5,10-14] Moreover, muscles that bridge axial and appendicular systems, such as the trapezius and latissimus dorsi that help to stabilize the forelimb, have been shown to be partly primaxial and partly abaxial.[10]

The development and maturation of MMPs occurs in a series of steps. First, the cells of the ventral dermomyotome undergo an epithelial-to-mesenchymal transition to dissociate from the somite. Second, individual undifferentiated cells migrate through the LPM to the site of the future muscle. Finally, at the site of the future muscle, they differentiate into muscle fibers.[6,11,15,16] Thus, MMPs migrate before differentiation into muscle. This mode of muscle development allows muscles to begin development at a distance from, and partially independent of the body axis. However, a developmental and evolutionary continuum exists between strict nonmigratory and MMP-derived muscle.[11,17,18] In chondrichthyans, fin muscle precursors delaminate, but then migrate as a compact cell aggregate.[18] Within amniotes, muscles that develop with features of both MMPs and epithelial extensions seem to be muscles that bridge the axial and appendicular systems, such as those of the pectoral girdle.[10,11]

In amniotes, MMPs express *Pax3*, *c-Met*, and *Lbx1* genes.[6,7,9,16,19] High levels of Pax3 activate *c-Met* and *Lbx1* in the ventrolateral dermomyotome of limb-level somites, from which MMPs develop.[20,21] *Pax3* mutant mice fail to develop most MMP-derived muscle, including limb muscle.[9] c-Met and its ligand, SF/HGF (scatter factor), are necessary for the epithelial-to-mesenchymal transition (EMT) that MMPs undergo when detaching from the dermomyotome.[6,21,22] In mice, Lbx1 is necessary for migration of MMPs into the limbs, and is likely a repressor of *MyoD* and thus of myogenesis.[22]

In teleosts, there are four muscles with evidence of MMP derivation: the esophageal (not addressed in this study), the sternohyoideus (SHM), the pectoral fin (PFM), and the posterior hypaxial (PHM).[23-25] The SHM is located in the head and connects the cleithrum to the hyoid, its fibers derive from somites 1 to 3.[24,26] SHM contraction depresses the hyoid, a movement crucial for early larval suction feeding.[27] The PFM is located at the head-trunk transition, and its muscle cells arise from somites 2 to 4.[25] When transplanted, cells from somites 1 to 6 are capable of forming PFM,[23] suggesting that all of these somites are capable of generating MMPs. Teleost PFM precursors express *lbx* genes, and *pax3b* morpholino disrupts normal pectoral fin muscle development in zebrafish.[24-26,28]

The PHM connects the trunk muscle and the cleithrum.[23] The PHM is defined as the two anterior-most segments of the obliquus inferioris, a ventral body wall muscle.[26,29] The two segments of the PHM correspond to and arise from somites 5 and 6.[23,29] PHM fibers are longer than myotomal fibers, and are oriented at a different angle.[26] The early attachment of the PHM to the cleithrum is critical for larval suction feeding, as it stabilizes the SHM.[26,27,29]

Based on the expression and necessity of key developmental genes, the PHM shares developmental characteristics with the SHM and PFM.[23,26,27] The PHM precursors express *lbx2* (previously known as *lbx1*), just like the SHM and PFM (Neyt et al[25]; Wotton et al[30]; Figure 1). In pearlfish, the *mef2* expressing cells in the PHM primordium are an "island" separate from the somites. This suggests that the PHM is formed from MMPs, because the physical gap in gene expression implies a distance traveled by the precursors prior to the activation of myogenic gene expression.[26] A similar argument was employed by Neyt et al[25] to support the MMP-derivation of the PFM.

The PHM, however, also shares developmental and morphological characteristics with the axial musculature, such as being continuous with the inferior oblique muscle.[26,29] Additionally, unlike the PFM and SHM, PHM precursors begin differentiating into muscle before reaching the cleithrum, and the primordium includes cells that express myogenic markers during migration.[23,26] The extent of LPM contribution to the PHM is unknown.

Here, we directly compare the PHM to the better-studied PFM and the SHM. We look specifically at the time course of expression of an MMP marker, a differentiation marker, and the development of elongated, differentiated fibers. Using genetic lineage tracing, we examine the extent of LPM contribution to cells associated with the PHM as compared to the PFM. We find that the development of the PHM is distinct not only from the MMP-derived SHM and PFM, but also from axial musculature. We conclude that the PHM develops in a mode that is intermediate between axial and appendicular muscle. Evidence of a continuum of developmental modes suggests there may be more evolutionary and developmental plasticity in muscle development than hitherto realized.

2 | RESULTS

2.1 | Timing of migration and myogenesis in the sternohyoideus, pectoral fin, and posterior hypaxial muscles

Muscle precursors begin migrating in a wave from anterior to posterior. The first *lbx2*-expressing cells appear anterolateral to somite 1 by 20 hours in the path of the future SHM, and likely contribute to the SHM and esophageal muscle.[24] By 24 hours, a second migratory stream appears just posterior to the first stream, presumably originating from somite 2 and contributing to the SHM and pectoral fin

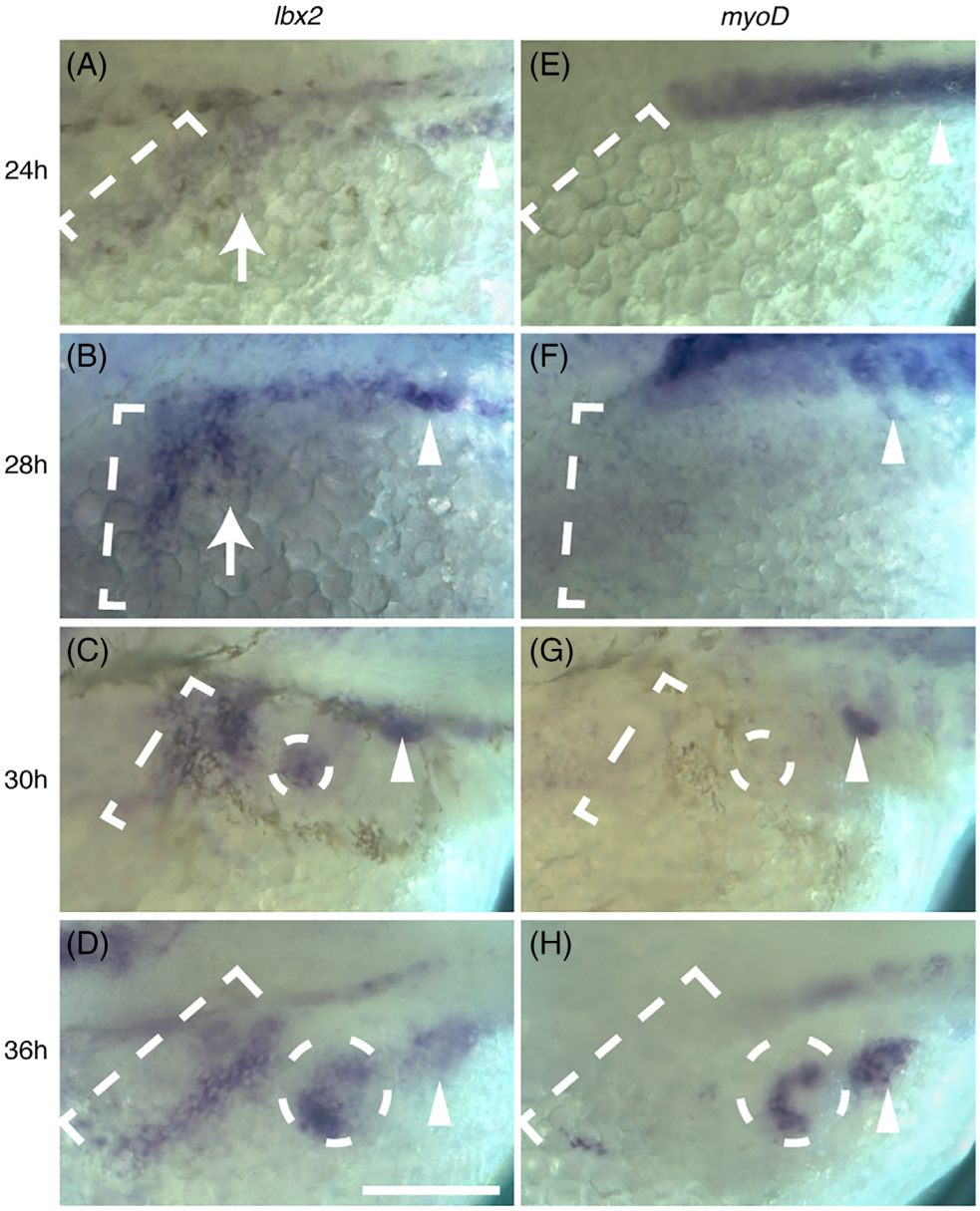

FIGURE 1 The PHM primordium differs in the timing of gene expression from both the SHM and PFM. Anterior is to the left. Arrow: second *lbx2* stream; arrowhead: PHM; dotted circle: PFM; dotted bracket: SHM. A-D, *lbx2* expression between 24 hours and 36 hours. A, dorsal view of *lbx2* expression at 24 hours, with two distinct anterior streams of MMPs. B, dorsolateral view of *lbx2* expression at 28 hours, with distinct anterior streams and dark ventral somite 5 (arrowhead). C, Dorsolateral view of *lbx2* expression at 30 hours, expressed in SHM, PFM, and PHM. D, Dorsal view of *lbx2* expression at 36 hours, expressed in SHM, PFM, and PHM. E-H, *myoD* expression between 24 hours and 36 hours. E, Dorsal view of *myoD* expression at 24 hours is restricted to the somites (arrowhead). F, Dorsolateral view of *myoD* expression at 28 hours showing the earliest evidence of PHM. G, Dorsolateral view of *myoD* expression at 30 hours showing continued expression in the PHM and still no expression in the SHM or PFM. H, Dorsal view of *myoD* expression at 36 hours showing continued PHM differentiation, and early SHM and PFM differentiation. Scale bar is 100 μm

muscle (Figure 1A).[24,25] By 28 hours, a cluster of *lbx2*-expressing cells extends from myotome 5 over the yolk in the area of the future posterior hypaxial muscle (Figure 1B). By 30 hours, a discrete spot of *lbx2* expression is visible in the fin bud, presumably originating from somites 2 to 4,[25,31] and the extension from ventral myotome 5 continues (Figure 1C). All of the muscles of interest contain *lbx2*-expressing cells at 36 hours (Figure 1D).

The first MMPs to differentiate are in the posterior hypaxial muscle. Early stages of myogenic differentiation, as indicated by *myoD* expression, begin in the somites before 24 hours[32] (Figure 1E). The expression of *myoD* in the PHM begins concurrent with *lbx2* expression at the ventral margin of myotome 5 at 28 hours (Figure 1F). Notably, *myoD* expression in the PHM at 28 hours is nearly continuous with *myoD* expression in the myotomes (Figure 1F). At 28 hours, there is no detectable *myoD* in either the PFM or the SHM (Figure 1F). At 30 hours, when MMPs have clustered in the fin bud as indicated by *lbx2* expression, they still do not express *myoD* (Figure 1G). The PFM begins to express *myoD* after 30 hours.[25] By 36 hours, all three muscles express *myoD* (Figure 1H).

2.2 | PHM fiber formation and growth

The presumptive cells of the PHM express detectable Myosin, a protein of mature muscle fibers, at around 40 hours, overlapping with the mid-to-dorsal expression of *myoD* (Figure 2A,B). The earliest PHM fibers express Slow Myosin (S58; Figure 2B). The first non-slow fiber appears by 42 hours, around the time the fin begins to express non-slow Myosin (data not shown). The first Slow Myosin expression

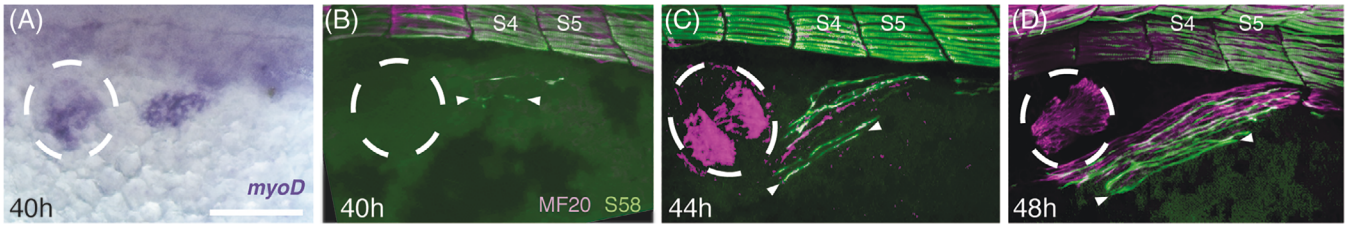

FIGURE 2 PHM differentiation begins with slow fibers elongating distant from the somites and then growing anteriorly and posteriorly. Anterior is to the left. Embryos are in dorsolateral view. Dotted circle represents the position of the pectoral fin bud. A, *myoD* expression at 40 hours showing the relative location of the earliest PHM fibers. B-D, Antibody labeling for S58 (slow myosin, green) and MF20 (all muscle myosin, magenta), arrowheads indicate the ends of the most recently formed muscle fibers at each stage, somite 4 and somite 5 are indicated (S4, S5). B, 40 hours Myosin at somites 2-6 shows the first PHM fibers separate from the axial myotome. C, 44 hours Myosin at somites 1-6 showing the PHM expanded anteriorly and posteriorly, and the second segment of the PHM forming at ventral somite 6. D, 48 hours Myosin at somites 1-6 (S4 and S5 labeled) showing fiber striations and further expansion. Scale bar in A is 100 μm. Scale bar in D, for B to D, is 50 μm

appears in a single, unstriated muscle fiber "floating" anterolateral to myotome 5, posterior to the fin bud, and discontinuous with myotome fibers (Figure 2B). The somite 6-derived PHM segment begins forming by 44 hours, also expressing Slow Myosin (Figure 2C). The somite 6-derived PHM segment appears to be closer to and more aligned with the myotome than the segment from somite 5, but there is a distinct gap separating it from the myotome (Figure 2C). By 48 hours, the SHM is expressing Slow Myosin (data not shown), while the PHM fibers are clearly striated, and extend from the ventral edge of myotome 5 to the base of the fin bud (Figure 2D). The shortest PHM fibers are those most distal from the myotome. This indicates that new fibers are added on the ventral/lateral edge of the PHM muscle (Figure 2C,D)—the PHM is growing from dorsal to ventral, in the same manner as the ventral myotome. Fibers labeled by MF20, which labels all Myosins, but not by S58, are fast fibers. These appear in each segment after slow fibers. In cross-sections through the PHM at 4d and 9.3 mm stained with phalloidin, smaller-diameter muscle fibers are located superficially and ventrally (see Figure 3B,C,E). The smallest of the superficial muscle fibers are likely to be new slow muscle fibers, while the smallest of the deep fibers are likely to be new fast fibers, contributing to the growth of this muscle, which maintains slow and fast fibers into adult life.[33] Myofibrils appear in the PFM about 4 hours after they do in the PHM, and in the SHM about 4 hours after that (Figure 2C, data not shown).

2.3 | Lateral plate mesoderm and posterior hypaxial muscle

We used *drl:creERT2*;*ubi:Switch* embryos (with *ubi:Switch* being *ubi:lox-GFP-lox_mCherry*) to genetically label cells that expressed *draculin* (*drl*) during early embryonic stages with mCherry. Early *drl* expression marks a cell population mainly comprised of LPM.[34] Activation of CreERT2 by the addition of 4OH-Tamoxifen at high stage (pregastrulation at ~3 hours of development) resulted in mCherry expression in about 70% of cells in tissues known to be primarily composed of LPM cells, such as the heart and the internal cartilage of the pectoral fin (data not shown). In the trunk myotome, there are occasional mCherry-positive muscle fibers, as seen in Mosimann et al.[34] This indicates that *creERT2* had been expressed in the precursor to one of the myoblasts that contributed a nucleus to that fiber. As the LPM does not give rise to skeletal muscle, we believe these mCherry-positive muscle fibers are a result of cells that in the blastula or early gastrula stage expressed very low levels of *drl:creERT2* but later became part of the paraxial mesoderm. All of the mCherry-positive cells within and on the surface of the PHM and PFM had the morphology of connective tissue cells (Figure 3). The quantitative and qualitative nature of the labeling was similar in all embryos examined.

High stage-activated *drl:creERT2;ubi:Switch* zebrafish show that the PFM has mCherry-positive cells both internally and superficially; the internal cells have the morphology of connective tissue cells (Figure 3). At 4 d, the anterior PHM has large numbers of mCherry-positive cells superficially (Figure 3B), while the posterior PHM has fewer mCherry-positive cells superficially (Figure 3C). There are no superficial or internal mCherry-positive cells in the trunk myotome that have the morphology of connective tissue (Figure 3D). We found no internal mCherry-positive cells in the PHM at this stage, but the PHM is only a few fibers thick (Figure 3B,C). At 9.3 mm (~7 weeks), the anterior PHM continues to have large numbers of mCherry-positive cells superficially, and a few internal mCherry cells, while the PFM is surrounded by and invested with mCherry-positive cells (Figure 3E). We conclude that some LPM cells remain on the lateral surface of the PHM, indicating a persistent somatopleure, as seen in the pectoral fin. Moreover, the PHM has far fewer internal lateral plate cells than the PFM.

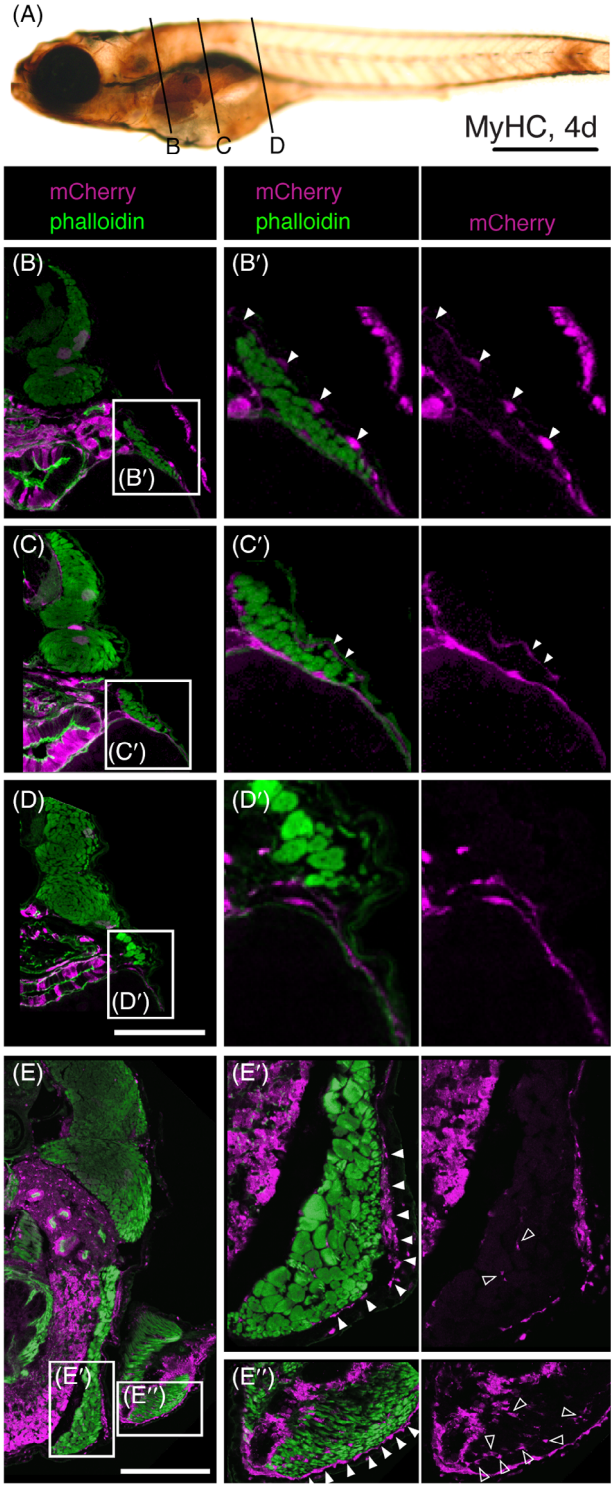

FIGURE 3 The PHM has superficial and internal mCherry-positive connective tissue cells. A, Whole-mount MyHC stain showing positions of sections shown in B-D. B-D, Transverse sections of regions indicated in A of *Tg(drl:creERT2);Tg(ubi:Switch)* embryos, labeled for mCherry (magenta) and phalloidin (green). mCherry expressing cells are derived from lateral plate mesoderm. B′-E′, magnifications of boxed regions in B-E. B-B′, Section at the level of the anterior PHM. mCherry-expressing cells are found along the entire superior surface of the PHM (arrowheads). C-C′, Section through more posterior PHM. Weak mCherry expression is seen along a portion of the superior surface of the PHM (arrowheads). D-D′, Section through trunk myotome. mCherry expression is absent from the surface of the trunk myotome. E-E″, Transverse section at the level of the anterior PHM in a 9.3 mm juvenile. mCherry positive cells are found along the entire superficial surface of the PHM (closed arrowheads). mCherry expression is present between muscle fibers in the PHM and PFM (open arrowheads). Scale bar in A is 1 mm. Scale bar in D, for B-D, is 100 μm. Scale bar in E is 500 μm

3 | DISCUSSION

We have demonstrated that the development and differentiation of the posterior hypaxial muscle (PHM) is distinct not only from other MMP-derived muscles but also from axial muscle (Figure 4). The anterior six somites all up-regulate expression of genes associated with MMPs at their ventral margins, but cells from these somites differentiate at very different times after this expression. Our work supports the hypothesis that the PHM has characteristics of both primaxial and abaxial muscles, similar to some extrinsic shoulder muscles in amniotes.[8,10] The intermediate nature of PHM development reflects its unique characteristic of bridging the somite-derived axial skeleton, and the almost entirely LPM-derived appendicular skeleton.[10,35,36]

The PHM is unlike MMP-derived muscles in the context and the timing of its differentiation. The PHM lies partially beneath a persistent somatopleure and has internal LPM-derived cells that are likely to be connective tissue cells, though these cells are fewer than seen in the PFM (Figure 3E). The PHM precursors begin differentiating almost as soon as they leave the somite, unlike the sternohyoideus (SHM) and pectoral fin (PFM) muscles that exhibit a 14-hour and 7-hour gap between first expression of *lbx2* and *myoD*, respectively. Additionally, whereas the differentiating (*myoD*-positive) muscle cells of the PHM are close to the myotome when they first appear and expand into the region of the future PHM, the differentiating (*myoD*-positive) muscle cells of the SHM and PFM appear distant to the myotome in areas where *lbx2*-positive cells have previously arrived. This implies that the PHM begins to differentiate while *lbx2*-positive cells migrate, whereas the SHM and PFM differentiate after *lbx2*-positive cells have completed their migration.

On the other hand, the PHM is not a simple ventral extension of myotome 5. The first PHM fibers begin to express Myosin after the cells have moved away from the axial myotomes, and then elongate posteriorly to contact the vertical myosepta and anteriorly to contact the cleithrum of the pectoral girdle. The PHM does not follow normal axial muscle development and growth, as the LPM-derived somatopleure is maintained around the PHM but not around axial muscle.

We propose that in zebrafish, as in *Xenopus*, the anterior somites produce an anterolateral flow of MMPs that starts in

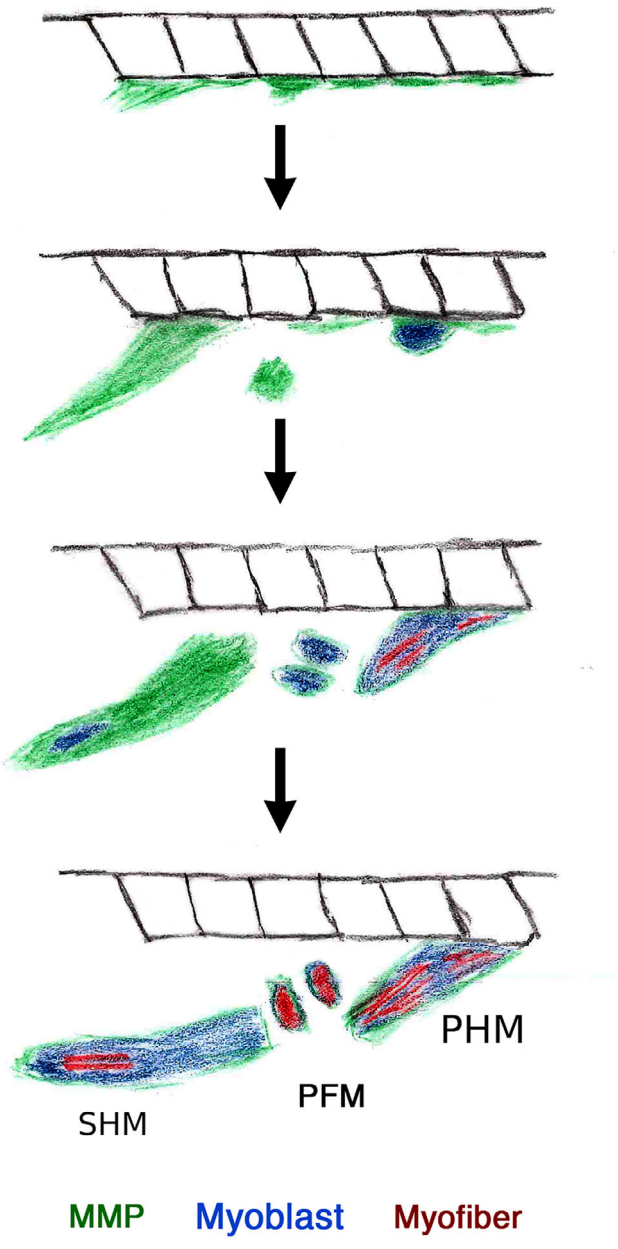

FIGURE 4 MMPs migrate first in the anterior, differentiate first in the posterior. Schematic lateral-dorsal views of the left side of the anterior trunk, during the maturation of the SHM, PFM, and PHM, from about 20 hours (top) to about 48 hours (bottom). Migratory muscle precursors (green) appear in the ventrolateral regions of somites 1-6, the first MMPs to move away from the somites are the most anterior. Migratory cells first begin the process of myogenesis (blue) in the most posterior of the paired muscles, the future PHM. Myogenesis (blue) then begins in the PFM, and later in the SHM. Elongated muscle fibers (red) first appear in the PHM, and then later in the PFM and the SHM

the anterior (Figure 4) and progresses to posterior (References 23-25,37; Figure 4). In contrast, muscle development progresses from posterior to anterior, as indicated by expression and presence of *myoD* and Myosin, (Figure 4). As the SHM, PHM, and axial muscle differentiate, slow muscle fibers appear before fast fibers and all fibers develop semi-parallel to the body axis. Moreover, all of these muscles exist in zebrafish mutants without pectoral fins,[35,38] and in amphibians prior to the development of paired appendages.[37]

In embryonic zebrafish, each of the six anterior somites is capable of generating pectoral fin muscle fibers.[23,31] This suggests that these six streams may be functionally equivalent. Lampreys have similar equivalent streams of MMPs and this was likely the condition in ancestral chordates before the evolution of paired appendages.[39] The developmental innovation of localized lateral outgrowth of the LPM, ultimately forming a fin bud, would interrupt the equivalence of the migrating MMPs. The redirection of a subset of these MMPs by the LPM would result in three different muscles—an ancestral SHM and PHM, and a derived PFM.

A central question raised by this study is how to define an MMP. Migration implies distance traveled prior to differentiation, but the cells that form the anterior segment of the PHM begin to differentiate virtually immediately after leaving somite 5. Despite this, the PHM is not a simple extension of myotome 5. In our model, the PHM precursor cells exit the ventral somite, but do not migrate as undifferentiated cells to their ultimate destination; rather, they expand as an aggregated whole. Precedents for this are muscle precursors that migrate following partial, or fleeting EMT,[18] and neural precursors that undergo neuronal chain migration.[40] Whether the PHM precursor cells undergo a complete or a partial epithelial to mesenchyme transition will require further ultrastructural and molecular analyses.

4 | EXPERIMENTAL PROCEDURES

4.1 | Zebrafish lines

All whole-mount in situ hybridizations and immunohistochemistry were done on wild-type zebrafish (*Danio rerio*) from Wesleyan University. *drl:creERT2* zebrafish (*Tg[−6.35drl:Cre-ERT2,cryaa:Venus]* ^{cz3333Tg})[34] and *ubi:Switch* zebrafish (*Tg[−3.5ubb:LOXP-EGFP-LOXP-mCherry]*^{cz1701Tg})[41] were maintained separately and offspring of a female *ubi:Switch* and a male *drl:creERT2* were used to label and follow the development of LPM. Embryos were staged and cared for using standard procedures.[42,43] All animal use was approved by the Wesleyan University Institutional Animal Care and Use Committee.

4.2 | In situ hybridization and immunohistochemistry

Fixations, whole-mount RNA in situ hybridizations, and immunohistochemistry were performed as previously described.[44] RNA in situ hybridizations were done on a minimum of 20 wild-type embryos each from 18 hours to

48 hours for *lbx2* and *myoD*. Plasmid containing *lbx2* was received from the Currie lab and confirmed with sequencing.[23] Immunohistochemistry was done on wild-type embryos from a range of 40-42 hours and 48 hours using MF20 (1:20) and S58 (1:10). *drl:creERT2;ubi:Switch* cryosections of 4d and 9.3 mm zebrafish were immunostained with Living Colors anti-dsRed (ClonTech 1:600) and phalloidin (1:100).

4.3 | Imaging

Whole-mount RNA in situ hybridized fish were imaged on a Zeiss Axioskop in PBS-Tw shortly after staining. Images were taken at multiple focal points and stitched together with ZereneStacker using the PMax and DMap algorithms, and we chose the clearer image resulting from those two options (Zerene Systems LLC 2009). Whole-mount immunostained fish embedded in 1.2% agarose and immunostained cryosections were imaged on a Zeiss LSM510 confocal microscope. Confocal z-stacks were flattened using a maximum intensity projection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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