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Evolution and Developmental Patterning of the Vertebrate Skeletal Muscles: Perspectives From the Lamprey

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The myotome in gnathostome vertebrates, which gives rise to the trunk skeletal muscles, consists of epaxial (dorsal) and hypaxial (ventral) portions, separated by the horizontal myoseptum. The hypaxial portion contains some highly derived musculature that is functionally as well as morphologically well differentiated in all the gnathostome species. In contrast, the trunk muscles of agnathan lampreys lack these distinctions and any semblance of limb muscles. Therefore, the lamprey myotomes probably represent a primitive condition compared with gnathostomes. In this review, we compare the patterns of expression of some muscle-specific genes between the lamprey and gnathostomes. Although the cellular and tissue morphology of lamprey myotomes seems uniform and undifferentiated, some of the muscle-specific genes are expressed in a spatially restricted manner. The lamprey *Pax3/7* gene, a cognate of gnathostome *Pax3*, is expressed only at the lateral edge of the myotomes and in the hypobranchial muscle, which we presume is homologous to the gnathostome hypobranchial muscle. Thus, the emergence of some part of a hypaxial-specific gene regulatory cascade might have evolved before the agnathan/gnathostome divergence, or before the evolutionary separation of epaxial and hypaxial muscles. *Developmental Dynamics 234:824–834, 2005.* © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Lampreys, a group of agnathans, are one of the extant animals retaining ancestral characteristics of vertebrates, such as the jawless mouth apparatus. Their anatomy, behavior, physiology, and evolutionary history have been summarized by Hardisty (1981). They share a basic body pattern with gnathostomes, such as a dorsal spinal cord, cranium, neural crest cells, notochord, bilateral rows of somites, and pharyngeal arches. Recent molecular phylogenetic analyses indicate that lampreys plus hagfishes are a monophyletic group branching outside the rest of the vertebrates (Mallatt and Sullivan, 1998; Kuraku et al., 1999; Takezaki et al., 2003).

Even though the life history of lampreys makes them less suitable for experimental embryology than such established models as *Xenopus* or the zebrafish, embryos can readily be obtained during the month-long breeding season in the spring. Like many lampreys, the Japanese river lamprey *Lethenteron japonicum* is anadromous. Adults live in the ocean and migrate up rivers to reproduce and die. The migrating adults can be caught and kept in tanks for a few weeks. Gametes are expressed by hand from the ripe adults and fertilized. Lamprey embryos develop relatively slowly: six hours to first cleavage, eight days to the pharyngula stage, and about one month to the ammocoete larva. After approximately four years, the ammocoete metamorphoses to an adult. Thus, it is not practical to rear embryos to reproductive maturity in the laboratory, and the animals are not suitable for genetic experiments. Even so, the large

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numbers of embryos that can be obtained have facilitated studies of early development.

Studies on the developmental mechanisms of lampreys began in the mid-1980s (Nakao and Ishizawa, 1987a–d; Langille and Hall, 1988). Many genes have been isolated from *L. japonicum* (Ueki et al., 1998; Ogasawara et al., 2000; Murakami et al., 2001; Kusakabe et al., 2004; Takio et al., 2004) and from the sea lamprey *Petromyzon marinus* (Tomsa and Langeland, 1999; Neidert et al., 2001; McCauley and Bronner-Fraser 2002, 2004), and their patterns of expression have been documented. The effects of exogenous secretory proteins have been analyzed using impregnated beads implantation followed by detection of tissue-specific gene expression (Shigetani et al., 2002). Microinjection of foreign genes into fertilized eggs revealed the function of gene regulatory regions driving reporter genes such as that for green fluorescent protein (GFP; Kusakabe et al., 2003). Cell fate tracing studies using tracer dyes such as DiI have revealed similarities and differences in the cell lineages of neural crest and mesodermal cells of early embryos compared with those of gnathostomes (Horigome et al., 1999; Shigetani et al., 2002; McCauley and Bronner-Fraser, 2003; Kuratani et al., 2004).

Comparisons of gene expression patterns between lamprey and gnathostomes have provided knowledge on the origins of evolutionary novelties, such as the jaw (reviewed by Shigetani et al., 2005; Kuratani, 2004), brain compartmentalization (Murakami et al., 2001, 2004), and endocrine systems (Uchida et al., 2003). One prominent example is the developmental and evolutionary origin of jaws. Lamprey larvae have upper and lower lips, the developmental origins of which can be found in embryonic pharyngeal arches. The mesenchyme of the upper and lower lips is derived from both premandibular and mandibular domains, which is not the case in the jaws of gnathostomes (Shigetani et al., 2002; Kuratani et al., 2004). Thus, the morphology of the lamprey oral apparatus is not only plesiomorphic, but results from lamprey-specific and independent developmental evolution.

Another remarkable and yet unexplored feature of lampreys is the unique morphology of its skeletal musculature, which might be developmentally related to the more recognizable characteristic of this animal: the lack of paired fins (Janvier, 1996). Based on our current molecular and embryological approaches, we have established a hypothetical scenario for the evolutionary pathway of the vertebrate skeletal muscles. In the following sections, we will discuss the importance of the lamprey in the study of the molecular developmental evolution of complex vertebrate skeletal muscles.

ESTABLISHMENT OF THE COMPLEX MORPHOLOGY OF GNATHOSTOME SKELETAL MUSCLES

Myotomes in gnathostomes are divided dorsoventrally into two parts, the "epaxial" region (dorsal to the notochord) and "hypaxial" region (ventral to the notochord), separated by a sheet of connective tissue called the horizontal myoseptum (Fig. 1A; Goodrich, 1930; Romer and Parsons, 1978). The hypaxial muscles give rise to the interconstal and body wall muscles. In addition, in four regions along the anteroposterior axis (Fig. 1B), the hypaxial part of the myotome also gives rise to migratory muscle precursors (MMPs; Alvares et al., 2003). In the occipital region, these MMPs give rise to the tongue muscle (Fig. 1B). In the cervical region, they give rise to the cucullaris muscles in fish (or the trapezius in tetrapods), the infrahyoid muscles, and the diaphragm. At the fore- and hindlimb levels of tetrapods, the MMPs give rise to the fin or limb muscles. Epaxial and hypaxial regions are innervated by dorsal or ventral rami of the spinal motor nerves, respectively (Fig. 1A).

Once specified, MMPs undergo epithelial-mesenchymal transition and migrate a long distance toward the sites where they differentiate into mature myotubes. The motor neuron innervation of these muscles corresponds to their origin; for example, the cucullaris/trapezius muscles and the diaphragm, both of which derive from the cervical somites, are innervated by the cervical spinal nerves. The cucullaris/trapezius muscle is also innervated by the accessory nerve. The rest of the hypaxial myotomes retain their epithelial status and give rise to the intercostal and body wall muscles.

Molecules and gene functions involved in the specification of MMP, delamination, and migration have been intensively studied in mammals (reviewed by Dietrich, 1999; Birchmeier and Brohmann, 2001). Briefly, within the somite *Pax3* acts as an upstream regulator of hypaxial muscle development. *Lbx1*, regulated by *Pax3*, is expressed in hypaxial myotomes only at the level where MMP cells appear (occipital, cervical, and limb levels) and is required for the delamination of MMPs (Gross et al., 2000). This localized activation of *Lbx1* is likely to be dependent on the differential expression of homeobox (*Hox*) genes along the rostrocaudal axis (Alvares et al., 2003).

Another *Pax3* target is the *c-Met*-tyrosine kinase receptor expressed in the lateral dermomyotome of all somites. Its ligand is SF/HGF (scatter factor/hepatocyte growth factor) expressed in the mesenchymal cells in the environment where MMPs migrate. Mutations in the loci encoding for these molecules cause disappearance of MMP-derived muscles in the fetus (Tremblay et al., 1998; Gross et al., 2000; Bredt et al., 1995). For example, the *Pax3* mutant *Splotch* mice lack tongue muscle, diaphragm, and appendicular muscle, and many elements of shoulder muscles (Tremblay et al., 1998).

Cognates of *c-Met, HGF*, and *Lbx1* have now been reported from nontetrapods, and the evolution of developmental mechanisms for MMP formation has been discussed (Neyt et al., 2000; Haines et al., 2004). In both the teleost zebrafish and the chondrichthyan dogfish, muscles of the paired fins derive from hypaxial myotomes (Neyt et al., 2000). Remarkably, different modes of myotome extension seem to be utilized in these two animal groups. As in amniotes, in teleosts, fin muscle precursors delaminate from the epithelial somites and migrate as mesenchymal cells, as in amniotes. Moreover, teleost fin muscles seem to be specified by similar molecular mechanisms to those in tet-

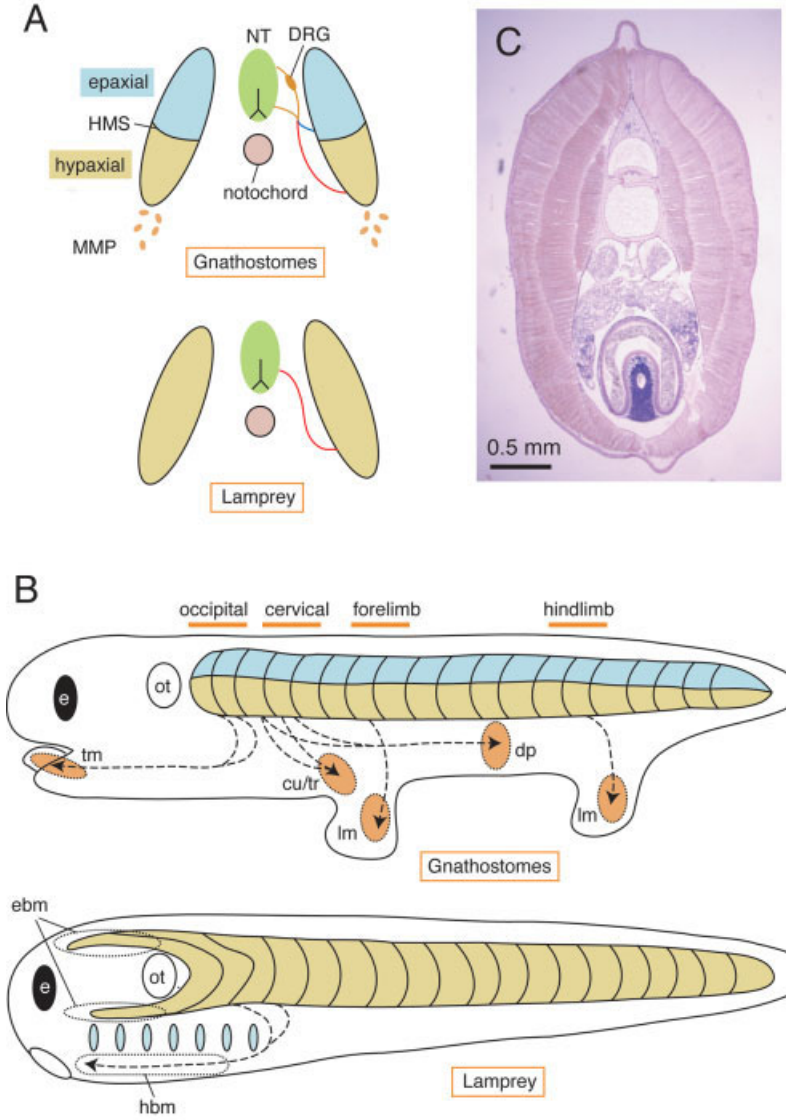

Fig. 1. A schematic representation comparing the skeletal muscle patternings between gnathostomes and the lampreys. **A:** A transverse view. The gnathostome myotomes (top) are morphologically divided into the epaxial (pale blue) and hypaxial (pale yellow) regions, which are innervated by dorsal (blue) and ventral (red) rami of the spinal cord, respectively. In contrast, there is no such distinction in lamprey myotomes (bottom). DRG, dorsal root ganglion; HMS, horizontal myoseptum; NT, neural tube; MMP, migratory muscle precursors. **B:** A lateral view. In gnathostomes (top), some of the hypaxial muscle cells, originating from the four different levels along the anteroposterior axis (indicated by bars at the top), undergo extensive migration (indicated by broken arrows) toward the periphery where they differentiate into the tongue muscle (tm), cucullaris or trapezius muscle (cu/tr), diaphragm (dp), and limb muscles (lm). The lamprey (bottom) lacks most of these muscles but possesses a hypobranchial muscle (hbm), which resembles the vertebrate tongue muscles. Epibranchial muscles (ebm) have a peculiar morphology, extending rostrally from the anterior myotomes. Ot, otic vesicle. **C:** A transverse section of a 55-mm ammocoete larva at the trunk region. Note that there is no horizontal myoseptum.

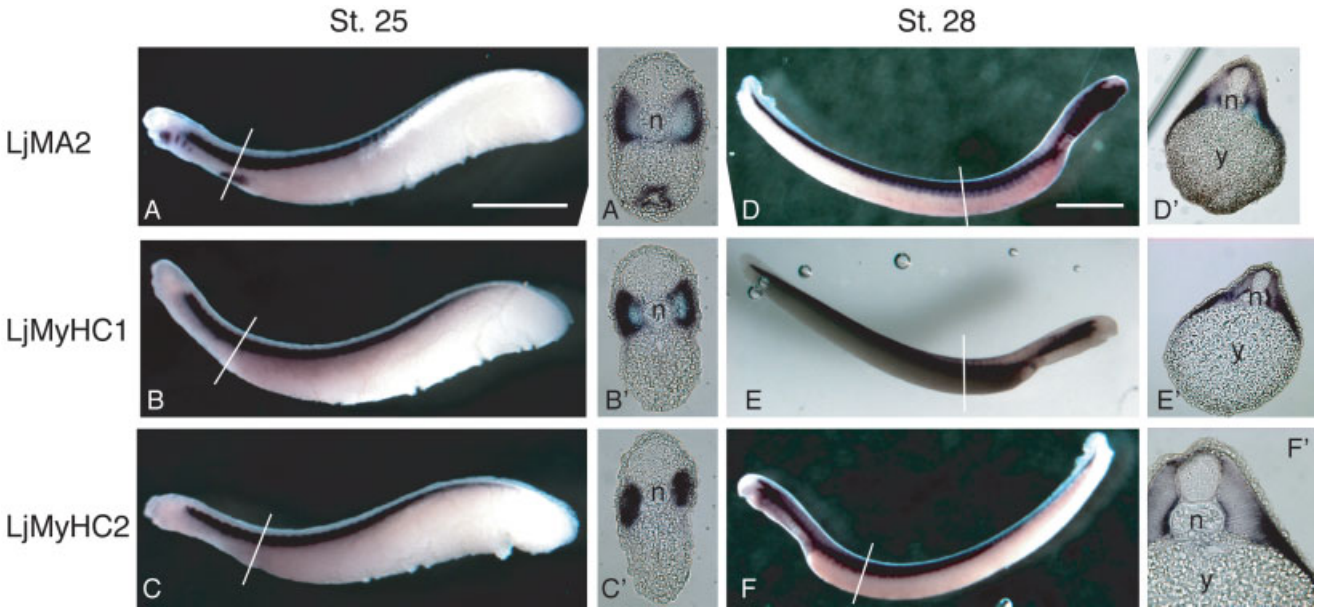

Fig. 2. Expression patterns of the contractile protein genes in *L. japonicum*, detected by in situ hybridization (Kusakabe et al., 2004). The probes are for the actin gene *LjMA2* (A, D, G) and for the myosin heavy chain genes *LjMyHC1* (B, E) and *LjMyHC2* (C, F). **A–C:** Lateral views of stage 25 embryos. **D–F:** Lateral views of stage-28 embryos. Scale bar = 1 mm. **A'–F':** Transverse sections corresponding to embryos in A–F at the levels indicated by lines. Stages are according to Tahara (1988).

rapod limb muscles, in which *Lbx1, c-met*, and *HGF* are involved (Neyt et al., 2000; Haines et al., 2004). In contrast, Chondrichthyan fins receive an epithelial extension from the hypaxial myotomes (Neyt et al., 2000), which is similar to the developing mammalian body wall and intercostal muscles. Consistently, *Lbx1* is not expressed in the fin muscle precursors in the dogfish (Neyt et al., 2000). Thus, the hypaxial muscle in ancestral vertebrates might have migrated as an epithelial extension, and the mesenchymal state might have been recruited only by MMP cells after the divergence of the Chondrichthyes from other vertebrates. It has also been speculated that the molecular mechanisms for mesenchymal MMP were established before the Sarcopterygian radiation (Neyt et al., 2000).

It is of particular interest to clarify the order of evolutionary events that may have led to the emergence of complex functions and morphologies of the skeletal muscles found in gnathostomes. In this regard, the emergence of the epaxial/hypaxial distinction of myotomes, and the appearance of mesenchymal MMP cells might be the major key events. In gnathostome embryogenesis, MMP specification apparently takes place only after the specification of the future epaxial/hypaxial domains, which first becomes apparent by the localized expression of *Pax3* in myotomes (Alvares et al., 2003). One of the possible evolutionary scenarios has been that, during evolution, the epaxial/hypaxial distinction of myotomes might have appeared before the emergence of MMPs in the hypaxial portion (Galis, 2001; Neyt et al., 2000; Haines and Currie, 2001; Hollway and Currie, 2003). Protochordates, such as amphioxus lack paired appendages and are considered to have no homolog of migratory population of hypaxial muscles (see Fig. 5B; Holland et al., 1999). Lampreys also lack paired appendages but are closer than amphioxus to the gnathostomes. Therefore, the molecular mechanisms controlling the skeletal muscle pathway in lampreys might be similar to those for skeletal muscle development in the ancestral vertebrate. Such study should also indirectly elucidate which molecular mechanisms are really new and unique to gnathostomes as the developmental background for gnathostome synapomorphies, as seen in the hypobranchial and cucullaris muscles.

THE LAMPREY AS A KEY TO UNDERSTANDING VERTEBRATE MUSCLE EVOLUTION

The lamprey trunk musculature derived from myotomes has a peculiar structure both at cytological and morphological levels. Myotomes of adult lampreys are W-shaped and each is folded behind the one in front, so that in a transverse section, layers of anteroposterior series of myotomes look like concentric circles surrounding the notochord and the spinal cord (Fig. 1C; Peters and Mackay, 1961). Since lamprey myotomes do not have a horizontal myoseptum, there is no epaxial/hypaxial distinction (Fig. 1A). They receive motor innervation only from the ventral roots of spinal neurons; the dorsal roots do not contain somatic motor neurons. Adult lampreys lack musculature that functionally corresponds to the MMP derivatives in gnathostomes. There are no trapezius-like muscles nor any hint of paired fin elements throughout development (Fig. 1B; also see Kuratani et al., 2002). No accessory nerve, which innervates the cucullaris/trapezius muscles in gnathostomes, exists in lampreys (see Kuratani et al., 1997). They do not possess the real tongue and its associated muscles, which in amniotes are innervated by the hypoglossal nerve. What appears like a "tongue" in lampreys is not homologous to the gnathostome tongue, as it is derived from the mandibular arch and innervated by the trigeminal nerves (Yalden, 1985). Thus, the somite-derived skeletal muscle pattern in the adult lamprey is much simpler than that in gnathostomes. Especially, the lack of pectoral fin girdle and cucularis muscle suggests that this animal group has not yet established the "neck region" comparable to that in gnathostomes (Matsuoka et al., 2005).

The arrangement of lamprey muscle fibers, both in the larvae and in adults, is also unlike that found in gnathostomes. In aminiotes, many skeletal muscles consist of intermingled mixture of two different main types of muscle fibers, "slow" and "fast," which have different contractile properties. The two fiber types are usually identified using antibodies to detect a specific isoform of myosin heavy chain (MyHC) expressed in each muscle fiber. Lamprey adult myotomes also consist of two types of myofibers, parietal and central, which are considered to correspond electrophysiologically to the slow and fast fibers of gnathostome skeletal muscles, respectively (Teräväinen, 1971). Staining of myosin ATPase has supported this notion (Meyer, 1979). However, there has been no report of detection of these fiber types using slow or fast muscle specific antibodies, or localized expression of the gene for MyHC. Unlike the situation in gnathostomes, these fibers are organized into "muscle units" (Peters and Mackay, 1961), in which about four thin layers of central fibers are sandwiched by two single layers of parietal fibers. Within myotomes, muscle units are horizontally arranged, stacking one above the other.

In larval lampreys, only a single type of muscle fibers seems to exist, arranged in a different configuration from that in the adults (Nakao, 1976). In each larval myotome, muscle cells are organized in triangular lamellae stacked horizontally. The lateral surface of the myotomes is covered by "lateral cells": flattened cells forming a thin layer (Nakao, 1976). A similar muscle fiber configuration can be found in the cephalochordate, amphioxus, in which the myotomes are comprised of lamellae (Peachy, 1961). In this animal, however, what appears to be the ventral nerve root that connects the muscle lamellae and the surface of the spinal cord is actually a cytoplasmic process extending from the muscle, not an axon from the central nervous system (Flood, 1966). Thus, although the myotomal morphology of larval lampreys might superficially resemble that of amphioxus, the relationships of motor neurons and muscles in lampreys do not correspond to those in cephalochordates, nor to the main group of vertebrates.

Zebrafish mutants in which the myoseptum does not form may give insights into how the partitioning of

gnathostome myotomes into hypaxial and epaxial portions evolved. Zebrafish fast and slow muscle fibers are spatially segregated and do not intermingle with each other (Devoto et al., 1996). Slow muscle fibers form a layer covering the lateral aspect of the myotomes, and fast fibers occupy the rest. This situation cannot be found in ammniotes, and might represent a diverged characteristic found only in teleosts. However, several mutant strains of zebrafish exhibit the skeletal muscle morphology that is reminiscent of lampreys. Such strains have mutations in molecules involved in the sonic hedgehog (Shh) signaling pathway, a molecular cascade known to induce slow muscle precursors (adaxial cells) lateral to the notochord.

One such strain is *yot* (*you-too*), in which *Gli2*, a downstream regulator of the Shh cascade, is mutated (Zeller et al., 2002). In *yot* embryos, no horizontal myoseptum forms, and thus no epaxial/hypaxial distinction of myotomes appear: a configuration similar to the lamprey myotomes. Moreover, the primary motor neurons that should innervate each of the epaxial and hypaxial portions of myotomes do not extend their axons normally (Zeller et al., 2002). Another remarkable characteristic of *yot* myotomes is the absence of slow muscle cells and their precursors, the adaxial cells. The resultant skeletal muscle consists only of fast muscle cells. These observations imply that, at least in teleosts, the establishment of epaxial/hypaxial division and the formation of slow muscle precursors are closely linked with each other in a developmental regulatory system.

The next question is whether the lampreys have the prototype developmental regulatory mechanism underlying the epaxial/hypaxial patterning of myotomes in gnathostomes. First, possible marker genes for embryonic lamprey myotomes were searched for, in order to detect multiple types of cells expressing different structural protein isoforms such as MyHCs (Kusakabe et al., 2004). Multiple muscle-specific genes of *L. japonicum* were characterized and were compared with that of gnathostome counterparts in the aspects of the phylogenetic relationships and expression domains of each gene, as discussed below.

EXPRESSION OF MUSCLE-SPECIFIC GENES IN THE DEVELOPING LAMPREY EMBRYO

In the lamprey, muscle-specific genes such as actin (*LjMA2*) and myosin heavy chain genes (*LjMyHC1* and *LjMyHC2*) are strongly expressed in developing skeletal muscles, analyzed by whole mount in situ hybridization (Kusakabe et al., 2004; Fig. 2A–C). It has been suggested that the regulatory mechanism for these contractile protein genes might be conserved between agnathans and gnathostomes (Kusakabe et al., 2003). When fertilized lamprey eggs were injected with the fusion gene constructs consisting of the 5′ regulatory sequence of medaka actin genes and the GFP coding sequence, GFP was specifically expressed in the developing skeletal muscles (Kusakabe et al., 2003). The GFP expression in each skeletal muscle follows exactly the temporal order of endogenous *LjMA2* expression, starting in developing myotomes, next in the cardiac muscle, and lastly in pharyngeal arch muscles, the derivative of the cephalic mesoderm. We expected that lamprey would possess regulatory mechanisms that differentially control genes encoding for muscle-specific protein isoforms, which in gnathostomes reflects the complexity of the skeletal muscle patterning (Marcucio and Noden, 1999).

Recently, we found that these contractile protein genes were expressed in restricted regions along the dorsoventral and mediolateral axis within the lamprey myotomes. At stage 25 (Tahara, 1988), in transverse sections, transcripts of *LjMA2* and *LjMyHC1* are detected in the lateral halves of the myotome, the dorsal edges adjacent to the neural tube, and the ventral edges (Fig. 2A' and B'). They are not expressed in the medial part of the myotome adjacent to the notochord. These expression patterns might reflect signaling between the myotomes and the notochord. The lamprey myotomal cells neighboring the notochord might represent to the lamprey adaxial cells, by analogy to the zebrafish adaxial cells.

Another MyHC gene, *LjMyHC2*, is expressed in all the myotomal cells including the adaxial cells (Fig. 2C'). At stage 28, *LjMA2, LjMyHC1*, and *LjMyHC2* are expressed specifically in the thin layer of adaxial cells but not in the deeper region of the somites (Fig. 2D'-F'). Transcripts are also seen in the ventrolateral lips of the myotomes, and a small population of cells at the dorsal extremities, but not in the deep region lateral to the adaxial cells or dorsomedial to the ventrolateral lips. The adaxial expression later disappears but the ventrolateral expression persists (data not shown). These observations indicate that the adaxial regions and the ventrolateral and dorsomedial extremities of the developing lamprey myotomes are under different transcriptional regulatory systems from those of the deep regions of the myotomes. This might be a similar situation to the early myotome configuration of zebrafish.

Further evidence for differential regulation of these lamprey contractile protein genes is found in the head region. The major muscular elements of the lamprey head region include the pharyngeal arch muscles associated with gill slits, and the extraocular muscles, both of which are presumably derived from unsegmented cephalic mesoderm (Koltzoff, 1901; Kuratani et al., 1999). They express the actin gene *LjMA2* (Kusakabe et al., 2004) and acetylcholinesterase (Kuratani et al., 1999), but these expressions start much later than in trunk myotomes, as in the chick (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002). Moreover, these muscles do not express either *LjMyHC1* or *LjMyHC2* (Kusakabe et al., 2004), both of which are abundantly expressed in myotomes.

The lamprey head contains another group of muscles, the epi- and hypobranchial muscles (EBM and HBM), derivatives of anterior trunk myotomes (Fig. 1B). EBM, located dorsal to the pharyngeal arches, forms from the anterior myostomes that become wedge-shaped as they elongate rostrally (Fig. 1B; Kuratani et al., 1999; Kusakabe et al., 2004), and eventually covers the dorsal and rostral aspects of the head. The segmentation patterns of EBM and HBM of lampreys apparently correspond to the locations

of gill pores, and they have been mistaken as evidence for head mesoderm segmentation (Sewertzoff, 1916; Kuratani et al., 1997, 1998). Accordingly, the EBM and HBM express the same set of contractile protein genes as the trunk myotomes, i.e., all of *LjMA2, LjMyHC1*, and *LjMyHC2* (Kusakabe et al., 2004). On the other hand, the HBM undergoes a different mode of differentiation from that of the EBM and postotic myotomes (Kuratani et al., 1997). The precursor cells of the HBM originate from the ventral region of the anterior myotomes and migrate ventrally along the caudal aspect of the pharyngeal arches (Neal, 1897; Kuratani et al., 1997). When they reach the lateral aspect of the arches, HBM cells undergo segmentation and start to express the genes for contractile proteins such as LjMA2 (Kusakabe et al., 2004). The origin and the migration pattern of HBM precursors are very similar to that of the gnathostome MMP, especially to that of the tongue muscle precursors. Other evidence of homology of lamprey HBM to the gnathostome tongue muscle is that HBM is innervated by the nerve termed the hypoglossal nerve based on its morphological position associated with the head/trunk interface (Kuratani et al., 1997).

THE LAMPREY PAX3/7 GENE IS EXPRESSED IN THE PROSPECTIVE DERMOMYOTOME AND MIGRATING HYPOBRANCHIAL MUSCLE

Although the morphology and behavior of HBM precursors appear similar to that of MMPs, almost nothing is known about the molecular mechanisms underlying HBM development. Among the few developmental transcription factor–encoding genes reported from lampreys, a member of the *Pax3/7* family is likely to be related to muscle patterning.

In mammals, *Pax3* plays important roles in multiple steps of skeletal myogenesis. Early in somitogenesis, it is expressed in the entire paraxial mesoderm and then dermomyotome (Goulding et al., 1991, 1994). Later, *Pax3* expression is restricted to the hypaxial region of the myotome. Within the hypaxial myotomes, *Pax3* is required for MMP specification (Tremblay et al., 1998; Relaix et al., 2003). The mouse mutant strain *Splotch* has spontaneous mutations in the *Pax3* locus, causing the loss of *Pax3* expression in the dermomyotome (Franz et al., 1993). *Splotch* mice do not develop any of the MMP-derived muscles, including the limb muscles, the trapezius, or the diaphragm. The hypoglossal cord fails to form, resulting in the absence of pharyngeal and tongue muscles (Auerbach, 1954).

The lamprey *Pax3/7* gene, designated here as "*LampPax3/7*," belongs to the vertebrate *Pax3/Pax7* clade (McCauley and Bronner-Fraser, 2002). This gene was originally named *"LampPax-7,"* since its amino acid sequence is most closely related to the gnathostome *Pax7* genes. However, it is expressed in the otic and trigeminal placodes (McCauley and Bronner-Fraser, 2002), an expression pattern more similar to mammalian *Pax3* than *Pax7* (Stark et al., 1997). To clarify whether the *LampPax3/7* gene is involved in muscle patterning, we traced its expression in developing embryos using in situ hybridization.

In addition to its expression in neural tube and neural crest derivatives, it is strongly expressed in the somites of stage 22 lamprey embryos (Fig. 3A). At stage 25, *LampPax3/7* is expressed in the lateral aspect of the somites, more intensively in the ventral edge (Fig. 3B–D). Later in development, it is expressed exclusively in migrating HBM (Fig. 3E,F). The expression in HBM continues until the migration is complete (Fig. 3G). At stage 30, myotomal expression is restricted to the thin layer of cells at the lateral surface (Fig. 3H,I), which might correspond to the location of the "lateral cells" (Nakao, 1976). The HBM has completed its rostral migration at this stage, but still maintains the expression of *LampPax3/7* (Fig. 3I, arrowhead).

During this process, *LampPax3/7* expression appears to follow the migration pathway from the ventral edges of anterior somites. In contrast to the structural protein genes such as *LjMA2*, *LampPax3/7* is expressed while the HBM precursors are still migrating and thus are still essentially undifferentiated. This suggests it is involved in long-distance migration of the HBM. The HBM-specific expression of the *LampPax3/7* gene is consistent with the homology of this muscle to the gnathostome tongue muscle, or to the hypobranchial series as a whole (including the infrahyoid and possibly the diaphragm in mammals).

The expression pattern of *LampPax3/7* in the lateral surface of embryonic myotomes is very similar to that of the *Pax3* gene in mammalian and amphibian dermomyotomes (Goulding et al., 1994; Martin and Harland, 2001; McCauley and Bronner-Fraser, 2002; Grimaldi et al., 2004). Because of the similarities in embryonic structure between lampreys and the urodele amphibian *Xenopus*, the expression domain in trunk mesoderm is easily compared between these two animals. Like *Xenopus Pax3*, *LampPax3/7* is expressed more strongly in ventral than in dorsal regions, and more strongly in the trunk than in tail regions (data not shown; Grimaldi et al., 2004). Moreover, it has been suggested that active myogenesis occurs in the dorsomedial and ventrolateral edges in the *Xenopus* dermomyotome (Grimaldi et al., 2004). Also in zebrafish, the dorsal and ventral extremities of myotomes, designated "progress zones," have been suggested as the potential source of new myofibers added during larval growth (Barresi et al., 2001). These regions largely match the *LjMA2*- and *LjMyHC*s-positive domain in the lamprey myotomes from stage 28 onwards (Fig. 2D'–F'). The *LampPax3/7*-positive lateral region of the lamprey myotome might represent the latent dermomyotome-equivalent domain (Fig. 4), and the lamprey somites might contain multiple myogenic foci that correspond to those in gnathostomes.

Although either dermatome or sclerotome has been found in amphioxus, this animal might have evolved the prototypic subdivision of dorsal mesoderm. *AmphiZic*, a member of Zic gene family, a closely related family to Gli, is expressed in the amphioxus dorsal mesoderm and ectoderm during gastrulation (Gostling and Shimeld, 2003). After neurulation, this gene is expressed in a dorsolateral sheet of cells, a region similar to vertebrate dermatome in position but with yet unclear fate and function. In vertebrates, *Zic1* is expressed in the dorsal dermomyotome and sclerotome, and is

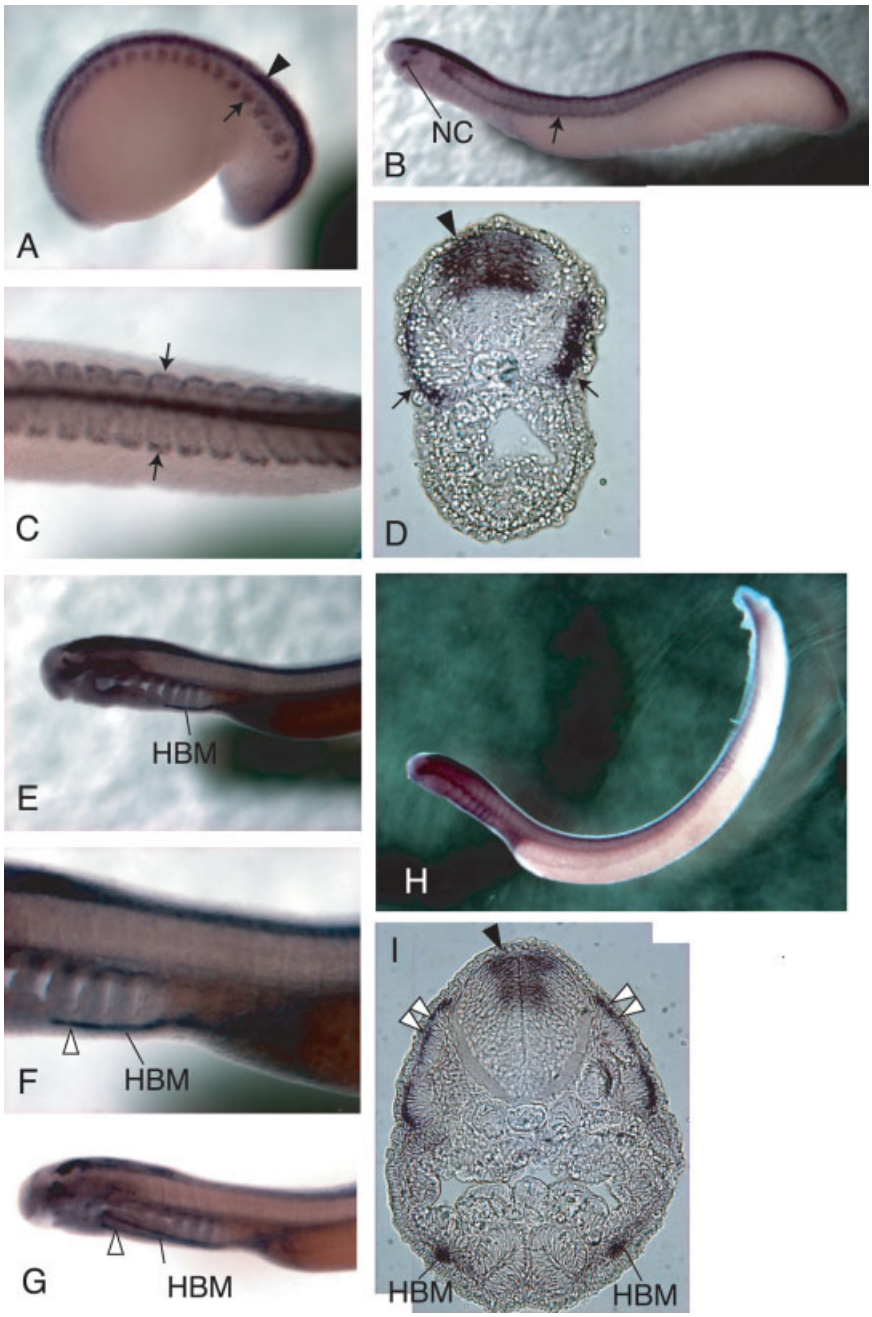

Fig. 3. Expression of *LampPax3/7* during development, as detected by in situ hybridization. *L. japonicum* embryos were hybridized with the RNA probe for the region spanning from paired domain to homeodomain of *LampPax3/7* (McCauley and Bronner-Fraser, 2002). **A:** A stage-22 embryo. *LampPax3/7* is strongly expressed in the brain, neural tube (black arrowhead), and the somites (arrow). **B:** At stage 24, the signal is stronger in ventral aspect of the somites (arrow). The trigeminal stream of neural crest cells (NC) is also visible. **C:** A dorsal view of the embryo in C. The somitic signal is stronger in the ventral edge (arrow). **D:** A transverse section of the pharyngeal region of a stage-25 embryo. The *LampPax3/7* signal is evident in the dorsomedial region of neural tube (black arrowhead), and in the ventrolateral edges of the somites (arrows). **E:** The head of a stage-28 embryo. **F:** Higher magnification of the embryo in F. *LampPax3/7* is expressed specifically in the migrating hypobranchial muscle (HBM) precursor cells. The white arrowhead indicates the leading edge of the HBM. **G:** At stage 29, *LampPax3/7* is still expressed throughout the HBM, which has completed migration. **H:** A stage-30 early ammocoete larva. **I:** A transverse section of the pharyngeal region of a stage-30 larva. *LampPax3/7* is expressed in the dorsomedial region of the neural tube (black arrowhead) and in a thin layer at the surface of myotomes (double white arrowhead), and in the hypobranchial muscles (HBM).

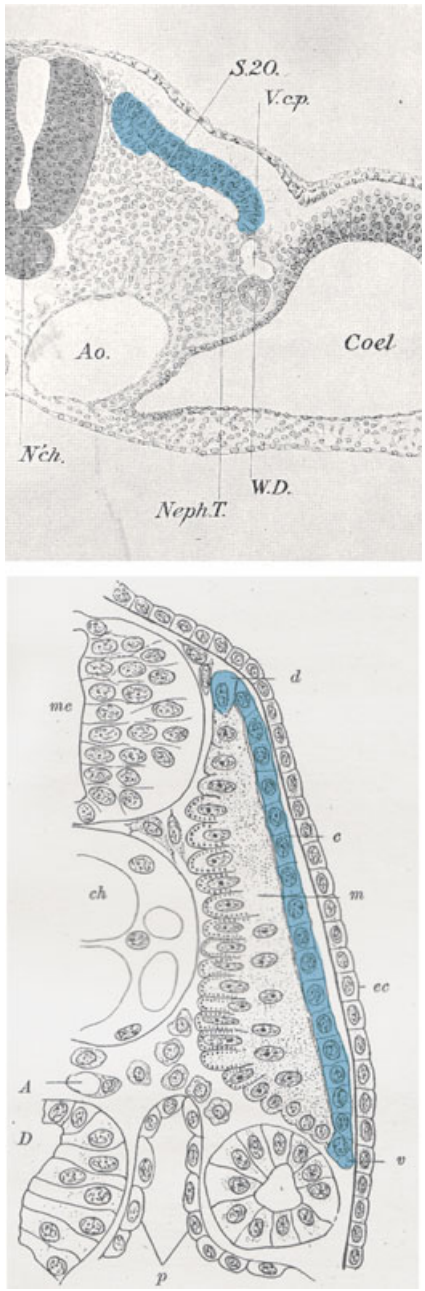

Fig. 4. The lamprey "lateral cells" might be homologous to the dermomyotome in the amniotes. In chick (left), the collection of epithelial somitic layers located beneath the epidermis is called demomyotome, and expresses *Pax3* and *Pax7*. In lamprey embryos at late stages and larvae (right), "lateral cells," a single sheet of cells covering the myotome (m), express *LampPax3/7*. Modified from Lillie (1919) and Maurer (1906).

later eliminated from the myotome (Nagai et al., 1997; Rohr et al., 1999), Thus, the identity of the dorsolateral somitic compartment, which later evolved into the vertebrate dermatome, might have been acquired in protochordates, before the emergence of agnathans.

PAX3/7 EXPRESSION PATTERNS SUGGEST AN ANCESTRAL GENE FUNCTION IN THE LAMPREY MESODERM

In mammals, the *Pax3* and *Pax7* genes, expressed in similar spatiotemporal patterns, have multiple and overlapping functions. Both genes have important roles in neural tube formation (Mansouri and Gruss, 1998), neural crest development (Le Douarin and Kalcheim, 1999), paraxial mesoderm development (Schubert et al., 2001; Goulding et al., 1991), and adult skeletal muscle development (Relaix et al., 2005). No defect in embryonic muscle development has been found in *Pax7*-single mutant (Mansouri et al., 1996; Relaix et al., 2005), suggesting a functional redundancy between *Pax3* and *Pax7* in somitic muscle formation. Recently, it has been reported that the MMP-derived muscles are lost in a transgenic mouse line in which *Pax3* is replaced by *Pax7*, although other developmental events in which *Pax3* and *Pax7* are involved are not significantly affected (Relaix et al., 2004). In this transgenic line, *Pax7* could substitute the function of *Pax3* in the dorsal neural tube, in the neural crest cells, and in the forming somites, but not in MMP specification. It seems likely that, after the evolutionary duplication of Pax3 and Pax7, the original functions of the ancestral *Pax3/7* appear to have been subfunctionalized so that the role for MMP patterning was retained only in *Pax3*, but not in *Pax7* (Relaix et al., 2004).

From molecular phylogenetic and developmental studies on various chordate species, the mesodermal function of *Pax3* is thought to have been recently acquired in evolution. In protostomes, ascidians, and amphioxus, a single ancestral gene for both *Pax3* and *Pax7*, designated *Pax3/7*, is retained as the cognate of vertebrate *Pax3* and *Pax7* (Fig. 5A). The ascidian *Pax3/7* gene is expressed only in the neural tube, and not in mesoderm (Wada et al., 1997). The amphioxus *Pax3/7* gene is expressed in both the central nervous system and the myogenic lineage of the mesoderm (Holland et al., 1999). Thus, during deuterostome evolution, mesodermal expression of the *Pax3/7* gene might have been acquired before the divergence of the cephalochordate lineage (Fig. 5A). However, because extant amphioxus species do not possess highly differentiated somatic skeletal muscles, it is unknown when the vertebrate *Pax3* function in MMP specification, which cannot be compensated by *Pax7*, was acquired in chordate evolution.

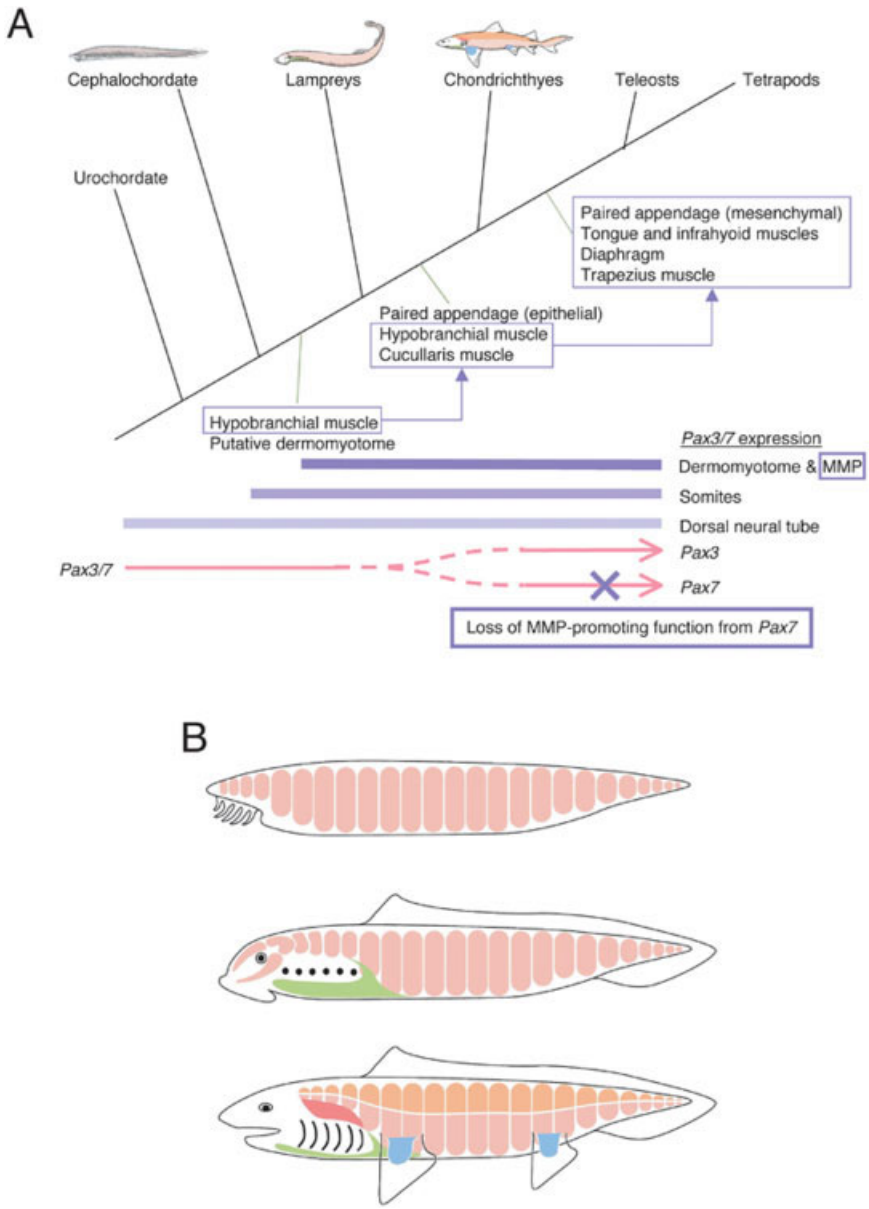

Fig. 5. A hypothetical scenario for vertebrate skeletal muscle evolution. **A:** A phylogenetic tree indicating the probable timing for acquisition of the morphological characteristics of vertebrates, in relation to the changes in the domains of expression of the *Pax3/7* genes (pale blue lines). The boxed characters are those in which mesenchymal modes of myoblast migration are involved. Changes in the structure of the *Pax3/7* family are indicated by pink lines and arrows. Here, the broken lines mean that the exact timing of gene duplication remains unclear. **B:** Diagrammatic illustrations of the representative animals are shown with colors indicating different developmental modes recruited by somitic skeletal muscles. In amphioxus (top), all the somitic muscle cells develop in situ (pink). The agnathan lampreys (middle) have acquired the hypobranchial muscle (pale green) that undergoes extensive migration, but there is no epaxial/hypaxial distinction. The horizontal myoseptum had been acquired before the divergence of Chondrichthyes (bottom), and the dorsal half of the myotomes was established as the epaxial domain (orange). Now the hypaxial muscle cells give rise to the paired fin muscle (pale blue) and the cucullaris muscle (red).

The lamprey provides an important key for this problem (Fig. 5A). The probable involvement of *LampPax3/7* in the development of the HBM, homologous to the gnathostome tongue muscle, suggest its function in the MMP might have already been present in the ancestral *Pax3/7* gene before the agnathan/gnathostome divergence. This MMP-regulating function might have been lost from the *Pax7* gene independently after the gene duplication of *Pax3/7*. However, there are more questions on the temporal relationships between these molecular changes and the divergence of species. First, it is not known when the *Pax3/7* gene duplicated after the evolutionary divergence of the cephalochordates. It has not been determined whether *LampPax3/7* is the unique member of the *Pax3/7* family (McCauley and Bronner-Fraser, 2002), and no *Pax3/7* gene has been reported from the Chondrichthyes. Secondly, the involvement of *Pax3* and *Pax7* genes in MMP development in teleosts and amphibians has yet to be characterized. In zebrafish, both *Pax3* and *Pax7* are expressed in the dorsal neural tube, but expression in somites is transient and weak (Seo et al., 1998). *Xenopus laevis Pax3* is expressed in the dermomyotome and the ventral body wall muscles, which undergo extensive ventral migration from hypaxial somites (Martin and Harland, 2001). To date, however, there has been no detailed report on the function of amphibian *Pax7*. More genetic and expression data are, therefore, required to elucidate the exact timing of molecular changes during the evolution of the *Pax3/7* family (Fig. 5A).

In lamprey embryos, the HBM is the only muscle that undergoes extensive, long-distance migration. Thus, during vertebrate evolution, agnathan HBM might represent the first skeletal muscle that had acquired MMP-type specification and differentiation (Fig. 5B). As there is no epaxial/hypaxial distinction in the lamprey, it is evident that MMP specification does not require a cellular environment that corresponds to the hypaxial myotomes of gnathostomes. Therefore, during vertebrate evolution, the establishment of a developmental regulatory system for MMP specification might have occurred in the ventral portion of the myotomes before the establishment of the horizontal myoseptum. In other words, the epaxial identity of myotomes might have been added onto the myotome (Fig. 5B), which initially contained cells only of MMP and non-MMP identities. The establishment of a horizontal myoseptum might have facilitated the independence of the epaxial and hypaxial muscles from each other with respect to their innervation, contractile properties, and development, which further might have led to the morphological and functional variety of hypaxial paired appendages of fish and tetrapods.

FUTURE PERSPECTIVES

With regard to the dorsoventral patterning of myotomes, a new context for categorization has recently been proposed (Nowicki et al., 2003; Burke and Nowicki, 2003). According to this scheme, the somites are divided into primaxial and abaxial domains, instead of epaxial and hypaxial. The primaxial domain represents somitic cells that develop in situ, whereas the abaxial domain consists of those that undergo differentiation under the regulation from connective tissue cells derived from the lateral plate mesoderm. The boundary between these two parts is termed the lateral somitic frontier. Importantly, this frontier does not correspond to the position of the horizontal septum. The primaxial domain includes all the epaxial muscles and the nonmigratory population of hypaxial muscles including all the intercostal muscles. The rest of the hypaxial muscles migrate as mesenchyme in the lateral plate mesoderm, and belong to the abaxial portion.

This categorization can be applied to both fish and tetrapods, whose adult muscle topologies are significantly different (Burke and Nowicki, 2003). In fish, a large portion of the somites gives rise to the trunk skeletal muscles without drastically changing their morphology, and is thus categorized as primaxial. The abaxial portion includes only a small population of muscle cells, which migrate to the fin bud. By contrast, the tetrapod abaxial muscles are more expanded and occupy a larger part of the body. These changes in the distribution of somitic elements according to this scheme suggest that tetrapod somitic derivatives might have come to receive more influence from the lateral plate mesoderm during evolution. This change might also have promoted further complexity in the morphology and regulation of limb and other muscles.

It is known that the lateral mesoderm serves as an environment where secreted factors such as bone morphogenetic proteins (BMPs) or hepatocyte growth factor (HGF) promote extension of hypaxial muscles from the adjacent myotomes. For the lampreys, the topology and fate of lateral plate mesoderm has not been well documented. Because HBM is the only muscle that undergoes an extensive migration, the rest of the lamprey muscles might be categorized as "primaxial." This would be true if these muscles are not under the regulation from the lateral plate mesoderm. A precise description for the developmental fates of different regions of lamprey trunk mesoderm would provide the solution to this problem. As in gnathostome fish, the myotome occupies much of the lamprey somite, and the locations of the sclerotome and the dermomyotome are not clear. Our observation suggests the "lateral cells" in lamprey larva (Nakao, 1976) might correspond to the gnathostome dermomyotome. To clearly indicate that these cells might represent the ancestral state of dermomyotome, it has to be shown that these cells give rise to the dermis and dorsally and ventrally extending, newly formed muscle fibers.

The lamprey myotome consists of multiple regions with distinct molecular properties, and lamprey HBM has recruited the migratory identity found in gnathostome MMPs. This prompted us to clarify the developmental mechanisms in lamprey skeletal musculature. It would be of particular interest to characterize the lamprey homologues of genes involved in gnathostome hypaxial muscle patterning, such as *Lbx1, c-Met*, and *HGF*. We have obtained some preliminary evidence that some of these molecules may not be involved in lamprey skeletal muscle development. The function of these molecules can be directly tested in developing lamprey embryos

by modern molecular embryological techniques, such as the injection of mRNAs or morpholino oligonucleotides, or the implantation of beads impregnated with secretory molecules.

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