

Hypaxial Muscle: Controversial Classification and Controversial Data?

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Abstract Hypaxial muscle is the anatomical term commonly used when referring to all the ventrally located musculature in the body of vertebrates, including muscles of the body wall and the limbs. Yet these muscles had very humble beginnings when vertebrates evolved from their chordate ancestors, and complex anatomical changes and changes in underlying gene regulatory networks occurred. This review summarises the current knowledge and controversies regarding the development and evolution of hypaxial muscles.

1 Introduction

Vertebrates evolved from chordate ancestors that lived in water (reviewed in (Clack 2002; Freitas et al. 2014)). Their mode of movement was a side-to-side undulation of body and tail, which can still be seen in extant chordates, but also in aquatic and semi-aquatic vertebrates (exception: aquatic mammals; see below). This side-to-side undulation is facilitated by reiterated (segmented) blocks of muscle—the myotomes (Goodrich 1958). The myotomes work against a central skeletal element, initially the notochord, later the vertebral column. The myotomes are innervated by somatic motor neurons which are connected with activating and inhibitory interneurons such that muscle contracts in an alternating fashion on either side of the

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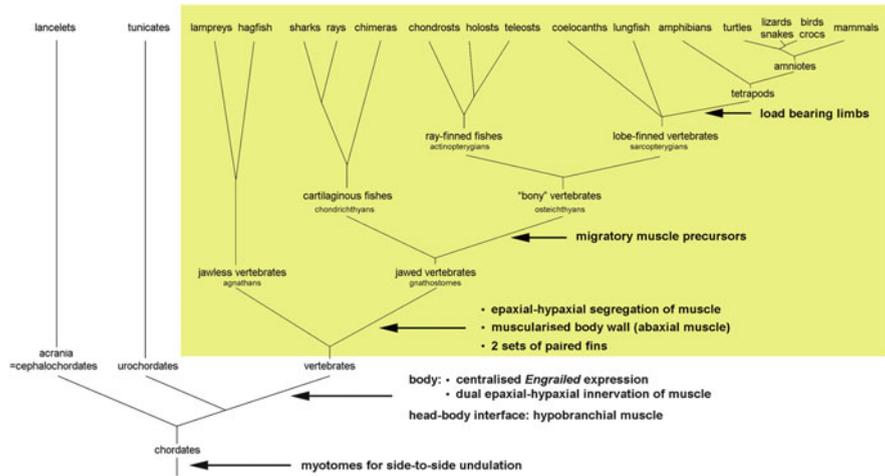


Fig. 1 Vertebrate phylogenetic tree, indicating the key changes in the organisation of their body musculature that underpinned changes in movement pattern. The basic chordate movement pattern is swimming via side-to-side undulations of the body and tail, relying on segmented muscle blocks—the myotomes—on either side of the body. The first steps towards three-dimensional mobility were taken in the shared ancestors of agnathans and gnathostomes, when *Engrailed* was recruited into the somite to facilitate the separate innervation of the dorsal/epaxial and ventral/hypaxial domain of the myotome. Likewise, in this ancestor the most rostral segments became incorporated into the head and muscle was deviated from a role in locomotion to role in pharyngeal support, respiration and food uptake. Specifically, the ventral/hypaxial muscle precursors were recruited to provide elaborate hypobranchial muscles. In the lineage leading to jawed vertebrates, epaxial–hypaxial muscle became fully segregated. Moreover, the lateral mesoderm developed two distinct leaves, facilitating the establishment of a muscularised body wall and the evolution of paired fins. Initially, muscle penetrated the outer, somatopleural aspect of the lateral mesoderm as a somitic outgrowth. Yet in the lineage leading to osteichthyans, a molecular program that allowed the de-epithelialisation and emigration of muscle precursors evolved. This program was first used to generate the muscles of the pectoral fins, but in the lineage leading to sarcopterygians, it was co-opted into the pelvic fins/hind limbs. It is thought that the resolution of segmental boundaries allowed the redistribution of muscle cells and, together with the more mobile insertion of the sarcopterygian fin/limb in the shoulder girdle, it facilitated the evolution of load-bearing limbs

body, and a wave of contractions runs from rostral to caudal. This creates a force against water as a viscous medium and propels the body forward (Kiehn 2011).

The myotomes were initially set up as dorsoventrally continuous half-rings (Goodrich 1958; Fetcho 1987). Yet in the ancestor of jawed vertebrates, muscle became split into distinct, separately innervated dorsal and ventral muscle blocks, which allowed full three-dimensional movements for the first time (Goodrich 1958; Fetcho 1987; Fig. 1). Moreover, when the lateral mesoderm evolved to form two distinct leaves, an inner splanchnopleura and an outer somatopleura, muscle penetrated the outer layer, thus leading to a muscularised body wall (Onimaru et al. 2011). Finally paired fins evolved. In most cartilaginous vertebrates (chondrichthyans) and

in ray-finned (actinopterygian) bony¹ vertebrates (osteichthyans), paired fins are mainly used for steering. Yet in lobe-finned animals (sarcopterygian osteichthyans), these fins eventually changed into load-bearing limbs that allowed the tetrapods to conquer land and to take to the air (Clack 2002). These amazing changes in body plans and locomotion took some 500 million years and allowed vertebrates to populate every ecological niche on Earth: land, air, fresh and marine waters. With that respect, vertebrates are one of the most successful animal group ever.

The anatomical changes that allowed the change of vertebrate movement patterns predominantly affected the lay-out of the ventral muscular system, traditionally referred to as “hypaxial”, and this review will retrace their development and evolution. It also will discuss the “primaxial–abaxial” subdivision of muscle that is often portrayed as contrasting concept. Finally, the review will provide an overview of a specialised type of hypaxial muscle precursors that evolved in the osteichthyan lineage and that is thought to have aided the evolution of load-bearing limbs—the migratory hypaxial muscle precursors.

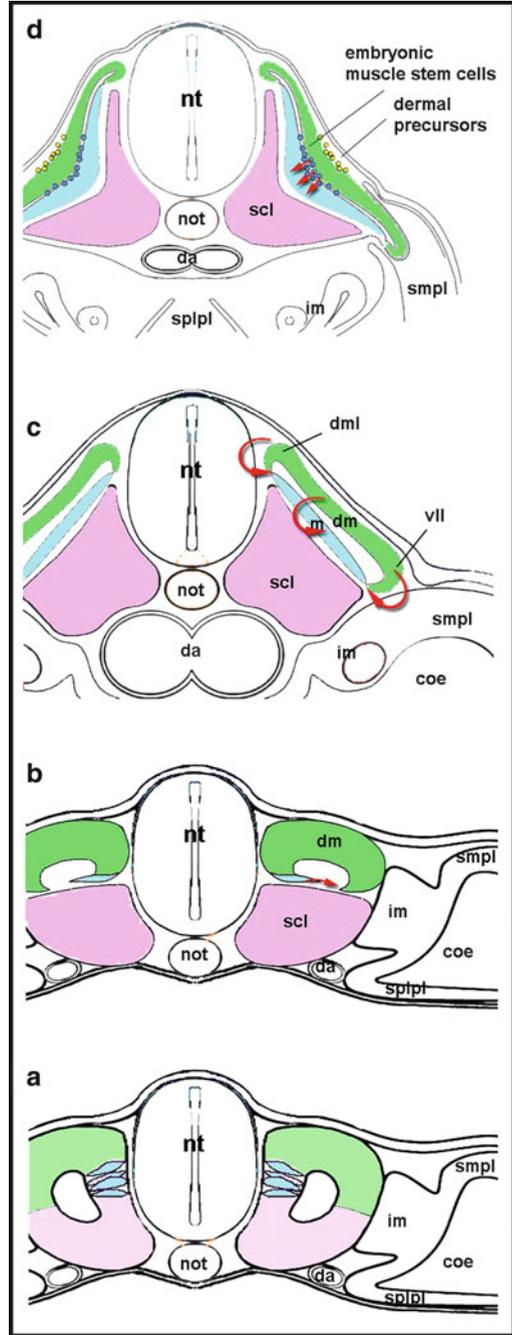
2 Developmental Anatomy of Dorso-Ventral Muscles and the Classical Epaxial–Hypaxial Concept

In all vertebrates, the skeletal musculature of the body and fins/limbs originates from the segmented paraxial (=next to the axial notochord) mesoderm termed somites, and muscle is laid down in waves (reviewed in: Bryson-Richardson and Currie 2008; Buckingham 2006; Fig. 2). The first muscle (primary myotomes) is immediately contractile. This is essential since anamniote vertebrates (as well as their chordate relatives) develop via free-feeding, motile larvae (Goodrich 1958). Yet, differentiated muscle fibres are postmitotic, thus limiting muscle growth to an increase in fibre size by hypertrophy. However, vertebrates have set aside muscle stem cells that drive hyperplastic muscle growth and muscle repair upon injury (reviewed in: Bryson-Richardson and Currie 2008; Buckingham 2006). These stem cells initially reside in a structure superficial to the myotome, the dermomyotome. However, eventually these cells populate the muscles, thereby establishing a resident pool of stem cells. In the adult, these cells are located underneath the basal lamina of muscle fibres and are referred to as satellite cells. In actinopterygians such as teleosts, muscle stem cells drive the continuous muscle growth typical for these animals. In amniotes, the satellite cells become quiescent and are only activated and proliferative upon injury.

In jawed vertebrates, the myotomes become segregated into dorsal and ventral components. This is achieved by the intercalation of a physical boundary, the

¹ The phrase “bony” vertebrate as a more colloquial term for osteichthyans is somewhat misleading since mineralised bone was already present in stem group gnathostomes and was secondarily lost in sharks and rays, (Zhu et al. 2013).

Fig. 2 Vertebrate muscle is generated in waves. Schematic cross sections of amniote flank somites, dorsal to the top, modelled after the chicken. In line with the rostrocaudal progression of somite formation and differentiation, the developmentally youngest somites are shown at the *bottom*, the most mature at the *top*. **(a)** Soon after the epithelial somite formed, its dorsal territory becomes specified as dermomyotome (*light green*), its ventral territory as sclerotome (*light pink*), and cells in the medial wall of the somite are specified as the first myogenic cells (*turquoise*). **(b)** The myogenic cells, also referred to as muscle pioneers, spread (*red arrow*) and form a scaffold between the now morphologically defined dermomyotome (*green*) and sclerotome (*pink*). **(c)** More cells are being added to the scaffold from the dorsomedial, ventrolateral, rostral and caudal edges of the dermomyotome (*red arrows*), leading to a morphologically well-defined, contractile myotome (*turquoise*). **(d)** Eventually, the dermomyotome de-epithelialises, releasing myogenic stem cells into the myotome (*red arrows*). These cells drive the foetal and perinatal growth of muscle and provide the adult muscle stem cells (satellite cells)



thoracolumbar fascia or horizontal myoseptum (Goodrich 1958; Gray 1995). In teleost fish, the dorsoventral subdivision of muscle occurs at an early time point and is organised by specialised, *engrailed* expressing slow muscle cells. These cells are somewhat misleadingly termed muscle pioneers since they express muscle structural genes at an early time point (Devoto et al. 1996; Currie and Ingham 1996). However, their key role is to serve as a first target for the axons of the three large, primary somatic motoneurons and organise the projection of one of them to the dorsal muscle block, one to the ventral muscle block, and one to innervate the slow-twitch muscle at the dorsoventral boundary (Beattie and Eisen 1997), reviewed by (Lewis and Eisen 2003). When *engrailed* function is disrupted, severe innervation defects occur (Ahmed et al. manuscript in preparation; Fig. 3).

In teleosts, eventually the smaller, secondary motoneurons outnumber the primary motoneurons and take over to drive muscle contraction (Fetcho 1987; Lewis and Eisen 2003). However, these neurons use the pre-existing scaffold for their axonal pathfinding. In amniotes in contrast, it is generally held that only secondary-type motoneurons are being used (Fetcho 1987). They are organised into two pools in the ventral spinal cord, with the medially located pool (medial motor column) destined to innervate the dorsal muscles, and the laterally located neurons (hypaxial motor column) innervating the ventral muscles (reviewed in: Tsuchida et al. 1994). Yet in all vertebrates, the physical segregation of muscle is matched by their separate innervation, such that the dorsal and ventral muscles can contract independently. According to their distinct innervation, muscles have classically been distinguished as epaxial (innervated by the medial motor column via the dorsal ramus of the spinal nerve) or hypaxial (innervated by the hypaxial motor column via the ventral ramus of the spinal nerve; Fig. 4a, Ai).

Interestingly, in amniotes, the dorsoventrally distinct innervation of body muscle occurs before the myotome is physically segregated (Tosney and Landmesser 1985; Tosney 1987; reviewed in: Fetcho 1987). Yet this innervation pattern also relies on *Engrailed* (*En1*; Ahmed et al., manuscript in preparation). *En1* is initially expressed in the central dermomyotome where it sets up a molecular and compartment boundary (Cheng et al. 2004). Expression of *En1* is brought into the myotome when the muscle stem cells arrive from the de-epithelialising dermomyotome (Ahmed et al. 2006). *En1* then supports the outgrowth of the dorsally projecting axons and suppresses the outgrowth of the ventrolaterally projecting axons (Ahmed et al., manuscript in preparation; Fig. 3). Thus, although *En1* function has shifted in time, it is remarkable that in jawed vertebrates, it is associated with epaxial–hypaxial boundary formation and innervation. Moreover, in all vertebrates investigated *Engrailed* expression is controlled by the Shh signalling molecule released from the notochord, suggesting the conservation of key parts of the underlying regulatory network (Cheng et al. 2004; Hammond et al. 2009; Maurya et al. 2011).

Jawless vertebrates such as the lamprey already have a dorsal and ventral innervation point of their myotome even though a horizontal myoseptum is absent (Fetcho 1987). Moreover, markers have been identified that distinguish the dorsal and the lateral edge of the somite, suggesting that the first steps towards an epaxial–hypaxial segregation of muscle had been taken before the agnathan–gnathostome

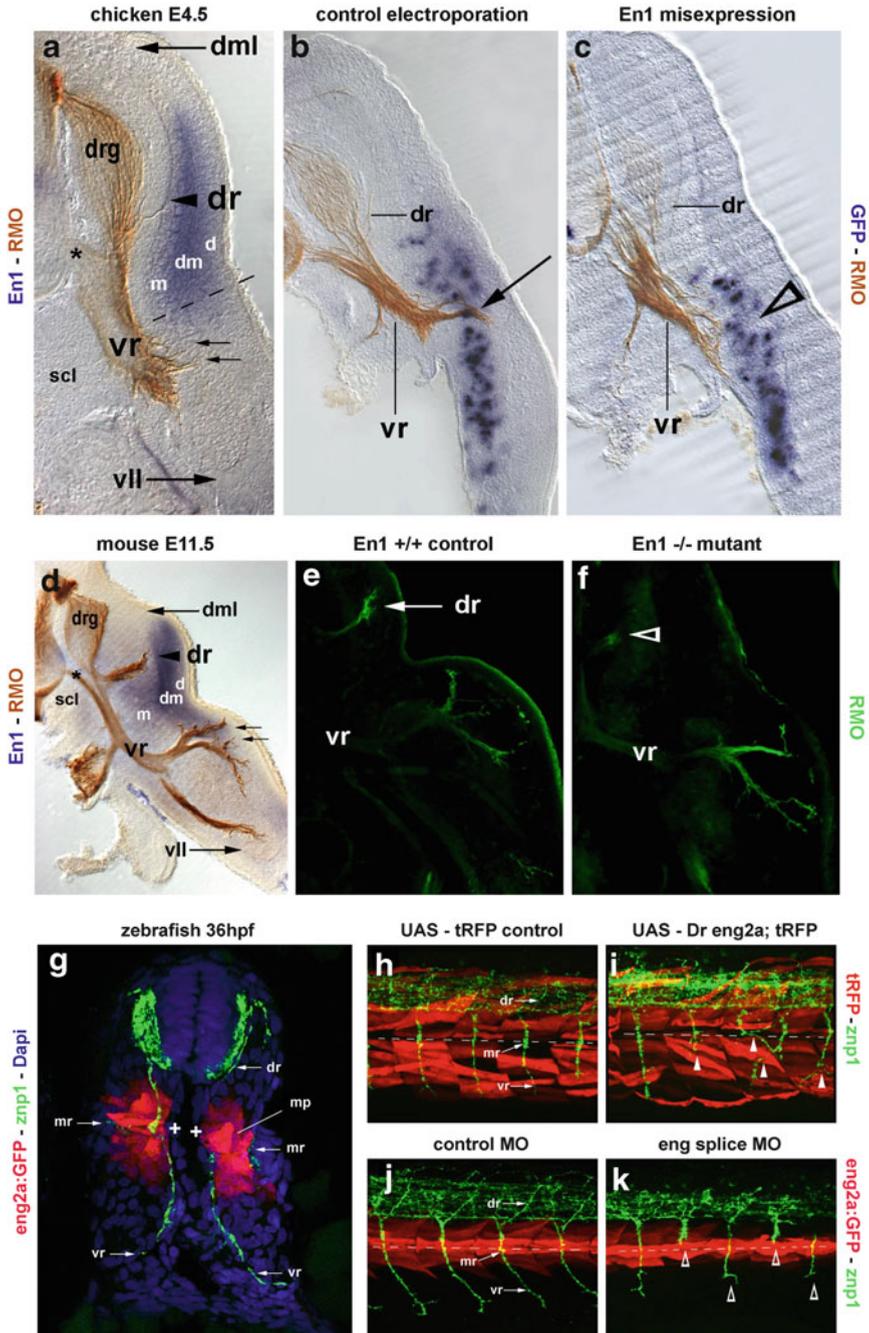


Fig. 3 Conserved role of *Engrailed* in organising the epaxial-hypaxial innervation of muscle. (a–c) Role of *En1* in the chicken embryo. (a) Cross sections of chicken flank somites at embryonic day E4.5 of development, dorsal to the *top*, medial to the *left*. *Engrailed 1* (*En1*) expression is shown in *blue*, intermediate neurofilaments of nerve axons are revealed with the RMO antibody in

divide (Kusakabe et al. 2011). Interestingly, also in the lamprey, one of its *engrailed* genes is expressed in the centre of the myotome, molecularly separating its dorsal and ventral aspect (Hammond et al. 2009; Matsuura et al. 2008). This suggests that already before the agnathan-gnathostome divide, *eng/En* had been

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Fig. 3 (continued) brown. Note that *En1* expressing cells are leaving the central dermomyotome and populate the central myotome underneath. The developing ventral ramus of the spinal nerve navigates around the *En1* expression domain and targets the hypaxial myotome; first contact with the myotome is made when axons of the cutaneous branch of the ventral ramus travel along the ventral boundary of the *En1* domain and project towards the dermis (*small arrows*). The dorsal ramus lags developmentally behind; its axons target the *En1* domain (*arrowhead*). **(b–c)** Gain-of-function experiment; phenotype displayed on cross sections. Flank somites were electroporated at E2.5 with **(b)** a *GFP* expressing control construct or **(c)** a bi-cistronic *GFP* and *En1* expressing experimental construct. 24 h later, expression of the construct was revealed by in situ hybridisation in *blue* and the position of the axons by RMO staining in *brown*. Note that in the *En1* misexpressing somites, the ventral ramus of the spinal nerve defasciculated and failed to form the cutaneous branch of this ramus (*open arrowhead*). **(d–f)** Role of *En1* in the mouse embryo. **(d)** Cross sections of mouse flank somites at E11.5 of development, dorsal to the *top*, medial to the *left*; *En1* expression in *blue*, RMO staining in *brown*. Note that marker gene expression and axonal projections in the mouse closely match that of the chicken. **(e–f)** Loss-of-function experiment, axons were revealed by RMO antibody staining on cross sections (green staining). Note that in wildtype litter mates, the dorsal ramus of the spinal nerve is well developed and innervates the epaxial myotome **(e)**. In *En1* deficient embryos, that dorsal ramus falls short of its target **(f, open arrowhead)**. **(g–k)** Role of *engrailed* genes in the zebrafish. **(g)** Cross section of a 36hpf zebrafish *eng2a:GFP* embryo. *engrailed* expression as revealed by the GFP transgene expression is shown in *red*; axons are stained with the *znp1* antibody (*green*) and cell nuclei with Dapi (*blue*). Initially, the primary motor neurons all project to the *eng* expressing muscle pioneers (+, *mp*) which organise the formation of the horizontal myoseptum. Subsequently, the primary and accompanying secondary motor neurons located next to the posterior somite half extend their axons ventrally to contribute to the ventral ramus of the spinal nerve and to innervate the hypaxial myotome. Motor neurons neighbouring the anterior somite half send their axons laterally along the developing horizontal myoseptum to form the fish-specific medial ramus and to target the superficial slow muscles. Motor neurons in the middle of each segment withdraw their connection to the muscle pioneers and project dorsally to form the dorsal ramus and to innervate the epaxial myotome. **(h–k)** Lateral views of 36hpf experimental zebrafish embryos, anterior to the *left*. The position of the developing horizontal myoseptum is indicated by a stippled line. **(h–i)** Gain-of-function experiment: Transgenic α actin-Gal4 embryos were used to drive expression of **(H)** a UAS-tRFP control construct or **(i)** a bi-cistronic construct encoding tRFP as well as zebrafish *engrailed 2a*. Cells expressing the constructs fluoresce in red; axons are revealed with the *znp1* antibody in *green*. Note that *engrailed* misexpression leads to severe misguidance of motor axons **(i, arrowheads)**. **(j–k)** Loss-of-function experiment: *eng2a:GFP* embryos (transgene expression shown in red, *znp1*-stained axons in green as in **(g)**) were treated with **(j)** a control morpholino or **(k)** a morpholino cocktail targeting the splice sites of *engrailed1a*, *1b* and *2a* which are all expressed in muscle pioneers. Note that this knock down of *eng* function blocked axonal outgrowth, and axons stalled or took up erratic paths in search for their targets **(k, open arrowheads)**. The data shown here are the work of Mohi U. Ahmed, Ashish K. Maurya, Louise Cheng, Erika C. Jorge, Frank R. Schubert, Pascal Maire, M. Albert Basson, Philip W. Ingham and Susanne Dietrich. *d* dermis precursors, *dm* dermomyotome, *dml* dorsomedial lip of the dermomyotome, *dr* dorsal ramus of the spinal nerve, *drg* dorsal root ganglion, *m* myotome, *mp* muscle pioneers, *mr* medial ramus of the spinal nerve, *scl* sclerotome, *vll* ventrolateral lip of the dermomyotome, *vr* ventral ramus of the spinal nerve. The asterisk in **(a,d)** marks the axons of motor neurons projecting out of the neural tube

recruited into the developing musculature where it paved the way for the dorso-ventral segregation and innervation of muscle and the evolution of full three-dimensional mobility. On the other hand, when mammals returned to the sea, they adapted to movement in water with fully segregated epaxial–hypaxial muscle in place. It can be speculated that this divide was the basis to evolve upwards-downwards undulations of the body and tail as seen best in dolphins and whales.

3 The Primaxial–Abaxial Concept and the Lateral Somitic Frontier

Skeletal muscle fibres are organised into anatomically defined muscles via several layers of connective tissue (Gray 1995). Moreover, muscle can only fulfil its role when anchored on skeletal elements via tendons or aponeuroses. Thus, functional muscle has an intricate relationship with the various types of connective tissue. Indeed, even though initially muscle and connective tissue develop independently, eventually both tissues rely on each other for function and survival (Murphy et al. 2011). Interestingly, in muscle-less limbs, connective tissue organises itself in the correct anatomical pattern, anticipating the position of muscle (Kardon et al. 2003). This indicates that the connective tissue directs the muscle cells to their defined places. Yet at different locations in the body, connective tissue is made from different cell types, and hence, muscle has to adjust to different partners. This has led to the primaxial–abaxial concept of muscle development (Fig. 4b,Bi).

Epaxial muscles, but also some hypaxial muscles—for example the sub-vertebral muscle of the neck and the muscles associated with the ribs—receive their connective tissue from the somites. Thus, the cells always remain in a paraxial—or primaxial—environment. On the other hand, hypaxial muscle precursors for muscles associated with the sternum, the body wall or the limbs enter a new environment, namely the dorsal leaf of the lateral mesoderm (somatopleura), and all the connective tissue is derived from this environment (Durland et al. 2008). When heterotopically grafted, muscle precursors entering the lateral plate environment switch *Hox* gene expression to match the position values found on site (Nowicki and Burke 2000). Thus, these hypaxial cells cross a boundary, termed “lateral somitic frontier”. They will settle far from their original position and are patterned by their new environment, and hence have been termed “abaxial” (reviewed in: Shearman and Burke 2009). In *Amphioxus* and in the lamprey, the lateral mesoderm of the body does not split into splanchnic and somatic lateral mesoderm, and muscle does not enter this environment (Onimaru et al. 2011; Tulenko et al. 2013). Thus, abaxial body muscle, i.e. a muscularised body wall and derivatives thereof (see below) may be a novelty that emerged in the lineage which eventually led to the jawed vertebrates (Fig. 1). How connective tissue cells communicate their positional values to muscle cells is not clear. It has been shown, however, that homeodomain transcription factors can act as short range signalling

molecules, both in vertebrates and in insects (reviewed in: Brunet et al. 2007; Layalle et al. 2011). Thus, it is tempting to speculate that a similar process may allow the alignment of *Hox* gene expression patterns. However, single cells versus group cell grafting performed in a cranial environment suggested that, if the grafted cells have enough neighbours of their own kind, they retain their original *Hox* code (Trainor and Krumlauf 2000). Thus, more work is needed to determine cell–cell communication at the lateral somitic frontier. Yet, it should be emphasized that the classical epaxial–hypaxial concept and the primaxial–abaxial concept are not necessarily exclusive; they simply refer to different aspects of ventrolateral muscle development (Fig. 4).

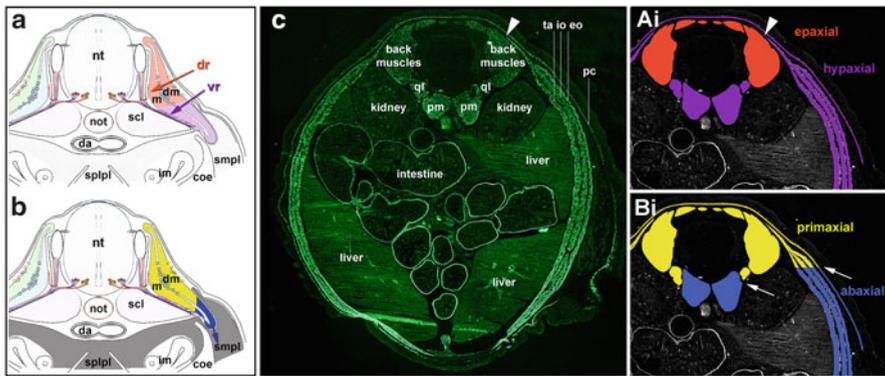


Fig. 4 Comparison of the epaxial–hypaxial and primaxial–abaxial concept. **(a)** Schematic cross section of an amniote abdominal somite at the time of innervation, modelled after the section shown in Fig. 4a. Motor neurons located in the medial motor column and contributing to the dorsal ramus of the spinal nerve are shown in orange. Motor neurons located in the hypaxial motor column and contributing to the ventral ramus of the spinal nerve are shown in purple. Areas of the somite targeted by the dorsal or ventral ramus of the spinal nerve are displayed in matching colours. Notably, the myotome is still dorsoventrally continuous at this stage. **(b)** Same schematic cross section as in **(a)**. Areas of the somite developing in association with the sclerotome-derived axial skeleton are shown in yellow, areas of the somite that grow out into the somatopleural leaf of the lateral mesoderm are shown in blue. The direction of outgrowth is marked by a blue arrow. The lateral mesoderm is shown in grey. **(c)** Cross section of the abdomen of a newborn mouse, muscle is stained for skeletal muscle Myosin in green. The arrowhead indicates the insertion of the body wall muscles at the thoracolumbar fascia. **(Ai)** Cross section as in **(c)**, with muscles colour-coded according to their innervation as in **(a)**. Muscles innervated by the dorsal ramus of the spinal nerve are the epaxial muscles (red). Muscles innervated by the ventral ramus are the hypaxial muscles (purple). **(Bi)** Cross section as in **(c)**; the arrows indicates the boundary between sclerotome and lateral mesoderm derived connective tissue as revealed by the lineage tracing of *Prx1*-expressing cells (Durland et al. 2008). Muscles developing in a sclerotome-derived environment are colour-coded yellow as in **(b)**. These are the primaxial muscles. Muscles developing in a somatopleura-derived environment are colour-coded blue as in **(b)**. These are the abaxial muscles. *coe* coelomic cavity, *da* dorsal aorta, *dm* dermomyotome, *dr* dorsal ramus of the spinal nerve, *eo* external oblique muscle, *im* intermediate mesoderm, *io* internal oblique muscle, *m* myotome, *nt* neural tube, *not* notochord, *pc* panniculus carnosus muscle, *pm* psoas muscle, *ql* quadratus lumborum muscle, *scl* sclerotome, *smp1* somatopleural leaf of the lateral mesoderm, *sp1pl* splanchnopleural leaf of the lateral mesoderm, *ta* transversus abdominis muscle, *vr* ventral ramus of the spinal nerve

4 Migratory Muscle Precursors for the Paired Fins and Limbs: An Osteichthyan Innovation

In tetrapods, even though the lateral swaying of the body is still an important part in the movement of amphibians and reptiles, locomotion clearly relies on load-bearing limbs and their associated musculature. Innervated by the lateral motor column a specialist group of hypaxial motor neurons only found at limb levels reviewed in Murakami and Tanaka 2011 and developing in the lateral mesoderm that provides the limb connective tissue and the limb skeleton, limb muscles are both hypaxial and abaxial. Embryological studies in the chicken established that in amniotes, limb muscles develop from cells that detach from the lateral lip of the dermomyotome and actively migrate into the limb, where they become organised into dorsal and ventral muscles masses to give rise to the extensor and flexor muscles groups, respectively (Chevallier et al. 1977; Christ et al. 1977; Hayashi and Ozawa 1995; Fig. 5a). Painstaking histological and lineage tracing experiments in various fish species showed that muscle precursors undertaking long-range migration muscularise the pectoral fins of teleosts (ray-finned “bony” vertebrate), while in cartilaginous vertebrates, somites form extensions that reach into the fin anlage in a similar fashion as they grow out into the body wall to form abdominal muscles (Neyt et al. 2000). The pelvic fins of lungfish (a lobe-finned relative of tetrapods), teleosts and paddlefish (both ray-finned osteichthyans) receive muscle precursors from somitic extensions that, when close to their target site, deepithelialise to release individual cells. The pelvic fin muscles of sharks and chimeras are made in the same way as their pectoral musculature, namely from somitic extensions (Cole et al. 2011). This has led to the view that hypaxial/abaxial muscle formation via somitic extensions is the evolutionarily older mechanism of muscle delivery, while migratory muscle precursors evolved later in the lineage leading to osteichthyans. They were first established for the pectoral fin/forelimb and subsequently for the pelvic fin/hind limb. In line with this view, a molecular program has been deciphered that specifically operates at teleost pectoral fin levels/tetrapod limb levels, and acts on top of the generic program for hypaxial myogenesis (Fig. 5b).

The generic program for hypaxial myogenesis is best researched in amniotes. Here, the lateral somite domain is specified by *Bmp4* released from the lateral mesoderm (Pourquié et al. 1996). Together with Wnt signals from the surface ectoderm, *Bmp* upregulates the expression of the pre-myogenic gene *Pax3* (Dietrich et al. 1998; Fan et al. 1997; Tajbakhsh et al. 1998). Six transcription factors, when activated by their *Eya* partners, contribute to the upregulation of *Pax3*, and *Pax3* enhances its own expression. Together, Six and *Pax3* transcription factors facilitate the generation of hypaxial skeletal muscle cells (Tremblay et al. 1998; Borycki et al. 1999; Grifone et al. 2005; Grifone et al. 2007). This occurs after *Bmp4* cooperated with Notch signalling to facilitate the release of smooth muscle and endothelial precursors (Ben-Yair and Kalcheim 2008).

At limb levels, muscle precursors destined to emigrate express the homeodomain transcriptional repressor *Lbx1*, and in animals as diverse as teleosts

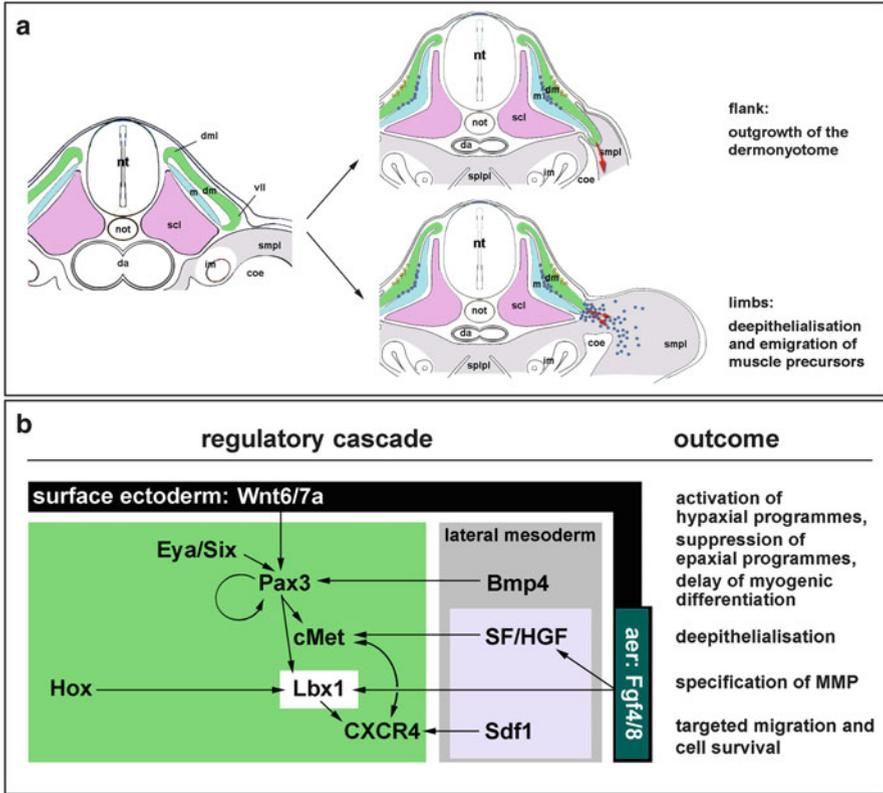


Fig. 5 The two modes of hypaxial muscle delivery—somitic outgrowth and targeted migration. (a) Schematic representation of maturing amniote somites at flank (*top*) and limb (*bottom* levels). At flank levels, the ventrolateral lip of the dermomyotome (*vll*) penetrates the somatopleura as a sheet. On its way, it deposits muscle precursors, thereby ensuring concomitant outgrowth of the myotome. At limb levels, the *vll* de-epithelialises, and cells actively migrate into the periphery. An intermediate mode of hypaxial muscle delivery is found in the teleost pelvic fins, where the *vll* of the outgrowing segment deepithelialises when the destination is reached. A similar mechanism has been reported for the formation of the ventralmost amniote abdominal muscle, the rectus abdominis. (b) Gene regulatory network for hypaxial muscle development. A generic program starting with *Wnt* signals from the surface ectoderm and *Bmp* signals from the lateral mesoderm operates at all axial levels. It upregulates the premyogenic gene *Pax3* which, together with premyogenic factors of the *Six* family, facilitates the generation of hypaxial myogenic cells. At limb levels, this generic program of hypaxial muscle formation is in operation. Yet, additional localised factors control the formation of migratory muscle precursors. Firstly, *Hox* gene expression in limb levels somites instructs these somites to activate the program of migratory rather than non-migratory muscle precursors (Alvares et al. 2003). In this context, *Pax3* activates the marker for migratory muscle precursors, *Lbx1*, which in turn activates the cytokine receptor *CXCR4* (Dietrich et al. 1999; Mennerich et al. 1998; Odemis et al. 2005; Vasyutina et al. 2005). Secondly, the limb lateral mesoderm provides the *cMet* ligand Scatter Factor/Hepatocyte Growth Factor (*SF/HGF*) and the *CXCR4* ligand *Sdf1* (Bladt et al. 1995; Prunotto et al. 2004; Vasyutina et al. 2005). Both signalling systems cooperate to control lip deepithelialisation, targeted cell migration and cell survival. Thirdly, the limb apical ectodermal ridge (*aer*) releases *Fgf* signalling molecules which are required for the expression of *SF/HGF* (Scaal et al. 1999). Importantly, *Fgf* molecules by themselves can override the program for non-migratory hypaxial muscle precursors, trigger *Lbx1* expression and serve as chemoattractants, thus ensuring that cells from the paraxial territory

(actinopterygians), lungfish and tetrapods (sarcopterygians), *Lbx1* (teleosts: *lbx1a*, *b*) is the bona fide marker for migratory muscle precursors (Cole et al. 2011; Dietrich 1999; Dietrich et al. 1999; Jagla et al. 1995; Martin and Harland 2006; Ochi and Westerfield 2009; Thisse et al. 2004); Figs. 5b and 6a, b, d and 7). Absence of *Lbx1* or misexpression of a dominant negative *Lbx1* construct prevents cell emigration into the paired fins/limbs, and only a subset of forelimb flexor muscle at the ventral junction to the limbs develops under the influence of local cues (Schäfer and Braun 1999; Gross et al. 2000; Brohmann et al. 2000; Ochi and Westerfield 2009; Martin and Harland 2006; Lours-Calet et al. 2014); Fig. 6f,Fi). Given this important role of *Lbx1*, the question of migratory muscle precursor formation has frequently been seen as a problem of localised *Lbx1* induction.

The mouse mutant *Spotch* is a well known model for muscle-less limbs (Franz et al. 1993; Bober et al. 1994; Tremblay et al. 1998), and in this animal, *Lbx1* expression is lost (Dietrich et al. 1999; Mennerich et al. 1998). *Spotch* mice carry a mutation for the paired box transcription factor *Pax3*, yet *Pax3* is expressed in the early somite, in the dermomyotome of more mature somites and is upregulated in the dorsomedial and ventrolateral dermomyotomal lips of all somites along the rostrocaudal body axis. Thus, while *Pax3* is necessary for *Lbx1* expression, it is not sufficient to position expression in limb-level somites. Interestingly, experiments in the zebrafish indicated that here, the duplicated *pax3b* gene had its expression restricted to pectoral fin somites and is required for *lbx* expression (*lbx2* in this case; Minchin et al. 2013). Thus, while displaying a variation on the theme, it suggests that the relationship of *Pax3* and *Lbx* is evolutionarily ancient.

It is well established that heterotopic transplantation of limbs or limb induction via implantation of Fgf beads in the flank of chicken embryos lead to the development of a muscularised and fully innervated ectopic limb (Chevallier et al. 1977; Christ et al. 1977; Hayashi and Ozawa 1995; Cohn et al. 1995). Moreover, the ectopic limb, its apical ectodermal ridge (aer) or the Fgf4/8 signalling molecule produced by the aer, all induce somitic *Lbx1* expression and the emigration of muscle precursors (Alvares et al. 2003). Furthermore, regulated by FGF from the aer, the limb mesenchyme produces the signalling molecule Scatter factor/Hepatocyte growth factor (Scaal et al. 1999). Its receptor cMet is found in all ventrolateral dermomyotomal lips, but the local activation of cMet leads to local lip deepithelialisation only (Bladt et al. 1995; Prunotto et al. 2004). Similarly, the cytokine Sdf1 which assists SF/HGF is expressed in the limb mesenchyme, and its CXCR4 receptor in the somitic dermomyotome (Odemis et al. 2005; Vasyutina et al. 2005). Together, this has led to the view that the limb overrides any pre-existing programme and is the key inducer of migratory muscle precursors.

Fig. 5 (continued) are recruited into the limb (Alvares et al. 2003). *coe* coelomic cavity, *da* dorsal aorta, *dm* dermomyotome, *im* intermediate mesoderm, *m* myotome, *MMP* migratory muscle precursors, *nt* neural tube, *not* notochord, *scl* sclerotome, *smp1* somatopleural leaf of the lateral mesoderm, *spl1* splanchnopleural leaf of the lateral mesoderm

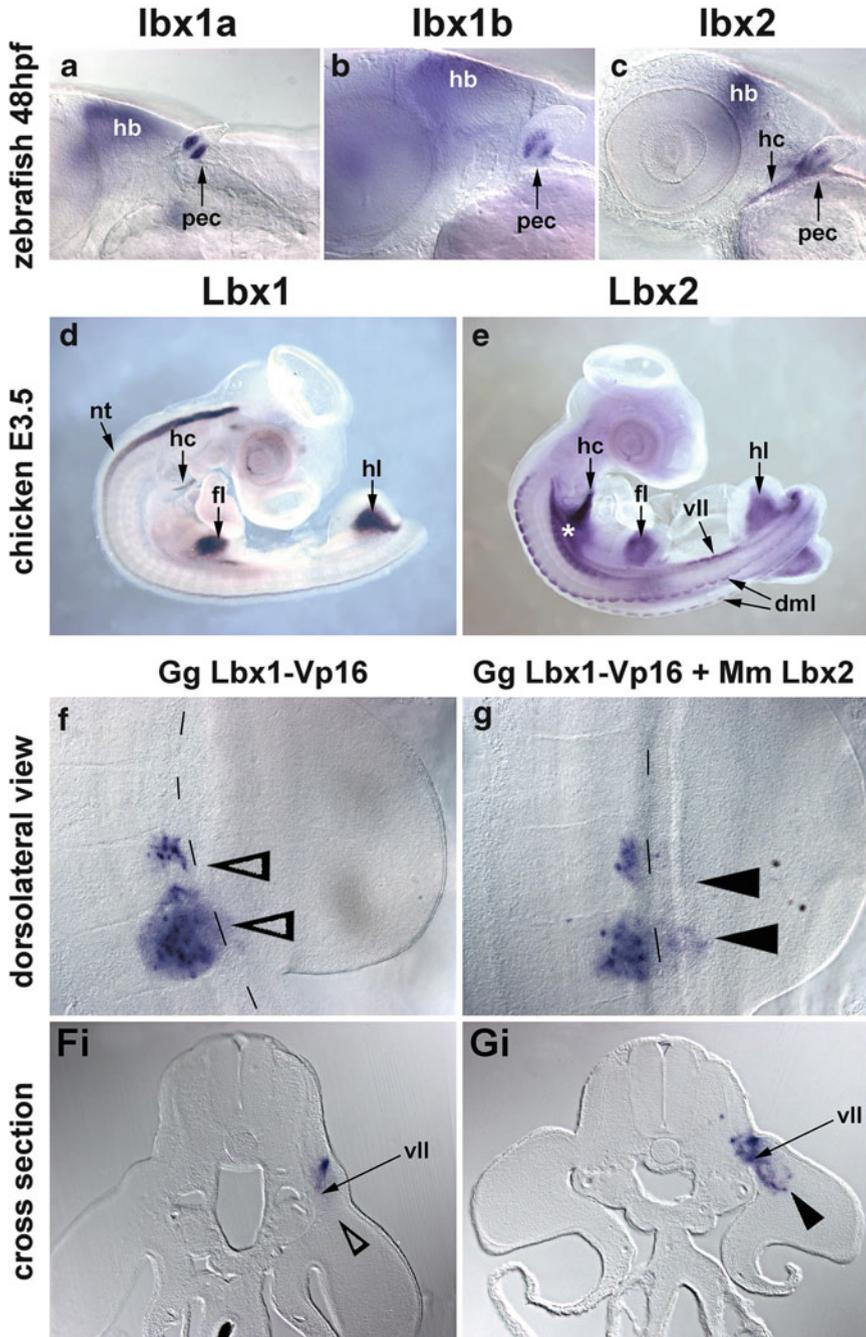


Fig. 6 Expression and function of *Lbx* genes. (a–c) Lateral views of zebrafish embryos, 48 h post-fertilisation (hpf), dorsal to the top, rostral to the left. Note that *lbx1a*, *lbx1b* and *lbx2* are all expressed in muscle precursors that have migrated into the pectoral fin. All are also expressed in the hindbrain (albeit *lbx2* in a smaller territory). Furthermore, *lbx2* is expressed in hypobranchial

However, evidence has accumulated that suggests the role of the limb has been overrated, and a more complex gene regulatory network has emerged (Fig. 5b).

A detailed characterisation of ectopic limbs revealed that the limb is not able to fully reprogram flank tissues since it does not force the spinal cord to generate a lateral motor column. Instead, the ectopic limb deviates axons originating from the flank hypaxial motor column to innervate the ectopic limb muscle rather than its normal target, the body wall musculature (Turney et al. 2003). Secondly, in cMet and SF/HGF mutants, while muscle precursors de-epithelialisation and emigration fails, *Lbx1* is well expressed (Dietrich et al. 1999), and in migratory muscle precursors, expression of CXCR4 is downstream of *Lbx1* (Vasyutina et al. 2005). This indicates that the specification of migratory muscle precursors is independent from cell de-epithelialisation. Thirdly and most importantly, *Lbx1* expressing muscle precursors develop when competent, limb-level somites are exposed to any type of lateral mesoderm, including lateral mesoderm from the flank (Alvares et al. 2003). When *Hox* genes were misexpressed to switch the axial identity of flank somites to that of limb somites, these somites faithfully expressed *Lbx1* (Alvares et al. 2003; Fig. 5b). This indicates that intrinsic, Hox-dependent cues predispose somites towards either generating non-migratory or migratory muscle precursors.

5 Migratory Muscle Precursors for the Paired Fins and Limbs: A Vertebrate Innovation?

In osteichthyans, *Lbx1* genes are exclusively expressed in cells detaching and migrating away from the somites (Cole et al. 2011; Dietrich 1999; Dietrich et al. 1999; Jagla et al. 1995; Martin and Harland 2006; Ochi and Westerfield 2009; Thisse et al. 2004); this article; Fig. 7). Possibly the most extreme case is

Fig. 6 (continued) muscle precursors coalescing in the hypoglossal cord (hc) and temporarily in the dorsal and ventral tips of the myotome (not shown). (d, e) Lateral views of chicken embryos at embryonic day E3.5 days of development, rostral to the *top-right*. (d) *Lbx1*, in addition to its expression in the neural tube, is expressed in the migratory muscle precursors populating the limbs and the hypoglossal cord. *Lbx2* is not expressed in neural tissues, but shows a widespread expression in myogenic cells including muscle precursors in all ventrolateral dermomyotomal lips (migratory and non-migratory), muscle precursors in all dorsomedial lips and the myogenic neck lateral mesoderm (asterisk). (f, g) Dorsolateral views and (Fi, Gi) corresponding cross sections of electroporated chicken somites at forelimb levels. (f, Fi) Misexpression of a dominant negative chicken *Lbx1* construct (Gg Lbx1-Vp16; blue staining) interferes with the emigration of muscle precursors into the forelimb (*open arrowheads*). (g, Gi) Co-expression of the dominant negative construct (blue staining) together with mouse (Mm) *Lbx2* rescues muscle precursor emigration even though Mm *Lbx2* is divergent and not expressed in myogenic cells. The data shown here are the work of Karl Wotton and Susanne Dietrich. *dml* dorsomedial lip of the dermomyotome, *fl* fore limb, *hb* hind brain, *hc* hypoglossal cord, *hl* hind limb, *nt* neural tube, *pec* pectoral fin, *vll* ventrolateral lip of the dermomyotome

Species	<i>lhx/Lbx1</i> -type genes				Reference	<i>Lbx2</i> -type genes				
	Gene	Expression in:				Gene	Expression in:			Reference
		all dml	all vll	vll producing MMP only			all dml	all vll	vll producing MMP only	
Lamprey	<i>lhx-a</i> *		✓		(Kusakabe et al. 2011)					
Zebrafish	<i>lhx1a</i>			✓	(Ochi and Westerfield 2009); this article					
Zebrafish	<i>lhx1b</i>			✓	(Thisse et al. 2004); this article	<i>Lbx2</i>	✓	✓		(Neyt et al. 2000; Ochi and Westerfield 2009); this article
Xenopus	<i>lhx1</i>		✓**	✓**	(Martin and Harland 2006)		No <i>Lbx2</i> gene in the Xenopus genome assembly			(Wotton et al. 2008)
Chicken	<i>Lbx1</i>			✓	(Dietrich et al. 1998)	<i>Lbx2</i>	✓	✓		(Kanamoto et al. 2006); this article
Mouse	<i>Lbx1</i>			✓	(Jagla et al. 1995; Dietrich et al. 1999)	<i>Lbx2</i>	Not expressed in somites			(Chen et al. 1999)

* Phylogenetic analyses did not fully resolve whether the lamprey *lhx-A* gene is an ortholog of gnathostome *Lbx1*, or whether the gene arose before the two rounds (teleosts: three rounds) of gnathostome genome duplication and hence, would be a homologue of both, gnathostome *Lbx1* and *Lbx2* (Kusakabe et al. 2011; Wotton et al. 2008).

** Frog body wall muscles seem to form from migratory cells (Martin and Harland 2006).

Fig. 7 Presence and myogenic expression of vertebrate *Lbx* genes

Extant gnathostomes show evidence of 2 rounds of whole genome duplication during evolution, with additional lineages, including that of the teleosts, undergoing a third. Yet, owing to the early loss of duplicates, gnathostomes genomes only have a *Lbx1* gene (teleosts: *lhx1a* and *1b*) and a *Lbx2* gene; frogs may have lost their *lhx2*. Of these, *Lbx1* genes are almost invariably associated with migratory muscle precursors. *Lbx2* genes have a more widespread expression, labelling the dorsomedial as well as ventrolateral lips. The exceptions are mammals that have lost somitic *Lbx2* expression.

It is currently controversial whether the aforementioned genome duplications occurred before or after the gnathostome-agnathan split, whether an independent genome duplication occurred in agnathans, or whether in the agnathan lineage numerous individual genes were duplicated. Thus, the phylogenetic relationship of the only lamprey *lhx* gene identified so far, *lhx-A*, is unclear. Yet it is remarkable that this gene is expressed in all vll's along the body.

dml dorsomedial lips of the dermomyotome, *MMP* migratory muscle precursors, *vll* ventrolateral lips of the dermomyotome

Xenopus laevis, where body muscle is made from *lhx1* expressing muscle precursors that detach from the lateral lip edge of the somite (Martin and Harland 2001, 2006). Yet, the lamprey *lhx* homologue is expressed along the ventrolateral lip of all somites (Kusakabe et al. 2011). This raised the question whether the program for migratory muscle precursors arose much earlier than previously anticipated. Indeed, it has been suggested that when the somatopleura evolved as a separate layer of lateral mesoderm, it recruited programmes previously used to generate the dorsal and ventral fins—which are present already in agnathans (Freitas et al. 2006; reviewed in Freitas et al. 2014). Likewise, marker gene expression in the dorsomedial and ventrolateral tips of somites suggested that specific somitic programmes originally used to supply the musculature of those fins were established at this stage. Notably, the list of markers expressed in both tips includes *cMet* and the *Lhx1* paralog *Lhx2* (Neyt et al. 2000; Ochi and Westerfield 2009; Kanamoto et al. 2006; Yang et al. 1996; Fig. 6c, e and Fig. 7), exceptions are the mouse which has shed somitic *Lhx2* expression (Chen et al. 1999) and perhaps the frog for which no *lhx2* gene has been identified in the genome (Wotton et al. 2008). In zebrafish, knockdown of *lhx2* interferes with pectoral fin muscle development (Ochi and Westerfield 2009), and more amazingly, when a dominant negative form of *Lhx1* is introduced into limb-level chicken somites, muscle precursor migration can be rescued by co-expressing mouse *Lhx2* (K. Wotton and S. Dietrich, unpublished observations, Fig. 6g, Gi). This suggests that possibly already in agnathans, a programme utilising *lhx* genes may have been present at the dorsal and ventral extremes of somites that allowed their local dissipation and the release of cells into the dorsal and ventral fins. Yet, evidence is inconclusive: phylogenetic studies have not yet established the relationship of the lamprey *lhx* gene with the two gnathostome *Lhx* paralogs that arose from two rounds of genome duplication and subsequent gene loss (Wotton et al. 2008; Kusakabe et al. 2011). Moreover, careful functional studies on gnathostome *Lhx1* genes pointed at roles in controlling precursor cell proliferation and suppressing premature differentiation both in development and in activated satellite cells, rather than roles specific to cell migration (Mennerich and Braun 2001; Martin and Harland 2006; Watanabe et al. 2007). Finally, already in the protostome *Drosophila melanogaster*, ladybird/*lhx* function is associated with myogenesis (Jagla et al. 1998). Thus, gnathostome *Lhx* genes may be rather overrated as markers and may simply have a generic association with the generation of muscle precursors.

6 Hitching a Ride: The Development and Evolution of Hypopharyngeal and Tongue Muscle

During vertebrate evolution, the most rostral (occipital) somites were incorporated into the head (Gans and Northcutt 1983). Their sclerotomes were recruited to accommodate for larger brain sizes by reinforcing the base of the skull, and a

proportion of muscle precursors were deviated from making muscle for locomotion. Instead, these cells were recruited to contribute to the caudal pharyngeal arches and to provide an elaborate hypopharyngeal muscular system, all crucial in ventilating the gills and in food uptake (Goodrich 1958), recently reviewed in (Sambasivan et al. 2011). In lung-breathing tetrapods, the pharyngeal arches are not required for respiration any more, but the role in particular of the hypopharyngeal and, as an important component, the tongue muscles, remained crucial. Interestingly, in osteichthyans the hypopharyngeal/tongue muscle precursors undertake long-range migration, and they all express *Lbx1* (Dietrich et al. 1999; Lours-Calet et al. 2014; Martin and Harland 2006). Yet, in mouse mutants for the upstream regulator *Pax3*, in *cMet* and *SF/HGF* mutants as well as in *Lbx1* mutants, hypobranchial muscle formation is reduced, not abolished (Bladt et al. 1995; Prunotto et al. 2004; Schäfer and Braun 1999; Gross et al. 2000; Brohmann et al. 2000; Lours-Calet et al. 2014). Similarly, misexpression of a dominant negative *Lbx1* construct in occipital somites in the chicken only delayed the formation of hypopharyngeal muscle (Lours-Calet et al. 2014). Likewise, when somites normally producing non-migratory muscle precursors only, or head mesoderm that is unable to read signals for somitic myogenesis, were grafted in place of the somites normally providing hypobranchial muscle, the grafts contributed cells to this musculature (Mackenzie et al. 1998; Lours-Calet et al. 2014). Finally, the hypopharyngeal musculature of the lamprey is thought to derive from somitic extensions, not migratory cells (Goodrich 1958). Together, this suggests that there is an alternative, evolutionarily ancestral mechanism of hypopharyngeal muscle precursor transport that does not require active migration. Intriguingly, molecular markers for all occipital tissues and cells types extend their expression along the floor of the pharynx along the same path that is taken by the muscle precursors, and markers for the lateral mesoderm and overlying ectoderm precede those of other tissues (Lours-Calet et al. 2014). Lineage tracing experiments revealed that this extension of marker gene expression is due to cells moving along this circumpharyngeal path (Lours-Calet et al. 2014; Fig. 8). Specifically, the lateral mesoderm originating from the level of the first somite leads the procession, with the lateral mesoderm originating from the level of somite 2 following and embedding the hypopharyngeal muscle precursors; more caudal lateral mesoderm becomes displaced caudally. Studies on cells moving as a tissue sheet have shown that the moving sheet is able to drag non-migratory cells along (reviewed in (Montell 2008)). Thus, it can be speculated that the newly discovered occipital cell movements, deep in the evolution of vertebrates, provided the original transport system for the initially non-migratory hypopharyngeal muscle cells.

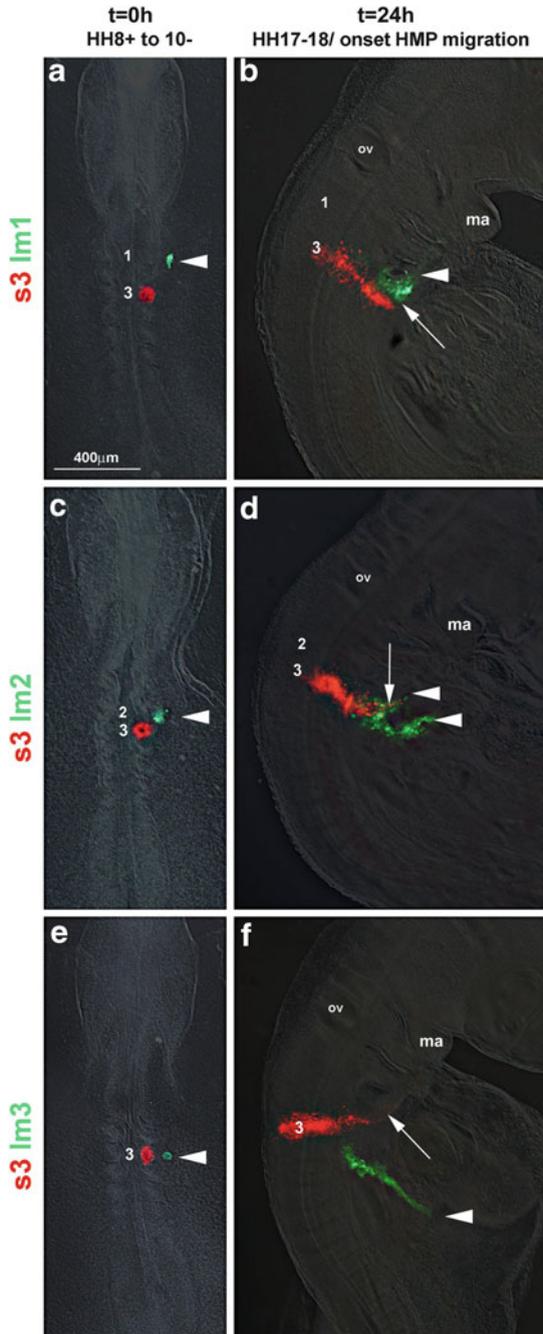


Fig. 8 An alternative mode of hypaxial cell transport at the head–neck interface. When genes required for the emigration of limb muscle precursors are mutated, limb muscles fail to form. Yet in most cases, hypobranchial muscle formation is merely delayed. This figure shows DiO-labelings (*green*) of the lateral mesoderm and DiI labelling (*red*) of a somite destined to produce

7 Outlook

Hypaxial muscles have been remodelled quite extensively during the evolution of vertebrates, and many aspects of their development have been deciphered. However, a number of questions, mainly surrounding migratory muscle precursors, remain: is it indeed possible that these cells evolved earlier than the emergence of osteichthyans, and is the underlying molecular programme an adaptation of programmes used for the dorsal and ventral fin muscles? And how does the formation of migratory muscle precursors for the fins/limbs relate to the release of individual cells to form the body wall muscle in frog (Martin and Harland 2001, 2006), the contribution of migratory cells to the rostral body wall in teleosts (Windner et al. 2011) or the de-epithelialisation of the somitic lips when the outgrowing somite reaches the ventral midline in amniotes (Christ et al. 1983)? Is it possible that, even though the cells destined to contribute to the body wall are mesenchymal, they migrate as sheet rather than individual cells? And what are the underlying molecular mechanisms? These questions may seem to have mainly academic merit, yet answers may provide knowledge and understanding for the therapy of birth defects such as gastroschisis or for the reconstruction or regeneration of muscle and limbs. Recently, it has been suggested that a mutation of the human *LBX1* gene may be responsible for the myopathy and severe vertebral column malformation in a patient (Fernandez-Jaen et al. 2014), reinforcing how basic research informs Medicine.

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Fig. 8 (continued) hypobranchial muscle precursors (HMP) in a 36 hours chicken embryo (**a,c,e**). The embryos were analysed 24 h later when hypobranchial muscle precursors start to emigrate (**b,d,f**). Notably, all occipital lateral mesoderm moves ventrolaterally. However, eventually the streams of cells deviate. Cells originating from a position next to the most rostral somite (somite 1) take a rostral path along the floor of the pharynx which anticipates the course of the HMP (**a,b**). Lateral mesoderm from the level of somite 2 contributes both to the rostrally and ventrally—caudally directed stream. HMP become embedded in the rostrally projecting stream (**c,d**). Cells from the level of the third somite project exclusively caudally and are out of the way when HMP start to emigrate (**e, f**). This suggests extensive cells movements at the head–trunk interface. It furthermore suggests that there is a rostrally directed stream that is suited to carry non-migratory cells along. This stream is conserved and may represent the evolutionarily ancestral way of muscle precursor transport. *lm* lateral mesoderm, *ma* mandibular arch, *ov* otic vesicle, *s* somite

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