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Distinct regulatory cascades for head and trunk myogenesis

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SUMMARY

Most head muscles arise from the pre-otic axial and paraxial head mesoderm. This tissue does not form somites, yet expresses the somitic markers *Lbx1*, *Pax7* and *Paraxis* in a regionalised fashion. The domain set aside by these markers provides the lateral rectus muscle, the most caudal of the extrinsic eye muscles. In contrast to somitic cells that express *Lbx1*, lateral rectus precursors are non-migratory. Moreover, the set of markers characteristic for the lateral rectus precursors differs from the marker sets indicative of somitic muscle precursors. This suggests distinct roles for *Lbx1*/*Pax7*/*Paraxis* in the development of head and trunk muscles. When grafted to the trunk, the pre-otic head mesoderm fails to activate *Lbx1*, *Pax7* or *Paraxis*. Likewise, somites grafted into the region of the lateral rectus precursors fail to activate the lateral rectus marker set. This suggests that distinct regulatory cascades act in the development of trunk and head muscles, possibly reflecting their distinct function and evolution.

Key words: Chick, Quail, Embryo, Head mesoderm, Somites, Skeletal muscles, Eye muscles, Lateral rectus, *Lbx1*, *Paraxis*, *Pax7*, *Myf5*, *MyoD*

INTRODUCTION

The striated or skeletal musculature serves crucial functions in the vertebrate body as it underlies the ability of movement. Head muscles, however, do not primarily participate in locomotion. Instead, they provide control over the gill apparatus and its derivatives in the branchial arches, they are crucial for mastication and, by rotating the eyeball, contribute to the function of the visual system. During vertebrate evolution, the cranial muscles experienced enormous diversification. It can therefore be assumed that they were as crucial for the success of vertebrates as the muscles providing mobility (reviewed by Goodrich, 1958).

Muscles in the trunk originate from somites: epithelially organised, metameric blocks of paraxial mesoderm (reviewed by Christ and Ordahl, 1995; Gossler and Hrabe de Angelis, 1998). In amniotes, the myogenic precursor cells reside in specialised somitic structures, the dermomyotomal lips. The medial dermomyotomal lips provide the non-migratory, epaxial muscle precursors, which generate the epaxial part of the myotome. The lateral dermomyotomal lips provide the non-migratory, hypaxial muscle precursors that constitute the hypaxial part of the myotome, along with muscle precursors that actively migrate to their target sites to generate the limb muscles and, in mammals, the muscular diaphragm (reviewed by Dietrich, 1999). The different epaxial and hypaxial precursor cells use distinct sets of control genes during their development. However, they all require the paired and homeobox-containing transcription factor *Pax3* as upstream regulator (reviewed by Dietrich, 1999). Likewise, in all lineages, differentiation is initiated by the transcription factors *Dach2*, *Six1* and *Eya2* (Heanue et al., 1999) and then governed by members of the *MyoD* family of transcription factors, which withdraw the cells from cell cycle, trigger the expression of muscle structural proteins, and finally permit the assembly of functional myofibres (reviewed by Molkentin and Olson, 1996).

Muscles in the head are heterogeneous with respect to both origin and regulatory mechanisms. Caudal to the otic vesicle, head muscles develop from the so-called occipital somites (Noden, 1983a; Wachtler and Jacob, 1986; Couly et al., 1992; Huang et al., 1999). These are the most cranial of the series and, during evolution, have been secondarily incorporated into the head (Gans and Northcutt, 1983). They provide the epaxial and hypaxial muscles of the neck, the pharyngeal and laryngeal muscles that develop in the caudal branchial arches and the musculature of the tongue (Noden, 1983a; Wachtler and Jacob, 1986; Couly et al., 1992; Huang et al., 1999). Despite their localisation in the head, myogenic precursors from occipital somites essentially follow the trunk programmes (E. H. Walters and S. D., unpublished). Cranial to the otic vesicle however, skeletal muscles develop from mesoderm that does not form appreciable somites, the pre-otic paraxial mesoderm and further cranially, the pre-chordal, axial mesoderm (Adelmann, 1926; Noden, 1983a; Jacob et al., 1984; Wachtler and Jacob, 1986; Couly et al., 1992; Hacker and Guthrie, 1998). These tissues provide the genuine head muscles, including all extrinsic eye muscles, and, in addition, the jaw, facial and the most anterior pharyngeal muscles, which develop in the core of the first three branchial arches.

Owing to the obscure organisation of the pre-otic head mesoderm, the development of its muscular derivatives has been conversely debated ever since 'head vertebrae' or head somites

were proposed by Oken (Oken, 1807) and Goethe (Goethe, 1820) (reviewed by Goodrich, 1958). In a modification of this model, vesicular structures within the head mesoderm of many vertebrate species, the 'head cavities', have been suggested as head somites. Here, cranial muscles are seen as head myotomes that are serially homologous with the somitic myotomes in the trunk (Adelmann, 1926). This model, however, has been rejected on the basis of profound morphological differences between head cavities and somites (reviewed by Wachtler and Jacob, 1986). Nevertheless, the segmentation model saw its revival when swirls of mesodermal cells visible on electron micrographs were interpreted as cryptic head somites or 'somitomeres' (Meier, 1979; Meier and Tam, 1982). Interestingly, genes that drive mesoderm segmentation in the trunk are absent from the pre-otic mesoderm in the head (G. Parkyn and S. D., unpublished). Thus, this mesoderm either never truly possessed metamerism inherent to trunk paraxial mesoderm or such properties have been shed from the head over the course of evolution. Despite the arguments for and against segmentation, it is clear that owing to the absence of somites, the pre-otic mesoderm never forms dermomyotomal lips. This suggests that the mechanisms that underlie somitic and non-somitic muscle development may be fundamentally different.

Unfortunately, the regulation of muscle development from pre-otic mesoderm is largely enigmatic. Cranial neural crest cells, which provide all the connective tissue and tendons in the head, have been suggested to pattern and shape the individual cranial muscle anlagen (Noden, 1983b; Noden, 1986; Köntges and Lumsden, 1996). Recent work has also established that cranial muscles, similar to their trunk counterparts, use MyoD family members to control differentiation (Hacker and Guthrie 1998; Noden et al., 1999). However, no candidate upstream regulators for these processes have been identified. Significantly, *Pax3* is not expressed in the pre-otic mesoderm (Hacker and Guthrie 1998) (this study), and no muscular defects are found in the head of splotch (*Pax3*$^{-/-}$) mutant mice (Franz et al., 1993; Tajbakhsh et al., 1997; Tremblay et al., 1998). Thus, entirely different regulatory cascades may serve to govern trunk (somitic) and cranial (pre-otic, non-somitic) myogenesis.

The aim of this study is to shed light onto the regulation of pre-otic muscle formation and to address, whether or not vertebrate myogenesis proceeds according to a universal scheme. We demonstrate for the first time that a set of upstream regulators for trunk myogenesis is present in the avian pre-otic mesoderm. This marker set labels a single head muscle only. Significantly, the combination of markers differs considerably from the marker combinations characteristic for epaxial or hypaxial myogenic programmes in the trunk. Despite the presence of somitic markers, the head mesoderm fails to read patterning cues in a somitic environment. Likewise, somites are unable to obey signals in the head properly. This suggests that head muscle formation is governed by head-specific regulatory cascades, which are fundamentally distinct from regulatory cascades in the trunk.

MATERIALS AND METHODS

Embryos and microsurgery

Fertilised hens' eggs (Winter Farm, Royston) and quails' eggs (Potter Farm, Woodhurst), were incubated at 38.5°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Four types of in ovo microsurgery were carried out, using flame-sharpened tungsten needles (Dietrich et al., 1997): (1) as a control, segmental plate or epithelial somites from forelimb levels of HH12 quail embryos were orthotopically grafted into stage-matched chick hosts (n=6); (2) As further control, pre-otic paraxial mesoderm from rhombomere 2 levels of HH8-10 quails was orthotopically grafted into stage-matched chick hosts (n=3); (3) HH8-10 quail head mesoderm was grafted in place of HH12 chick forelimb paraxial mesoderm (n=12); (4) HH12 quail forelimb paraxial mesoderm was grafted in place of HH8-10 chick head mesoderm at the level of rhombomere 2 (n=17). The eggs were then incubated for further 24-48 hours to reach HH18-20.

DiI injections

DiI labelling experiments were performed on HH8-8^{+} embryos, i.e. before the onset of cranial neural crest cell migration (Lumsden et al., 1991). Fixable DiI (Molecular Probes) at 3 mg/ml in dimethylformamide was pressure injected into the right pre-otic paraxial mesoderm. The axial level was recorded by labelling the neural plate on the left side of the embryo. The eggs were re-incubated for further 36-48 hours to reach HH16-18.

In situ hybridisation

Double whole-mount in situ hybridisation was carried out according to Dietrich et al. (Dietrich et al., 1997; Dietrich et al., 1998), with a detergent mix (1% IGEPAL, 1%SDS, 0.5% deoxycholate, 50 mM Tris pH8, 1 mM EDTA, 150 mM NaCl) replacing proteinase K. Probes and their expression patterns are detailed elsewhere: *Dach2* (Heanue et al., 1999); *Isl1* (Tsuchida et al., 1994); *Lbx1* (Dietrich et al., 1998); *Myf5* (Saitoh et al., 1993); *MyoD* (Bober et al., 1994); *Noggin* (Hirsinger et al., 1997), *Paraxis* (Šošić et al., 1997); *Pax3* and *Pax7* (Goulding et al., 1994); *Pitx2* (Yoshioka et al., 1998); *Pitx3* (unpublished probe, kindly provided by S. Noji); *R-Cadherin* (unpublished PCR product); *Sim1* (Pourquié et al., 1996); *Six1* (Heanue et al., 1999); *Tbx3* (Huang et al., 1999); and *Wnt11* (Tanda et al., 1995).

Immunohistochemistry

Upon in situ hybridisation, whole-mount immunohistochemistry was carried out according to Guthrie and Lumsden (Guthrie and Lumsden, 1992). Axonal staining was performed using the RMO-270 antibody (Zymed) which recognises the 155 kDa intermediate neurofilament subunit. Quail tissues were identified using the QCPN antibody (Developmental Studies Hybridoma Bank). Primary antibodies were detected using anti mouse IgG conjugated with horseradish peroxidase (Dako).

Sectioning

Embryos were embedded in 20% gelatine at 4°C, fixed in 4% paraformaldehyde and sectioned at 50 μm on a Pelco 1000 Vibratome.

Photomicroscopy

After in situ hybridisation/immunohistochemistry, embryos were cleared in 80% glycerol/phosphate-buffered saline. Whole-mounted embryos older than HH18 were split midsagitally prior to analysis. Embryos and sections were photographed on a Zeiss Axiophot, using Nomarski optics.

RESULTS

Regulatory cascades for muscle development in the trunk, i.e. from somites, are well characterised. However, the formation of genuine head muscles, i.e. those derived from the pre-otic,

non-somitic mesoderm, is obscure, the main obstacle being that upstream regulators over their development have not been identified. We therefore set out to discover possible regulators for cranial myogenesis, and further, to establish whether universal or unique head specific cues are necessary for their development.

Cranial expression pattern of the migratory muscle precursor marker *Lbx1*

The homeodomain containing transcription factor *Lbx1* is the only known marker specific for somite-derived, migratory muscle precursors (reviewed by Dietrich, 1999). In addition to this expression in the trunk, we have recently demonstrated that *Lbx1* identifies a subset of hindbrain interneurons (Schubert et al., 2001). During the course of our analyses, we uncovered a further prominent site of *Lbx1* expression in the avian head: from HH16 onwards, *Lbx1* labels a small territory within the cranial chick and quail mesenchyme, midway between the otic vesicle and the mesencephalon (Fig. 1A, and not shown). This pre-otic mesenchyme never forms somites (reviewed by Wachtler and Jacob, 1986). However, the restricted expression of *Lbx1* infers that from HH16 onwards, the pre-otic cranial mesenchyme is regionalised.

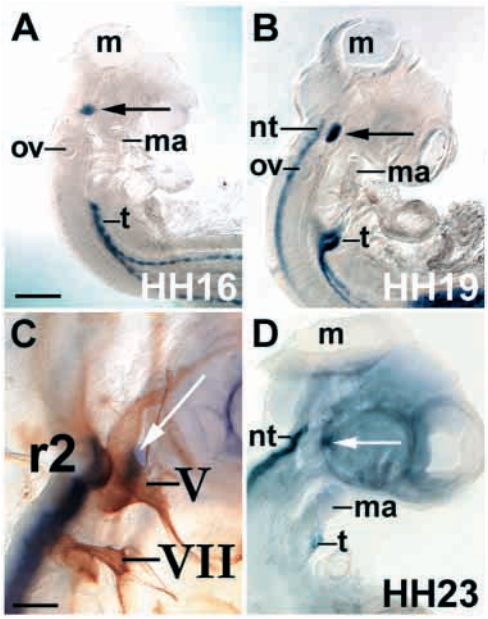

Fig. 1. Expression of *Lbx1* in the pre-otic head mesenchyme. (A-D) HH16-23 chick heads stained for *Lbx1* expression; lateral views, anterior towards the top. (A) *Lbx1* expression in the head mesenchyme rostral to the otic vesicle commences at HH16 (arrow). (B) At HH19, while *Lbx1*-expressing, somitic tongue muscle precursors (t) migrate towards the mandibular arch, *Lbx1* cells in the cranial mesenchyme remain in residence (arrow). (C) Higher magnification of the *Lbx1* domain at HH20. Using an anti neurofilament antibody to identify the cranial ganglia (brown), we located the *Lbx1*-positive cells (arrow) beneath the developing trigeminal (Vth) ganglion at the axial level of rhombomere 2 (r2). (D) The cranial *Lbx1* spot is still in the same location by HH23, by which time it has been overgrown by the eye. m, midbrain; ma; mandibular arch; nt, neural tube; ov, otic vesicle; r2, rhombomere 2; t, tongue muscle precursors; V, trigeminal ganglion; VII, facial ganglion. Scale bars: 500 μm in A,B,D; 200 μm in C.

In the trunk, somitic cells expressing *Lbx1* migrate into the periphery as shown for the tongue muscle precursors at HH19 (Dietrich et al., 1998) (Fig. 1B, t). By contrast, the *Lbx1* domain seen in the pre-otic head mesenchyme remains in residence at least until HH23 (Fig. 1A-D, arrows). Hence, in the head, *Lbx1*-expressing cells are non-migratory. Tracing out the developing nervous system with an anti-neurofilament antibody precisely mapped the expression domain of *Lbx1* to beneath the trigeminal ganglion, adjacent to rhombomere 2 (Fig. 1C). By HH23, the eye has grown in size considerably, and by this point has come to overlie this site (Fig. 1D). This suggests that cells expressing *Lbx1* may play a role in the assembly of the visual apparatus.

Origin of *Lbx1* cells in the pre-otic mesenchyme

At the time that *Lbx1* is expressed, the pre-otic head mesenchyme comprises cells from three sources, the pre-otic paraxial mesoderm, the pre-chordal axial mesoderm and also neural crest cells. Cells from each lineage have distinct fates (Noden, 1983a; Wachtler and Jacob, 1986; Couly et al., 1992). To uncover which processes *Lbx1* may be involved in, we needed to establish the exact derivation of the *Lbx1*-expressing cells. Therefore, we injected the fluorescent cell tracer DiI into the head mesoderm at the right side of HH8 embryos, before the onset of cranial neural crest cell emigration. To record the axial level, a second injection was made in the left side of the neural plate at the corresponding position, carefully avoiding the neural crest cell precursors in the neural folds (Fig. 2A). Thirty-six to 48 hours later, we analysed by in situ hybridisation which of the labellings coincided with *Lbx1* expression (Fig. 2B-E). Injections delivered at the level of the prospective posterior midbrain labelled cells cranial to our target area (n=8; Fig. 2B). Injections at the level of the future rhombomeres 3-4 labelled cells caudal to the *Lbx1* domain, eventually entering the hyoid arch (n=21; Fig. 2C). For injections placed adjacent to rhombomere 1, fluorescence was detected at the cranial margin of the *Lbx1* domain (n=6; data not shown). Finally, injections at the level of rhombomere 2 (n=44) coincided with the site of *Lbx1* expression, provided the injections was made close to the neural epithelium (n=13; Fig. 2D,E). Thus, the *Lbx1*-expressing cranial mesenchyme stems from the medial aspect of the pre-otic paraxial mesoderm at the level of rhombomere 2.

Comparative expression analysis of *Lbx1* and markers for somitic mesoderm

In the trunk, paraxial mesoderm expressing *Lbx1*, namely the migratory hypaxial muscle precursors, co-expresses other markers (reviewed by Dietrich, 1999). These are markers for the somitic dermomyotome, for the hypaxial or lateral somite half, and for cells in the dermomyotomal lips committed to a myogenic fate. Despite the absence of somite formation in the head and the fact that in the pre-otic mesoderm, *Lbx1* labels medial non-migratory cells, it still remains possible that a similar set of regulatory genes acts together with *Lbx1* throughout the paraxial mesoderm as a whole. With this in mind, we performed a comparative expression analysis (Fig. 3) with *Lbx1* expression shown in the centre (Fig. 3I,J), using whole-mount in situ hybridisation and vibratome sectioning. To visualise anatomical landmarks, the cranial nerves were labelled in red using a probe for *Islet1* (Tsuchida et al., 1994).

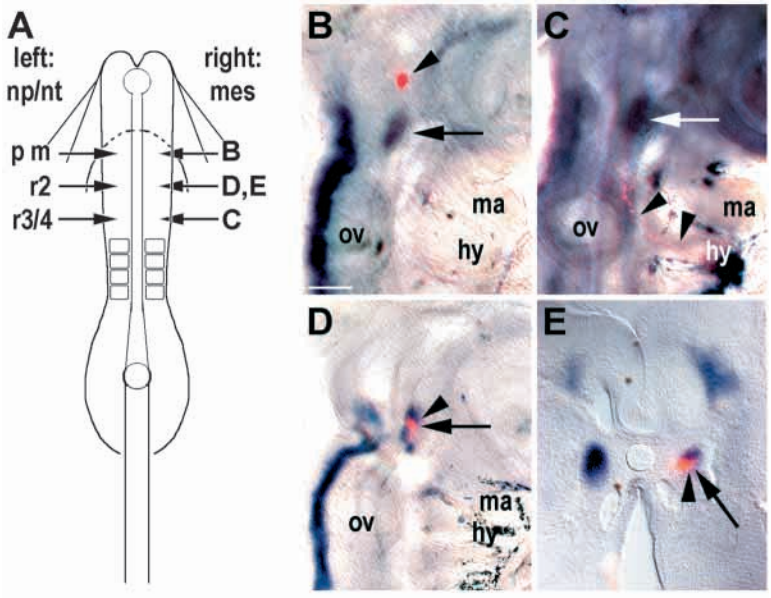

Fig. 2. Mesodermal origin of the *Lbx1*-expressing pre-otic mesenchyme. (A) DiI labellings at HH8-8^{+}, before cranial neural crest cell migration. The cranial paraxial mesoderm was labelled on the right side at the axial levels indicated. To record the position of the injection, a further injection was made in the neural plate. (B-D) Lateral views on the trigeminal region of chick heads, analysed for *Lbx1* expression (blue) 36-48 hours after DiI injection (red). (B) Mesoderm labelled at the level of the posterior midbrain (arrowhead) resides anterior to the *Lbx1* domain (arrow). (C) Mesoderm labelled at the level of rhombomeres 3/4 is seen posterior to the *Lbx1* domain (arrow), migrating into the hyoid arch, (hy, arrowheads). (D) The fluorescent signal coincides with *Lbx1* expression when mesoderm was labelled at the level of rhombomere 2 (arrowhead and arrow). (E) Vibratome cross-section through the embryo shown in D, confirming co-localisation of *Lbx1* and DiI signals. hy, hyoid arch; np/nt, neural plate/neural tube; ma, mandibular arch; mes, mesoderm; ov, otic vesicle; r, rhombomere. Scale bar in B: 200 μm in B-D; 100 μm in E.

Our analysis focused on HH19/20 embryos, at which point cranial *Lbx1* expression is firmly established and readily detectable.

Comparison with markers for the somitic dermomyotome

In the somitic dermomyotome, *Lbx1* expression in the lateral dermomyotomal lips overlaps with the expression domains of the paired- and homeodomain transcription factor *Pax3*, its paralogue *Pax7*, and the basic helix loop helix transcription factor paraxis (Goulding et al., 1994; Šošić et al., 1997), with *Pax3* serving as upstream regulator for *Lbx1* (reviewed by Dietrich, 1999). In the pre-otic paraxial mesoderm, however, we failed to observe any expression of *Pax3* (Fig. 3A,B), in line with previous studies (Tajbakhsh et al., 1997; Hacker and Guthrie, 1998; Tremblay et al., 1998). Nevertheless, *Pax7* (Fig. 3C,D) and *Paraxis* (Fig. 3E,F) were expressed. Significantly, expression of both markers coincided with *Lbx1* signals beneath the trigeminal, with all other areas in the pre-otic head mesoderm negative. Thus, it is possible that in the cranial paraxial mesoderm, the same dermomyotomal regulators act upstream of *Lbx1*, with *Pax7* substituting for *Pax3*.

Comparison with markers for the epaxial and hypaxial programmes of the somite

In the trunk, the function of *Lbx1* lies within the hypaxial programme of the somite. Cells that follow this express the lateral somite marker *Sim1*, a basic helix-loop-helix transcription factor (Pourquié et al., 1996), but lack the signalling molecule *Wnt11* and the BMP antagonist *Noggin* which mark epaxial muscle precursors in the medial dermomyotomal lips (Hirsinger et al., 1997; Marcelle et al., 1997). In the head, neither gene showed expression that was similar to their trunk profiles. *Sim1* displayed ubiquitous staining throughout the pre-otic head mesenchyme (Fig. 3G). The highest levels of expression were seen immediately adjacent to the ventral neural tube, while the *Lbx1* positive area beneath the trigeminal showed insignificant expression levels only (compare Fig. 3H with 3J). *Wnt11* was absent from cranial mesoderm. Instead, signals were found in the surface ectoderm, in a crescent around the eye (Fig. 3K,L). Finally, no appreciable levels of expression were found for *Noggin* (not shown). Therefore, it appears that in the pre-otic paraxial mesoderm, no trunk-like, molecular distinction is established between medial and lateral territories.

Comparison with markers for myogenic precursor cells

In the trunk, myogenic cells in both the medial and lateral dermomyotomal lips express the transcription factors *Tbx3* (Huang et al., 1999), *Dach2* (Heanue et al., 1999), *Six1* (Oliver et al., 1995; Heanue et al., 1999) and *Pitx2* (L. Cheng, R. C. M. and S. D., unpublished). Expression of *Six1* and *Pitx2* continues when the cells enter the myotome while *Lbx1*-positive, migratory limb muscle precursors harbour *Six1* transcripts only, with *Dach2* and *Pitx2* joining in once the target sites are reached (Heanue et al., 1999) (L. Cheng, R. C. M. and S. D., unpublished). Ultimately, *Dach2* and *Six1* cooperate with the transcription factor *Eya1*, to trigger myogenic differentiation (Heanue et al., 1999). In the pre-otic mesoderm, *Lbx1* expression was not associated with a particular combination of markers for myogenic precursor cells. *Tbx3* was not expressed in the mesoderm at all, but similar to *Pax3*, stained the trigeminal ganglion (not shown). *Dach2* labelled the interface between the trigeminal ganglion and the hindbrain (Fig. 3M,N, arrows) and a crescent of mesenchyme around the eye. *Six1* was evenly expressed throughout the head mesenchyme and the trigeminal ganglion (Fig. 3O,P) as opposed to restricted expression beneath the trigeminal. The only marker to share the *Lbx1* domain was *Pitx2* (Fig. 3Q,R, arrows), which in addition labelled the mesenchyme around the eye, and further domains beneath the eye and within the mandibular arch, in line with findings in the mouse (Gage et al., 1999; Kitamura et al., 1999).

Comparative expression analysis of *Lbx1* and markers for myogenic differentiation

Our analysis revealed so far that despite the presence of some dermomyotomal markers, the *Lbx1*-expressing pre-otic mesoderm subscribes neither to the epaxial nor the hypaxial

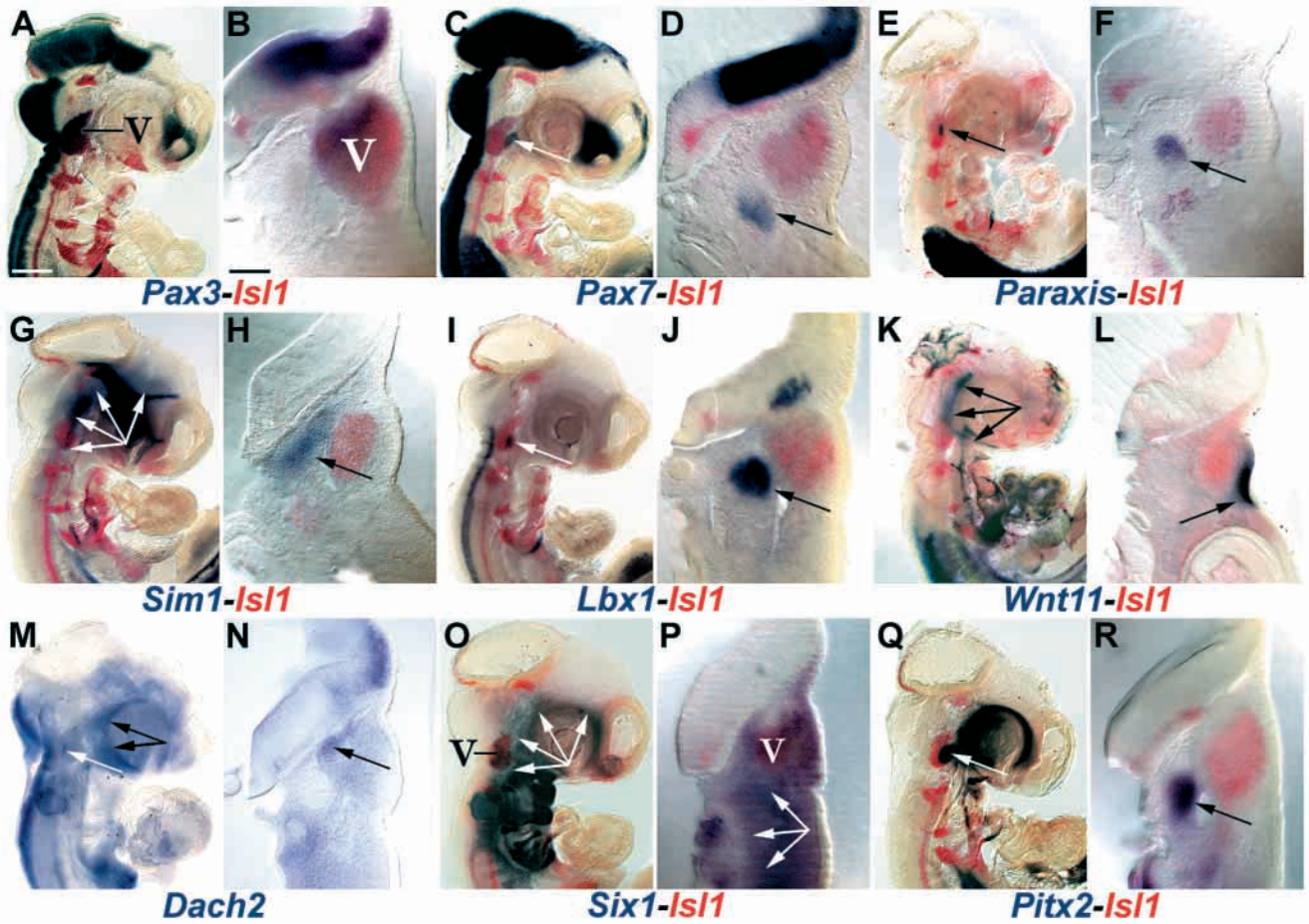

Fig. 3. Comparison between *Lbx1* and markers for the somitic dermomyotome, epaxial/hypaxial programmes and myogenic precursors at HH19/20. Lateral views of chick heads (A,C,E,G,I,K,M,O,Q) and cross sections at rhombomere 2 levels (B,D,F,H,J,L,N,P,R). To facilitate comparison, *Lbx1* expression is shown in the centre of the figure (I,J). To provide anatomical landmarks, *Isl1* was used to stain the cranial ganglia in red (except M,N). (A,B) *Pax3*, a master regulator of trunk myogenesis, is not expressed in the head mesoderm. (C,D) *Pax7* and (E,F) *Paraxis*, co-expressed with *Lbx1* during hypaxial muscle precursor migration in the trunk, coincide with *Lbx1* in the pre-otic mesoderm (arrows). (G,H) The hypaxial programme marker *Sim1* is expressed throughout the cranial mesenchyme (arrows). (H) Note that *Sim1* expression is highest next to the neural tube, avoiding the territory beneath the Vth ganglion (arrow). (K,L) The epaxial programme marker *Wnt11* is absent from the head mesoderm, instead labelling in the ectoderm around the eye (arrows). (M-R) Cranial expression patterns for the myogenic markers *Dach2*, *Six1* and *Pitx2*. (M) *Dach2*, besides signals in the peri-optic mesenchyme (black arrows) appears to be expressed underneath the trigeminal ganglion (white arrow). Cross-sections show that staining resides at the interface between the trigeminal ganglion and the hindbrain (N, arrow). (O,P) *Six1* shows widespread expression throughout the head mesenchyme (arrows). (Q,R) *Pitx2*, besides the peri-optic mesenchyme, shows prominent expression in the pre-otic mesoderm under the trigeminal (arrows). V, trigeminal ganglion. Scale bars: in A, 500 μm for A,C,E,G,I,K,M,O,Q; in B, 100 μm in B,D,F,H,J,L,N,P,R.

programme of myogenesis (summarised in Table 1). Significantly, factors initiating myogenic differentiation are also absent. Therefore, it remained open whether the *Lbx1* positive head mesoderm awaits a myogenic or alternatively, a skeletogenic fate (Noden, 1983a; Couly et al., 1992). To discriminate between both possibilities, we compared the expression pattern of *Lbx1* and markers for differentiating myoblasts: *Myf5*, *MyoD*, *Pitx3* and *R-Cadherin*. In the trunk, the helix-loop-helix muscle determining factors *Myf5* and *MyoD* label differentiating, post-migratory and post-mitotic myoblasts (Pownall et al., 1992). The homeodomain-containing transcription factor *Pitx3* stains the myotome, and in addition the rostral and caudal lips of the dermomyotome which have been proposed to generate a late wave of mitotically active myoblasts (Cinnamon et al., 2001) (L. Cheng, R. C. M. and S. D., unpublished), while the cell adhesion molecule *R-Cadherin* marks the mediolateral dermomyotomal lips and the myotome, thus more closely resembling *Myf5* (Inuzuka et al., 1991; Rosenberg et al., 1997). In developing head muscles, all four genes are active (Hacker and Guthrie, 1998; Noden et al., 1999) (this study). The first to be expressed is *Myf5* which at HH19-20, highlights all head muscle precursors in the process of differentiation, encompassing the muscle primordia of the first three branchial arches, and four of the six extrinsic eye muscles (Noden et al., 1999) (Fig. 4A,B). Double staining with *Isl1* revealed that one of the *Myf5* sites resided beneath the trigeminal ganglion, in the same position as the *Lbx1* signal (Fig. 4A,B, arrows). *MyoD* (Fig. 4C,D), *Pitx3* (Fig. 4F,G) and *R-Cadherin* (Fig. 4H,I), all closely resembled this result albeit with a delay in initiation of

Table 1. Comparison of marker gene expression in trunk (somitic, post-otic) and genuine (non-somitic, pre-otic) head muscle precursors

Marker	Predominant site(s) of expression	Epaxial	Hypaxial nonmigratory	Hypaxial migratory	Lateral rectus	Other oculorotatory or branchial arch muscles
Markers for skeletal muscle precursors						
Pax3	DM	+	+	+	–	–
Pax7	DM	+	+	+	+	–
Paraxis	DM	+	+	+	+	–
Sim1	Lateral somite*	–	+	+	+†	+†
Lbx1	MMP	–	–	+	+	–
Wnt11	m DML	+	–	–	–	–
Noggin	m DML	+	–	–	–	–
Tbx3	m,l DML	+	+	–‡	–	–
Dach2	m,l DML	+	+	–§	–	–
Six1	m,l DML; M	+	+	+	+†	+†
Pitx2	m,l DML; M	+	+	–‡,§	+	+
R-Cadherin	m,l DML; M	+	+	–‡	+	+
Pitx3	r,c DML; M	+	+	–‡	+	+
MyoD family members						
Myf5	m,l DML; M	+	+	+§	+	+
MyoD	M	+	+	+§	+	+

*Lateral aspect of somitic dermomyotome and sclerotome
†Ubiquitous expression in head mesenchyme
‡Expression in tongue muscle precursors only
§After migration is completed
c, caudal; DM, dermomyotome; DML, dermomyotomal lips; l, lateral; m, medial; M, myotome; r, rostral.

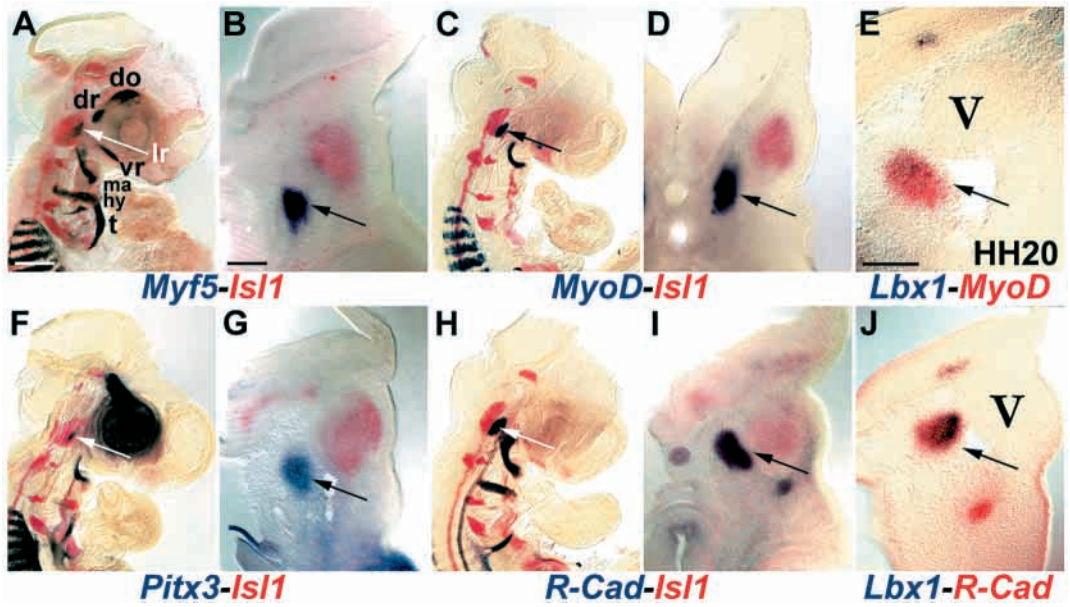

Fig. 4. Myogenic differentiation markers co-localise with cranial *Lbx1* expression. Lateral views and cross sections of HH19/20 chick heads as in Fig. 3. Cranial ganglia in A-D,F-I are highlighted with *Isl1* (red). (A,B) *Myf5* labels all cranial muscle precursors in the process of differentiation. Note that beneath the trigeminal ganglion, *Myf5* stains the anlage of the lateral rectus muscle (arrow, lr). (C,D) *MyoD*, (F,G) *Pitx3* and (H,I) *R-Cadherin* all resemble the expression pattern of *Myf5*, with expression evident in the cells beneath the trigeminal ganglion, and in the mandibular and hyoid arches. (E,J) Double labelling depicting *Lbx1* in blue and *MyoD* (E) or *R-Cadherin* (J) in red. Note that transcripts for both myogenic markers and *Lbx1* co-localise (arrows). do, dorsal oblique; dr, dorsal rectus; hy, hyoid arch; lr, lateral rectus; ma, mandibular arch; t, tongue muscle precursors; vr, ventral rectus. Scale bars: in A, 500 μm in A,C,F,H; in B, 100 μm in B,D,G,I; in E, 100 μm in E,J.

expression. To provide direct evidence that *Lbx1* and the myogenic differentiation markers colocalise, we simultaneously detected the transcripts for *Lbx1* together with *MyoD* (Fig. 4E) or *R-Cadherin* (Fig. 4J). We found expression of the three genes confined to the same location beneath the trigeminal (Fig. 4E,J, arrows), confirming that *Lbx1* indeed highlights muscle precursors. Significantly, *Lbx1* and the myogenic differentiation markers overlap at this site

only, indicating that by the means of *Lbx1* expression, a subpopulation of cranial muscle precursors is singled out.

Identity of *Lbx1*-expressing cranial muscle precursors

All head muscles are innervated in a distinct manner by the cranial nerves (reviewed by Goodrich, 1958). Hence, we used this information to establish the identity of the *Lbx1*-expressing subpopulation of cranial muscle precursors. The best candidate was the caudal-most extrinsic eye muscle, the lateral rectus, which in birds divides into the lateral rectus proper, and the muscles moving the nictitating membrane, the pyramidalis and quadratus muscles. These muscles have been shown to arise from a common primordium beneath the trigeminal ganglion (Adelmann, 1926; Noden, 1983a; Jacob et al., 1984; Wachtler and Jacob, 1986; Couly et al., 1992; Hacker and Guthrie, 1998). They are innervated by the abducens nerve, cranial nerve VI, with the abducens proper innervating the lateral rectus, and its branch, the accessory abducens, innervating the pyramidalis and quadratus muscles (Wahl et al., 1994).

Visualising the axons of the developing nervous system with an anti-neurofilament antibody after whole-mount in situ hybridisation for *Lbx1*, we found that at HH18, the *Lbx1* domain was yet to be innervated (not shown). From HH20 onwards, however, the abducens nerve was directly connected to the *Lbx1*-positive cells (Fig. 5A,B). Internal views of bisected heads verified that the abducens nerve whose roots lie in rhombomeres 5 and 6 (Fig. 5A, arrowheads), targeted the further anterior *Lbx1* domain at the level of rhombomere 2 (Fig. 5A,B arrow). This is clear proof that *Lbx1*-positive cells in the cranial paraxial mesoderm are the progenitors of the lateral rectus muscle. Higher magnification shows that some axons leave the abducens proper to form the accessory abducens (Fig. 5B, small arrows). These axons circumvent the *Lbx1* domain. Thus, the precursors for the quadratus and pyramidalis muscles either downregulated or never expressed *Lbx1*, so that by HH20, *Lbx1* labels the lateral rectus exclusively.

Localisation of signals required for cranial muscle development

Thus far, we have shown that among the muscles developing from the pre-otic, non-somitic mesoderm, the lateral rectus anlage selectively expresses a set of upstream regulators for somitic myogenesis: namely *Lbx1*, *Pax7* and *Paraxis* (Table 1). This suggests that essentially the same regulatory cascades control the development of trunk muscles and, at the very least, of one head muscle. This idea implies that somitic programmes were initially present throughout the vertebrate paraxial mesoderm, but during evolution were lost from the pre-otic head mesoderm, with the exception of the lateral rectus anlage. Alternatively, regulators for trunk myogenesis may have been secondarily recruited into the head for a specific and solitary aspect of cranial muscle development. In this instance, cascades for trunk and head myogenesis would be fundamentally different. To discriminate between both possibilities, we heterotopically grafted head mesoderm into the trunk (n=12; Fig. 6B-E), and vice versa, trunk mesoderm into the head (n=17; Fig. 6G-J). In case regulatory cascades were shared between head and trunk, the grafts would express the common set of markers correctly. However, if regulatory cascades leading to *Lbx1/Pax7/Paraxis* expression were distinct, heterotopic grafting would prevent marker gene expression at the new site. As controls, head mesoderm (n=3) and trunk mesoderm (n=6) were grafted orthotopically, leading to wild-type expression patterns (Fig. 6A,F). To facilitate the detection of the grafted tissues, transplants were taken from quail embryos.

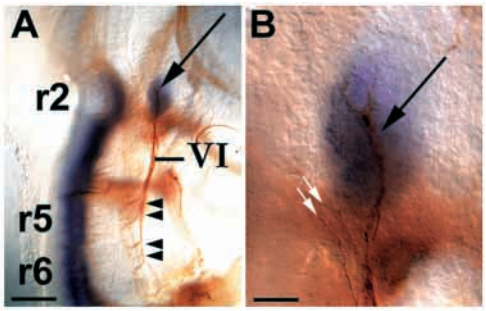

Fig. 5. Identity of *Lbx1*-expressing head muscle precursors. Internal views of HH20 bisected chick heads, stained for *Lbx1* (blue) and an anti-neurofilament antibody (brown). (A) The abducens nerve (cranial nerve VI) axons, with nerve rootlets in rhombomeres 5 and 6 (arrowheads), has innervated the *Lbx1* domain (arrow). (B) Higher magnification of the same embryo demonstrates that the accessory branch of the abducens (small arrows) avoids the *Lbx1* domain (large arrow). This indicates that at HH20, *Lbx1* labels the lateral rectus extrinsic eye muscle, but not the pyramidalis and quadratus muscles. Rhombomeres denoted by r2, r5 and r6; VI, abducens nerve. Scale bars are: 200 μm in A; 50 μm in B.

When at forelimb levels, pre-otic head mesoderm was grafted in place of somitic mesoderm, *Lbx1* (Fig.6B) *Pax7* (Fig.6C) and *Paraxis* (Fig. 6D) were not expressed in the graft. Moreover, *Myf5* signals were also absent (Fig. 6E), as were signals for the trunk-specific dermomyotomal marker *Pax3*, and the sclerotomal marker *Pax1* (data not shown). Despite this, the quail-specific antigen detected by the QCPN antibody was always present (Fig. 6, brown staining), demonstrating the viability of the grafted tissue. Thus, in the trunk environment, the grafted head mesoderm failed to interpret the surrounding patterning cues, and neither head-specific nor trunk-specific programmes was activated. Overall, this suggests that head specific as opposed to universal regulatory cascades govern myogenesis in the head.

When epithelial somites (n=10) or segmental plate mesoderm (n=7) from forelimb levels was grafted into the position of the lateral rectus precursors next to rhombomere 2, *Pax7* (Fig. 6H), *Paraxis* (Fig. 6I) and *Myf5* (Fig. 6J) were expressed throughout the graft, accompanied by *Pax3* and *Pax1* (Hacker and Guthrie, 1998) (data not shown). All markers were expressed in a segmented fashion as opposed to a localised signal typified by the lateral rectus primordium. Moreover, *Lbx1*, normally expressed in forelimb somites, was consistently absent (Fig. 6G). Thus, signals that are interpretable by the somitic mesoderm can be found in the head. However, the combination and pattern of markers present in the graft suggests that the medial/epaxial programmes of somite development were activated, while both the hypaxial somitic programmes and the programme for lateral rectus development failed. We conclude then that despite the fact that ‘trunk genes’ are used during the development of a single head muscle, the

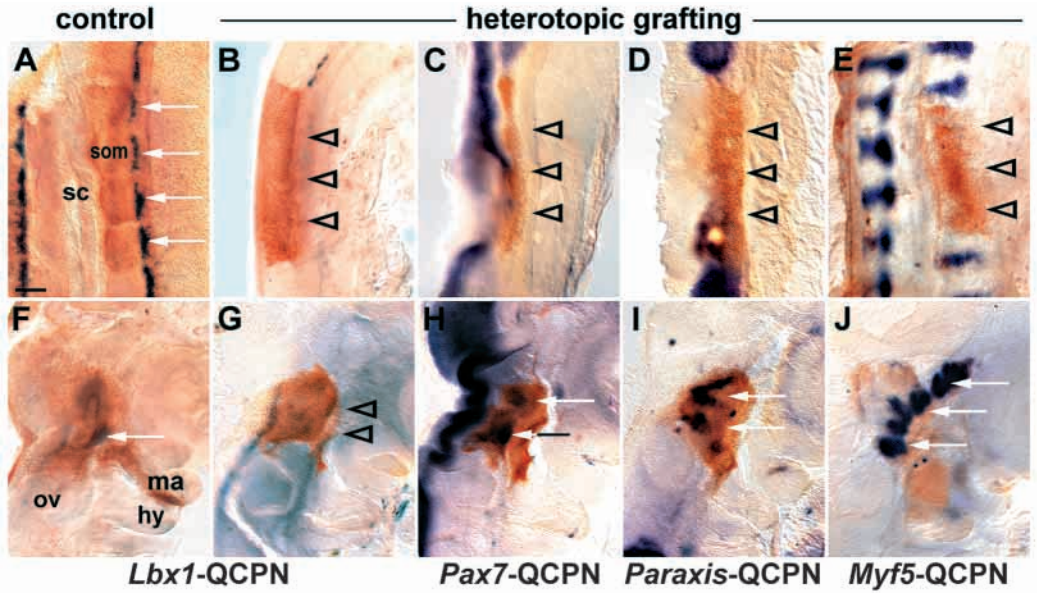

Fig. 6. Heterotopic grafting experiments reveal head-specific cues for lateral rectus development. (A-E) Dorsolateral view of chick embryos whose somites at forelimb levels were replaced (A) orthotopically with quail somites or (B-E) heterotopically with quail pre-otic mesoderm. (F-J) Lateral view of the trigeminal area of chick embryos whose head mesoderm at the level of rhombomere 2 was (F) replaced orthotopically with quail head mesoderm or (G-J) heterotopically with quail somites from forelimb levels. Quail tissues were detected in brown, using the QCPN antibody, in addition to blue staining for *Lbx1* (A,B,F,G), *Pax7* (C,H), *Paraxis* (D,I) and *Myf5* (E,J). Note that orthotopic grafting results in normal marker gene expression (A,F, arrows). Heterotopic grafting of head mesoderm into the trunk prevents marker gene expression, indicating that the graft is deaf to signals that pattern the somite (B-E, arrowheads). Somites transplanted into the head express *Pax7* (H), paraxis (I) and *Myf5* (J) in a segmented fashion (arrows) with *Lbx1* always absent (G, arrowheads). Thus, the ectopic somites show marker gene expression reminiscent of the epaxial half of the somite. hy, hyloid arch; ov, otic vesicle; ma, mandibular arch; sc, spinal cord; som, somites. Scale bar: 200 μm.

lateral rectus, these genes act as part of a distinct regulatory network.

DISCUSSION

The vertebrate head muscles are classically grouped according to the anatomical structures they associate with (reviewed by Goodrich, 1958). Thus, the six extrinsic eye muscles that liaise with the eyeball fall in one group, the branchiomeric muscles constitute the second group, the tongue muscles associated with the floor of the branchial arches form the third and the head-borne muscles that connect to the shoulder girdle form the fourth group. In contrast to the situation in the trunk muscles, the connective tissue and tendons in all head muscles are generated by neural crest cells (Noden, 1983a; Noden, 1983b; Couly et al., 1992; Köntges and Lumsden, 1996). However, the individual head muscle anlagen are distinguished based on their distinct innervation pattern (reviewed by Goodrich, 1958). The most fundamental difference between cranial muscles however is their embryonic origin (Adelmann, 1926; Noden, 1983a; Jacob et al., 1984; Wachtler and Jacob, 1986; Couly et al., 1992). Muscle precursors that provide the oculomotor innervated eye muscles originate from the axial, pre-chordal mesoderm underneath the forebrain. Non-somitic paraxial mesoderm reaching from midbrain to otic levels provides the remaining two extrinsic eye muscles, together with the muscles of the first three branchial arches. All further muscles in the head develop from occipital somites, located caudal to the otic vesicle.

Muscles that stem from occipital somites largely follow the epaxial or hypaxial programmes present in the trunk (E. H. Walters and S. D., unpublished), possibly reflecting their secondary enrolment with the head (Gans and Northcutt, 1983). Myogenesis from pre-otic mesoderm however differs considerably: this mesoderm does not form somites, therefore lacking myogenic dermomyotomal lips (reviewed by Wachtler and Jacob, 1986). Moreover, no known upstream regulators of trunk myogenesis have been sighted in the pre-otic head mesoderm to date. Thus, the regulation of genuine head muscle development is enigmatic, and, as a consequence, it cannot be decided whether vertebrate myogenesis proceeds according to a universal regulatory scheme or whether distinct programs are installed to control muscle formation from somitic and non-somitic mesoderm.

In this study we provide evidence that, despite the absence of somites, a region within the pre-otic paraxial mesoderm is set aside by the means of ‘trunk marker’ expression. This regionalisation coincides with the formation of a solitary cranial muscle. Despite expressing regulators for trunk myogenesis, this pre-otic head mesoderm is not able to read myogenic cues present in the trunk. Likewise, somitic mesoderm fails to follow cues residing in the head correctly. This implies that trunk (somitic) and head (non-somitic) muscle formation are distinct, the latter depending on regulatory mechanisms specific to the head.

The pre-otic paraxial mesoderm is regionalised

After the merger of pre-chordal and paraxial mesoderm early in development, the pre-otic head mesoderm forms a

continuous strip of mesenchyme on either side of the neural tube. Subsequently, this mesenchyme associates with the eye or with the first three branchial arches, owing to cranial flexure and arch outgrowth, respectively (reviewed by Goodrich, 1958). Despite localised expression of MyoD family members which demarcates sites of muscle differentiation (Hacker and Guthrie, 1998; Noden et al., 1999), neither morphological boundaries nor factors driving trunk mesoderm segmentation (G. Parkyn and S. D., unpublished) is present, and mesodermal cells seem promiscuous in the choice of muscles to which they will contribute (Noden, 1986; Hacker and Guthrie, 1998). However, we detected restricted expression of the transcription factors *Lbx1*, *Pax7* and *Paraxis* that, in the trunk, coincide with migratory muscle precursors (reviewed by Dietrich, 1999), that are in the pre-otic mesenchyme subjacent to the trigeminal ganglion. DiI labelling experiments confirmed that the labelled cells are of mesodermal origin and born at the level of rhombomere 2. Significantly, while the genes show additional expression domains located in the cranial neural tube and neural crest cells (Goulding et al., 1994; Schubert et al., 2001), no further head-mesodermal territory was stained. Thus, the three somitic markers depict the pre-otic head mesoderm beneath the trigeminal only.

Interestingly, regionalised expression in the pre-otic mesoderm has also been reported for the transcription factor engrailed 2 (En2), which labels the developing jaw closure muscles in the mandibular arch (Hatta et al., 1990; Gardner and Barald, 1992; Logan et al., 1993). However, *En2* does not bear relevance for trunk myogenesis as there is a lack of somitic expression and *En2* knockout mice do not display any myogenic phenotype (Joyner et al., 1991). Further, in the head, *En2* and upstream regulators of trunk muscle formation do not coincide. This suggests that *En2*, on one hand, and *Lbx1/Pax7/Paraxis*, on the other, are employed in distinct processes during cranial myogenesis. Moreover, it suggests that the regions set aside by the means of marker gene expression are not serially homologous, arguing against metamerism in the pre-otic head mesoderm. Nevertheless, the restricted expression pattern of these markers underlines that the pre-otic head mesoderm is regionalised, possibly compartmentalised.

Head mesoderm regionalisation coincides with the formation of the lateral rectus eye muscle

The pre-otic mesoderm demarcated by *Lbx1*/*Pax7*/*Paraxis* co-expresses the muscle determining factors *Myf5* and *MyoD*, along with further markers for newly born or differentiating muscle precursors, including *Pitx2*, *Pitx3* and *R-Cadherin* (Inuzuka et al., 1991; Rosenberg et al., 1997; Hacker and Guthrie, 1998; Gage et al., 1999; Kitamura et al., 1999; Noden et al., 1999) (this study). Thus, this mesoderm gives rise to muscle rather than cartilage. The restricted expression of the three trunk markers suggests however that a solitary head muscle anlage is singled out. Anatomical studies at the beginning of the last century suggested that the mesoderm beneath the trigeminal ganglion yields the precursors for the abducens-innervated lateral rectus muscle, the caudalmost of the six extrinsic eye muscles, responsible for horizontal movement of the eye (Adelmann, 1926) (reviewed by Goodrich, 1958). In birds, the lateral rectus anlage splits into the lateral rectus proper, as well as the pyramidalis and quadratus muscles that are innervated by the accessory abducens and move the nictitating membrane (Noden, 1983a; Jacob et al., 1984; Wachtler and Jacob, 1986; Couly et al., 1992; Wahl et al., 1994). We found that at HH20, the abducens proper headed for the *Lbx1*/*Pax7*/*Paraxis* domain, while the accessory branch diverged away, presumably seeking the pyramidalis and quadratus. As marker gene expression preceded innervation, we cannot exclude that transiently, also the latter two muscles expressed the set of genes. However, the cells showing persistent *Lbx1*, *Pax7* and *Paraxis* expression will ultimately give rise to the lateral rectus muscle.

Head muscle development is distinct from epaxial or hypaxial myogenesis in the trunk

In the trunk, *Lbx1* activity is confined to migratory muscle precursors which co-express markers for the dermomyotome (*Pax3*, *Pax7* and *Paraxis*) and markers for the lateral somite half (*Sim1*), while markers for epaxial muscle precursors are absent (*Wnt11*, *Noggin*) (Table 1) (reviewed by Dietrich, 1999). Both epaxial and hypaxial muscle precursors, before leaving the dermomyotomal lips, upregulate *Tbx3*, *Dach2* and *Six1*, the latter two acting with *Eya2* in the initiation of differentiation (Oliver et al., 1995; Heanue et al., 1999; Huang et al., 1999). In the head, however, the *Lbx1*-expressing cells are non-migratory: they remain in residence while the eye is brought close due to growth and the increase in cranial flexure. Nevertheless, the lateral rectus precursors seem the most trunk-like as they co-express *Pax7* and *Paraxis*. Considering that *Pax7* and *Pax3* are paralogues, and that *Pax7* can partially compensate for the absence of *Pax3* (Goulding et al., 1994; Borycki et al., 1999), it is conceivable that *Pax7* may replace *Pax3*, which is not expressed in the head, thereby installing a trunk-like regulatory cascade. However, all other trunk-markers are either not expressed in the pre-otic mesoderm (*Wnt11*, *Noggin*, *Tbx3*, *Dach2*) or show a ubiquitous expression throughout the cranial mesenchyme, not restricted to any particular muscle anlage (*Sim1*, *Six1*). Thus, the epaxial-hypaxial distinction is not established in the pre-otic mesoderm. Moreover, the striking absence of *Dach2*, together with the ubiquitous expression of *Six1* suggests that differentiation is initiated differently in head and trunk. This infers that despite the superficial similarity of lateral rectus and trunk muscle precursors, the developmental cascades employed for their development are distinct. This hypothesis is supported by the recent discovery of separate promoter elements controlling cranial and somitic expression of *Myf5* (Hadchouel et al., 2000; Summerbell et al., 2000; Carvajal et al., 2001).

Head muscle development depends on signals specific to the head

Despite the obvious differences between head and trunk myogenesis, we could not exclude that similar extrinsic cues were employed to initiate head and trunk myogenesis. We therefore exchanged pre-otic and somitic mesoderm by heterotopic grafting. Head mesoderm placed into the trunk at forelimb levels failed to express any of the markers shared by lateral rectus and somites, with the trunk-specific markers *Pax3* and *Pax1* not expressed either. Therefore, pre-otic mesoderm, despite possessing the ability to express certain trunk genes, clearly cannot read out trunk signals that pattern the somite.

When segmental plate or epithelial somites were transplanted from forelimb levels into the head, expression of the dermomyotomal marker *Pax3* and the medial sclerotomal marker *Pax1* was initiated, in line with data from Hacker and Guthrie (Hacker and Guthrie, 1998). Additionally, signals for *Pax7*, *Paraxis* and *Myf5* were observed. Importantly, *Lbx1*, which is normally expressed in these somites (reviewed by Dietrich, 1999), was absent at all times. Moreover, the grafted somites showed segmental expression for all the markers as opposed to localised expression beneath the trigeminal nerve. This suggests that the somitic mesoderm, instead of properly interpreting patterning cues in the head, activated somitic programmes of development.

The presence of *Pax1* and the absence of *Lbx1* indicate that the grafted somites had activated the medial/epaxial programme of development. This is in line with the observation that the signalling molecules sonic hedgehog and Wnt1/Wnt3a, which in the trunk induce medial sclerotome and medial/epaxial myotome formation, are also present in the head (reviewed by Christ and Ordahl, 1995; Molkentin and Olson, 1996; Gossler and Hrabe de Angelis, 1998). This fact implies that the three signalling molecules, while readily available to the pre-otic mesoderm, are not sufficient or unable to induce muscle formation in a head specific pattern. Therefore, we conclude that head-specific cues are a requisite for appropriate pre-otic muscle formation.

What function can trunk markers accomplish during head muscle development?

Our study shows that both the intrinsic and the extrinsic cues for trunk and head myogenesis differ considerably. This points at a fundamental difference between the somitic and non-somitic mesoderm throughout vertebrate evolution: the purpose of the metameric mesoderm was to generate muscles that propelled the body forward. However, the muscles emanating from non-somitic mesoderm were never recruited for locomotion, but instead assisted breathing, mastication and vision, by moving the gills and their derivatives, and by mobilising the eyeball (reviewed by Goodrich, 1958). For the latter, expression of *Lbx1*/*Pax7*/*Paraxis* holds much significance, but their role during lateral rectus development is not immediately clear. Considering that the *Drosophila Lbx1* homologue *ladybird* acts in the specification of particular somatic muscles (reviewed by Jagla et al., 2001), it is possible that in the vertebrate head, *Lbx1* serves a similar purpose. This implies that combinations of factors yet to be identified may specify the remaining pre-otic muscles. As a second possibility, *Lbx1*/*Pax7*/*Paraxis* may be employed to separate the lateral rectus precursors and the precursors for the pyramidalis and quadratus muscles, thereby ensuring that the correct connections with the eye are made. Interestingly, in reptiles and most mammals, the lateral rectus anlage, similar to avians, undergoes subdivision, thereby providing the muscle retracting the eyeball (reviewed by Goodrich, 1958). In this context, *Lbx1*/*Pax7*/*Paraxis* function might either represent a derived character of birds, or a more primitive condition shared by all amniotes. Third, *Lbx1*/*Pax7*/*Paraxis* may ensure development of the lateral rectus muscle by preventing the precursor cells from joining the more lateral precursors for the jaw closure muscles which ultimately enter the mandibular arch (Noden, 1983a; Couly et al., 1992; Hacker and Guthrie, 1998). Finally, the expression of *Lbx1*/*Pax7*/*Paraxis* may facilitate target recognition of the abducens nerve whose motorneurons originate in substantially caudal positions within rhombomeres 5 and 6. Given that the innervation of the lateral rectus occurs significantly later than expression of the marker set commences, this last prospect may be the most likely. Incorrect innervation of the extrinsic eye muscles is a frequent cause of squint in humans (misalignment of the optical axes, strabismus) which, if left unchecked results in loss of binocular vision (reviewed by Adams and Hubbard, 1999). Thus, innervation of the extrinsic eye muscles is a crucial event in the construction of a fully functional visual system, for which *Lbx1*/*Pax7*/*Paraxis* may play a role.

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