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The sub-lip domain – a distinct pathway for myotome precursors that demonstrate rostral-caudal migration

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SUMMARY

We have previously reported that the myotome is formed by a first wave of pioneer cells generated from all along the dorsomedial portion of the epithelial somite and a second wave of cells issued from all four edges of the dermomyotome. Cells from the extreme rostral and caudal edges directly generate myofibers that elongate towards the opposite pole of each segment and along the pre-existing myotomal scaffold. In contrast, cells from the dorsomedial and ventrolateral lips first reach the extreme edges and then contribute to myofiber formation. The mechanism by which these epithelial cells translocate remained unknown and was the goal of the present study.

We have found that epithelial cells along the dorsomedial and ventrolateral lips of the dermomyotome first delaminate into the immediate underlayer of the corresponding lips, the sub-lip domain, then migrate longitudinally along this pathway until reaching the extreme edges from which they differentiate into myofibers. Cells of the sub-lip domain are negative for Pax3 and desmin but express MyoD, Myf5 and FREK, suggesting that they are specific myogenic progenitors.

Key words: Quail embryo, Cell delamination, Dermomyotome, Epithelial-mesenchymal conversion, FREK, MyoD, Myf5, Pax3

INTRODUCTION

Skeletal muscles comprising epaxial, intercostal and abdominal components are derived from the transient embryonic myotome. The mechanisms underlying initial myotome formation and growth have been the matter of a long-lasting debate. In recent years, a combination of genetic analysis of tissue-specific molecular markers and cell lineage tracing using vital dyes made it possible to begin approaching the mode by which the cytoarchitecture of the myotome is established (Brand-Saberi and Christ, 1999; Cossu et al., 1996; Currie and Ingham, 1998; Kalcheim et al., 1999).

We have found that the ontogeny of the myotome is a complex process that can be subdivided into at least three distinct waves separable in time, topographical origin and mechanism of colonization. A first wave of pioneer cells originates from all along the dorsomedial quarter of the still epithelial somite. These cells express MyoD and then progressively withdraw from the cell cycle. Upon somite dissociation, the postmitotic pioneers delaminate and migrate towards the rostral lip of the newly formed dermomyotome. From this region, myofibers are generated that elongate in a caudal direction, leading to formation of a primary myotome that spans the entire dorsomedial to ventrolateral length of each segment (Kahane et al., 1998a).

A second wave of cells that colonize the myotome emanates from all four dermomyotome lips. Progressive intercalation of cells from these edges among pioneer fibers of the first wave leads to a highly patterned expansion of the myotome in the dorsomedial to ventrolateral aspect (see model in Cinnamon et al., 1999). However, the mechanism by which cells enter the myotome from rostral and caudal lips (R and C) differs from that used by cells originating at the dorsomedial and ventrolateral lips (DML and VLL). Cells that originate from the entire mediolateral extent of R and C lips directly generate myofibers in the myotome by elongating in a direction that is parallel to the pre-existing pioneers (Kahane et al., 1998b). In contrast, as assessed by whole-mount confocal analysis of dye-labeled lips, cells from the DML and VLL initially spread as discrete DiI-labeled spots all along these edges, subsequently generating partial-length myofibers that always have one end attached to either the R or the C lips, followed by the formation of full-length myofibers. These results led us to propose that DML- and VLL-derived cells contribute indirectly to the myotome by first translocating into the R and C lips from which they, in turn, generate myofibers (Kahane et al., 1998b; Cinnamon et al., 1999). In view of the epithelial nature of the DML and VLL, the precise mechanism accounting for this longitudinal translocation remained to be explored.

A widespread morphogenetic phenomenon during embryonic development is the stereotypic migration of progenitors, in many cases following a structural conversion

from an initial epithelial sheath into freely moving mesenchymal cells. Furthermore, in some cases it has been shown that proper cell translocation depends on both the state of cell specification and extrinsic cues, either of an attractive or repellent nature, that guide the cells or their processes along their migratory pathways (see, for example, Kalcheim, 2001; Tessier-Lavigne and Goodman, 1996).

In the case of striated skeletal muscles, cell migration has been shown to play an important role in the formation of specific types of hypaxial muscles. These include precursor cells of the limb, tongue, diaphragm and shoulder, which undergo an epithelial-to-mesenchymal conversion from the VLL of the dermomyotomes at cervical, brachial and lumbosacral levels of the axis (reviewed by Christ and Ordahl, 1995). The transcription factor Pax3 is normally expressed along the entire dermomyotome, at particularly high levels in the DML and VLL (Goulding et al., 1994; Williams and Ordahl, 1994). *Pax3* gene activity has been found to be essential for development of long-range migratory muscles; in the Splotch mutant, VLL cells of the dermomyotome fail to delaminate and thus development of tongue, limb, diaphragm and shoulder muscles is halted (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996; Tajbakhsh et al., 1997; Tremblay et al., 1998). Scatter factor/hepatocyte growth factor (SF/HGF) has been shown to induce mesenchymalization and proliferation in a variety of epithelial cells (Nakamura et al., 1989; Sonnenberg et al., 1993; Stocker et al., 1987). This factor is secreted from mesenchymal tissues in close proximity to epithelial cells that express its cognate receptor – Met. In mice that lack Met or SF/HGF, initial specification of ventrolateral cells is normal in the dermomyotome epithelia, but cells fail to engage in long range migration (Bladt et al., 1995; Dietrich et al., 1999). Notably, in the Splotch mutant, SF/HGF seems to be normally expressed but the Met receptor is absent (Daston et al., 1996; Epstein et al., 1996; Yang et al., 1996), suggesting that Pax3 regulates delamination and migration by modulating responsiveness to SF/HGF.

In contrast to the well-studied mechanisms by which long-range migratory progenitors give rise to muscles, the role of cell migration in development of the myotome remains unclear. In the present study, we have addressed the mechanism of cell translocation that accounts for the contribution of the DML and VLL epithelia to myotome growth. We report on the existence of a new migratory pathway that develops underneath the DML and VLL, which we term the 'sub-lip domain' (SLD). We show that muscle precursors from the DML delaminate into the SLD and migrate longitudinally as mesenchymal cells towards the R and C lips. Only when reaching the latter edges of the dermomyotome do migrating muscle progenitors begin differentiating into myofibers and colonizing the myotome as part of the second wave. Further characterization reveals that mesenchymal cells along the SLD are both devoid of *Pax3* mRNA and of desmin immunoreactivity, suggesting that they constitute an intermediate stage between their Pax3-positive epithelial ancestors and the later derivatives that express muscle differentiation markers. Instead, migrating SLD cells are positive for FGF tyrosine kinase-like receptor (FREK), MyoD and Myf5, indicating that they are specified myogenic progenitors.

MATERIALS AND METHODS

Embryos

Fertile quail (*Coturnix coturnix Japonica*) eggs from commercial sources were used in this study.

CM-DiI labeling of dorsomedial lip cells

Embryo preparation

Quail embryos were at the 28-30 somite stage at the time of dye labeling. Following removal of the vitelline membrane, a unilateral slit was made in the ectoderm over the DML of the cervical somites (somites 10-13). The ectoderm was then reflected with a microscalpel. Within 1 hour of labeling, the ectoderm completely regenerated over the somites.

Dye labeling

Borosilicate tubes with filament (OD, 1.0 mm; ID, 0.5 mm) were pulled using a vertical puller (Sutter model P-30). Micropipettes were backfilled with Chloromethylbenzamido-DiI (Cell-Tracker CM-DiI C-7001, Molecular Probes) dissolved in 100% ethanol (1 mg/ml) and further diluted in a 1:3 ratio with tetraglycol (Sigma). Micropipettes were then mounted on a Zeiss micromanipulator. Dye injections were performed by iontophoresis. Current was applied through an Ag/AgCl wire placed in a 2 M LiCl solution immediately before somite injections, and a 3 second pulse of 100 nA of current was used to deliver the dye. Injections were performed under an upright Zeiss Axioscope microscope adapted for holding eggs and equipped with long working distance objectives (LD-Achroplan X20) and epifluorescence. Embryos were viewed with oblique lighting from a fiber optic light source. The injections were directed to the center of the DML. In order to label small amounts of cells, the microinjector was pulled back immediately after the first labeled cells were detected. The accuracy of labeling was monitored throughout the procedure by observation under a total magnification of X200 with combined bright-field and epifluorescence optics. Following dye labeling, embryos were further incubated for various lengths of time ranging from time 0 to 25 hours. At the end of incubation, embryos were removed from the egg, washed in PBS and fixed in 4% formaldehyde in PBS.

Laser-scanning confocal microscopy

All embryos were scanned as a whole-mount preparations in order to verify the accuracy of the labeling and the amount of cell translocation. The same embryos were further embedded in paraplast and serial 10 μm transverse sections were mounted on gelatinized slides. Sections were further analyzed using regular fluorescence microscopy (Axioscope, Zeiss) or with confocal microscopy.

Fluorescent samples were analyzed using an LSM410 scanning confocal microscope (Zeiss, Jena, Germany) with a He-Ne laser with an excitation wavelength of 543 nm attached to an Axiovert 135M microscope. In the whole-mount preparations, dye-labeled cells were visualized with 10× or 20× Plan-Neofluar objectives. Dye-labeled cells in the serial sections were visualized using 40× oil Plan-Neofluar or 100× oil Fluar objectives. The whole-mounted samples and the serial sections were optically scanned at 2 μm and 1 μm increments, respectively through the *z*-axis and sequential images were collected on-line. The confocal images represent cumulative serial sections that include, in each case, all fluorescently labeled cells. Adobe Photoshop was used for image processing.

In situ hybridization and immunostaining

Whole mount in situ hybridization was performed as described by Kahane et al. (Kahane et al., 1998a; Kahane et al., 1998b) with avian-specific probes for FREK (Marcelle et al., 1994), MyoD and Myf5 (kindly provided by Charles Emerson – Pownall and Emerson, 1992) and Pax3 (a gift from Peter Gruss) followed by paraffin embedding and serial sectioning at 7 μm. Double labeling with desmin and

laminin antibodies was performed by combining the methods described in Kahane et al. (Kahane et al., 1998b) and Brand-Saberi et al. (Brand-Saberi et al., 1996). Following immunolabeling, nuclei were visualized with Hoechst staining. When *Pax3* mRNA and desmin protein localizations were combined, and whole-mount in situ hybridized embryos were embedded in paraffin wax, sectioned and treated for desmin immunolabeling as described above.

RESULTS

DML cells delaminate into a sub-lip domain through which they migrate longitudinally towards the extreme edges of individual segments

In previous studies we have shown that the DML and the VLL of the dermomyotome (the latter at specific levels of the axis) generate myofibers that contribute to the expansion of the myotome in the dorsomedial and ventrolateral directions, respectively. DiI labeling studies and confocal analysis of whole-mount preparations suggest that lip-derived cells first redistribute along the rostrocaudal extent of a segment and only upon reaching the extreme dermomyotome edges give rise to partial-length and then full-length myofibers (Kahane et al., 1998b; Cinnamon et al., 1999). These studies already suggest that a simple mechanism of cell proliferation cannot account for the rapid translocation observed to occur into the extreme edges. However, they still left unanswered the question of whether cells migrate along the plane of their respective epithelia (DML or VLL) or, alternatively, through another pathway.

To further characterize the precise cellular mechanism by which DML and VLL cells arrive at the extreme edges, lip cells were labeled with CM-DiI, which allowed us to examine cell behavior in both whole mounts and in serial transverse sections of paraffin wax-embedded embryos. The center of the DML of cervical segments in embryos age 28-30 somites was labeled with CM-DiI. Consistent with our previous results, analysis of whole embryos revealed that labeling was confined to the central target area immediately after injection, followed by spreading of discrete spots along the rostrocaudal aspect of the lips by 10 hours and formation of partial- and full-length fibers by 18 and 24 hours, respectively, but not at earlier stages (Fig. 1 and see also Kahane et al., 1998b; Cinnamon et al., 1999).

Serial section analysis in the transverse plane revealed that immediately after labeling, dye-positive cells were found exclusively in the outer part of the DML epithelium in a single or in two adjacent sections thus spanning a length of about 10-20 μm of a total rostrocaudal somitic length of 90-100 μm at this stage (17.3% of total somite length, *n*=9, Figs 2A, 3B). After 6 hours of incubation, some labeled cells had translocated in the transverse plane into a region immediately subjacent the DML (Fig. 2B, arrow), in addition to residual signal in the epithelium (Fig. 2B arrowheads). At this stage, the percentages of segment length with fluorescent cells in both DML and subjacent area were similar (31±6%, *n*=11; 20±8%, *n*=11, respectively; Fig. 3). Notably, 10 hours after labeling, cells that had translocated underneath the DML were detected over a very considerable length of individual segments (60%, *n*=10; Figs 2C,D, 3 and 4). In contrast, the proportion of segment length with labeling in the DML epithelium remained unchanged when compared with earlier time points examined (26±9%, *n*=10, Fig. 3B). Thus, when sections from the center of the somite were examined, CM-DiI-labeled cells were apparent in both the epithelium of the DML as well as in the subjacent region (Figs 2C, 4C, arrowheads and arrows, respectively). Nevertheless, rostral or caudal to the center of the segments, labeled cells were apparent exclusively in the area subjacent to the DML with no labeled cells in the epithelium itself (Figs 2D, 4A,B,D-F).

Altogether, these results suggest that cells located in the epithelium of the DML first undergo an epithelial-to-mesenchymal conversion and populate a region subjacent to the DML prior to translocating in the longitudinal plane. Subsequently, cells in this pathway migrate either rostrally or caudally with respect to the initial labeling site until reaching

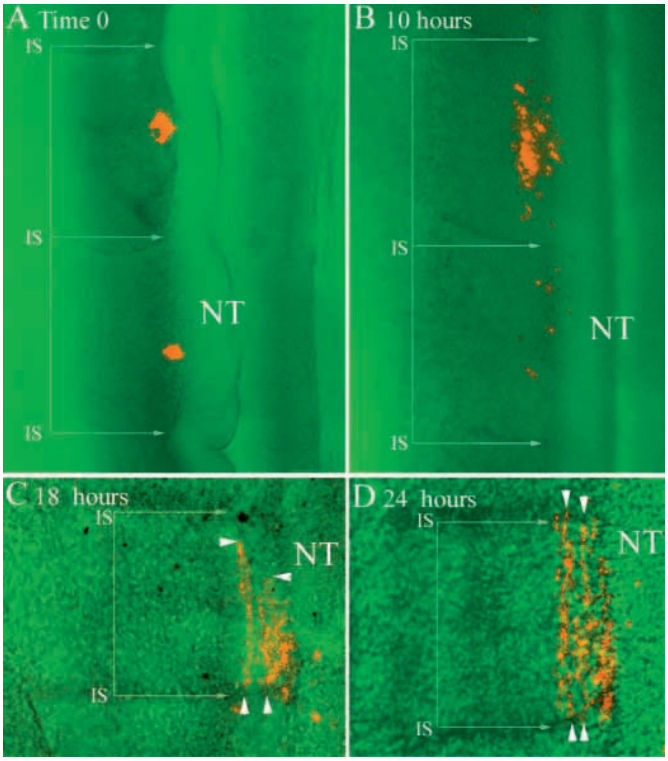

Fig. 1. Time-dependent distribution of DML-derived cells labeled with CM-DiI. CM-DiI was iontophoretically delivered to the center of the dorsomedial lip (DML) of cervical somites in embryos age 28-30 somite pairs. Timecourse analysis using confocal microscopy showing that CM-DiI labeled cells translocate in a longitudinal direction towards the rostral and caudal dermomyotome lips. This translocation is followed by formation of partial length and then full-length myofibers. (A) Discrete spots of CM-DiI are apparent immediately after injection into the center of the DML. (B) After 10 hours of incubation, labeled cells are spread along a considerable extent of the DML, between the two intersomitic regions (IS). No fibers are observed at this stage. (C) 18 hours after labeling, partial-length fibers are apparent (between arrowheads) along with labeled cells along the lip area. The myofibers are always anchored to either the rostral or caudal edge of the dermomyotome (see also Kahane et al., 1998b; Cinnamon et al., 1999). (D) 24 hours after injection, myofibers extend along the entire length of the myotome (delimited by arrowheads). Notably, the cells originating from the DML give rise to the dorsal-most fibers of the myotome. Arrows indicate intersomitic spaces. NT, neural tube.

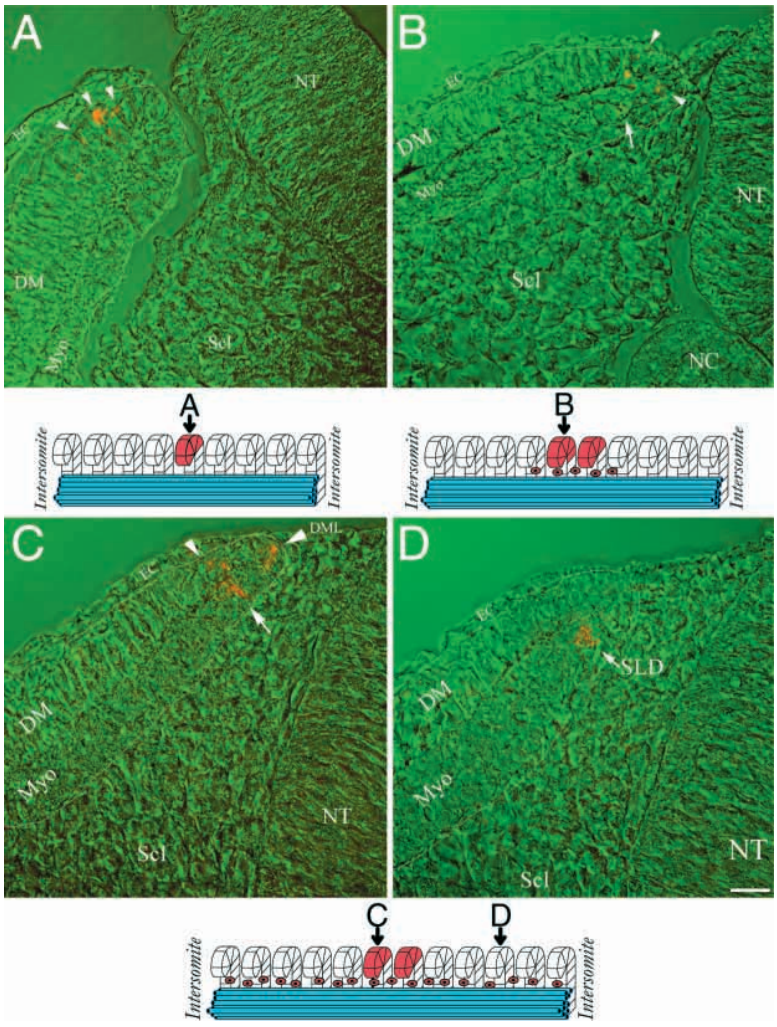

Fig. 2. Time-dependent delamination of CM-DiI-labeled DML progenitors into the sub-lip domain. A timecourse analysis on transverse sections was performed with confocal microscopy. Cells in the center of the DML of cervical somites were labeled with CM-DiI as described in the Materials and Methods. (A) At time 0, the labeled cells were exclusively found in the epithelium of the DML (arrowheads). The schematic underneath indicates that the section was taken from the middle of the somite corresponding to the site of injection. (B) 6 hours after CM-DiI injection, labeled cells are still present in the epithelium of the DML reaching a more medial position (arrowheads) and also ventral with respect to the DML (arrow). The ventral cells at this stage are found over a similar number of sections as the overlying labeled epithelial cells, close to the injection site. (C) Transverse section taken from the middle of the somite of an embryo incubated for 10 hours. Cells in both the epithelium (arrowheads) and the region subjacent to the DML (arrow) are seen, similar to B. (D) By contrast, in sections located in a para-median plane, the labeled cells are found solely in the region subjacent to the DML, termed sub-lip domain (SLD, arrow). The schematic illustrations indicate the relative location of the sections with respect to the rostrocaudal axis of the injected segments. CM-DiI-labeled cells are marked in red, and the dorsalmost fibers of the myotome in blue. DM, dermomyotome; EC, ectoderm; Myo, myotome; NC, notochord; NT, neural tube; Scl, sclerotome. Scale bar: 12 μm.

the extreme edges of individual segments. Hence, the delamination and further movement of DML-derived cells operationally define, at this stage, a new migratory pathway between the epithelial lip and the dorsal aspect of the myotome (see Fig. 6). We designate this region the 'sub-lip domain' (SLD).

Characterization of the SLD

Mesenchymal cells of the SLD are negative for Pax3 and desmin

To begin characterizing the SLD as a migratory path for second-wave progenitors, we defined some of its histological and molecular features, in comparison with those of the overlying dermomyotome lips and underlying myotome. To this end, transverse sections of relevant stages were triple-stained for desmin and laminin protein, and Hoechst nuclear staining (Fig. 5). In addition, desmin immunostaining was performed in combination with *Pax3* mRNA localization (Fig. 6). Serial section analysis of embryos aged 30-35 somites at cervical levels of the axis, revealed that the SLD, through which lip-derived cells migrate longitudinally (Figs 1-4), can be further defined as a discrete group of cells negative for both Pax3 and desmin. These cells are located underneath a Pax3-positive DML and dorsal to a desmin-immunoreactive myotome (Figs 5D, 6A). Likewise, a SLD displaying similar features was observed dorsal to the VLL in flank regions of the axis (Fig. 6B).

Notably, the basal lamina underlying the desmin-positive myotome appeared well defined and continuous, as revealed by anti-laminin immunostaining. In contrast, the basal lamina underlying the SLD was discontinuous (Fig. 5E) and sometimes a complete lack of laminin reaction product was noticed in that restricted area (data not shown). An additional salient feature of the SLD was the appearance of its nuclei. Whereas most nuclei in the DML epithelium (or VLL, not shown) were aligned parallel to each other and generally oval in shape (a stereotypic arrangement of epithelial nuclei), those in the SLD appeared less organized, and relatively smaller and rounder (Fig. 5F,H, compare nuclei in the SLD with those in overlying dermomyotome), further reflecting the mesenchymal nature of this group of cells. Moreover, when comparing the rostrocaudal distribution of myotomal nuclei with that of the overlying nuclei in the SLD, it became evident that in the latter, nuclei were present along the entire somite (Fig. 5G,H). In contrast, most myotomal nuclei at this stage were still concentrated in the center of the myofibers and only few are apparent near the segment edges (compare Fig. 5H with 5G; see also Kahane et al., 1998a; Kalcheim et al., 1999). In addition, cells in the SLD displayed frequent mitoses (10% to 20% of total BrdU-labeled nuclei, following a 1 hour pulse;

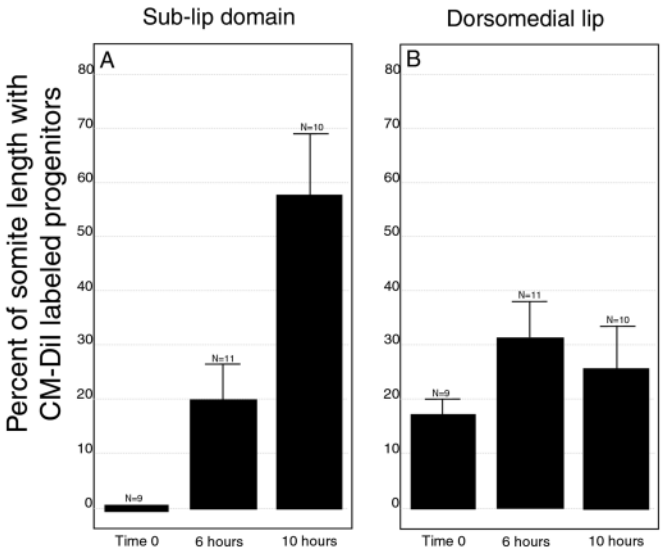

Fig. 3. Quantification of the time-dependent distribution of labeled DML progenitors in the sub-lip domain. Bar graph representing the percentage of somite length that contains CM-DiI-labeled progenitors after injections to the middle of the DML (see Methods and legends to Figs 1,2 and 4). Distribution of CM-DiI-labeled cells was monitored along the rostrocaudal aspect of the DML and sub-lip domains. (A) Appearance of labeled cells in the sub-lip occurs by 6 hours post labeling. By 10 hours, labeled cells have migrated longitudinally to occupy about 60% of the segment length. (B) Distribution of CM-DiI labeling in the injected DML. Note that there is no significant change with time in the relative length of DML that contains labeled cells, while the distribution of cells along the sub-lip domain dramatically increased from 20% to about 60% of segment length between 6 and 10 hours after labeling. Percentages were calculated by counting the number of serial sections with labeled cells over the total number of sections for a given segment at the different time points. Numbers above bars represent the number of cases scored and results are given as the mean±s.d.

see also Fig. 5I,J arrow) when compared with the postmitotic nature of myotomal nuclei. Altogether, these data show that according to several criteria, the SLD differs from the overlying epithelium of the DML and from the underlying myotome.

The onset of the SLD

To define the initial appearance of the SLD, we used the criteria described in the previous section. Embryos age 20-40 somites were serially analyzed following triple staining for laminin, desmin and Hoechst. At cervical levels of 22 somite-stage embryos, the epithelium of the DML and the primary myotome lay on a continuous laminin-containing basal lamina (Fig. 5A,B). Notably, desmin immunoreactivity reached the DML and no intervening region lacking desmin was yet apparent (compare Fig. 5A with 5D). This desmin-positive region is mostly accounted for by the presence of the pioneer myotomal cells, which develop from an inward bending of the dorsomedial epithelium at the time of somite dissociation (Kahane et al., 1998a). Hence, at early stages of myotome formation, the primary myotome composed of pioneer fibers is immediately subjacent to the DML epithelium (Fig. 5A-C). The first appearance of an intervening SLD between the DML and the desmin-positive myotome, with characteristics like

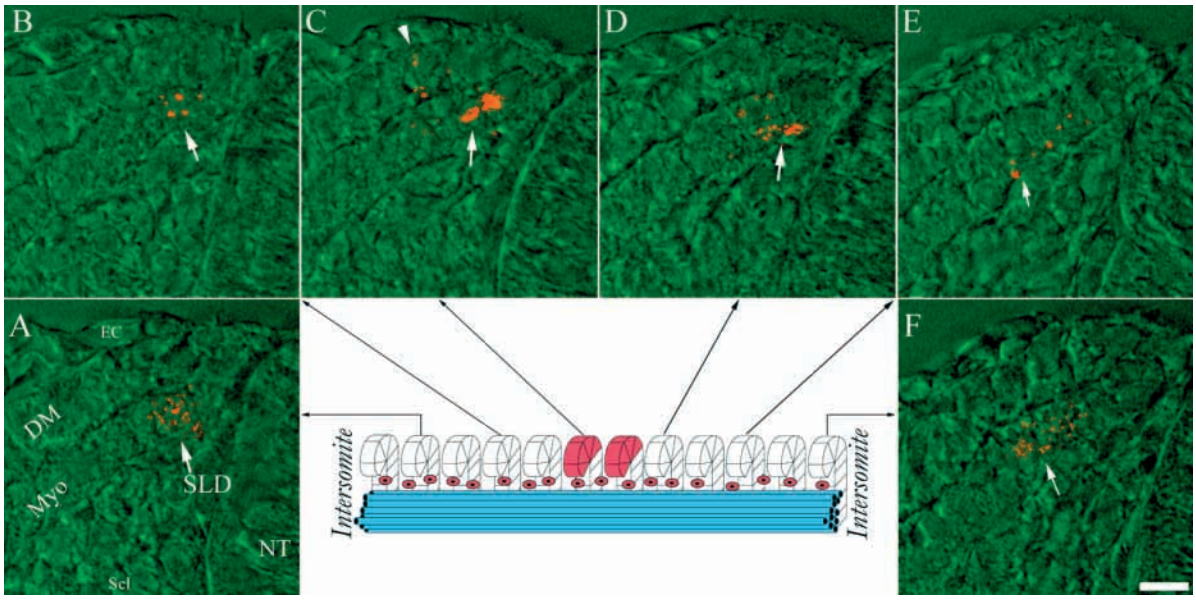

Fig. 4. Distribution along the somite of DML-derived cells 10 hours after CM-DiI labeling. The center of the DML in cervical somite 12 of a 29-somite embryo was labeled with CM-DiI. 10 hours later, embryos were serially sectioned in the transverse plane and analyzed with confocal microscopy. The figure shows selected sections taken sequentially from such a segment. In sections from para-median positions (A,B,D-F) the labeled cells were located in the SLD region exclusively (arrows). In a section from the middle of the somite (C), the labeled cells are in the DML (arrowhead) as well as in the SLD (arrow). In the schematic, arrows pointing at the various panels indicate the relative location of the presented section. CM-DiI labeled cells are colored red and the dorsalmost fibers of the myotome are blue. DM, dermomyotome; EC, ectoderm; Myo, myotome; NT, neural tube; Scl, sclerotome; SLD, sub-lip domain. Scale bar, 10 μm.

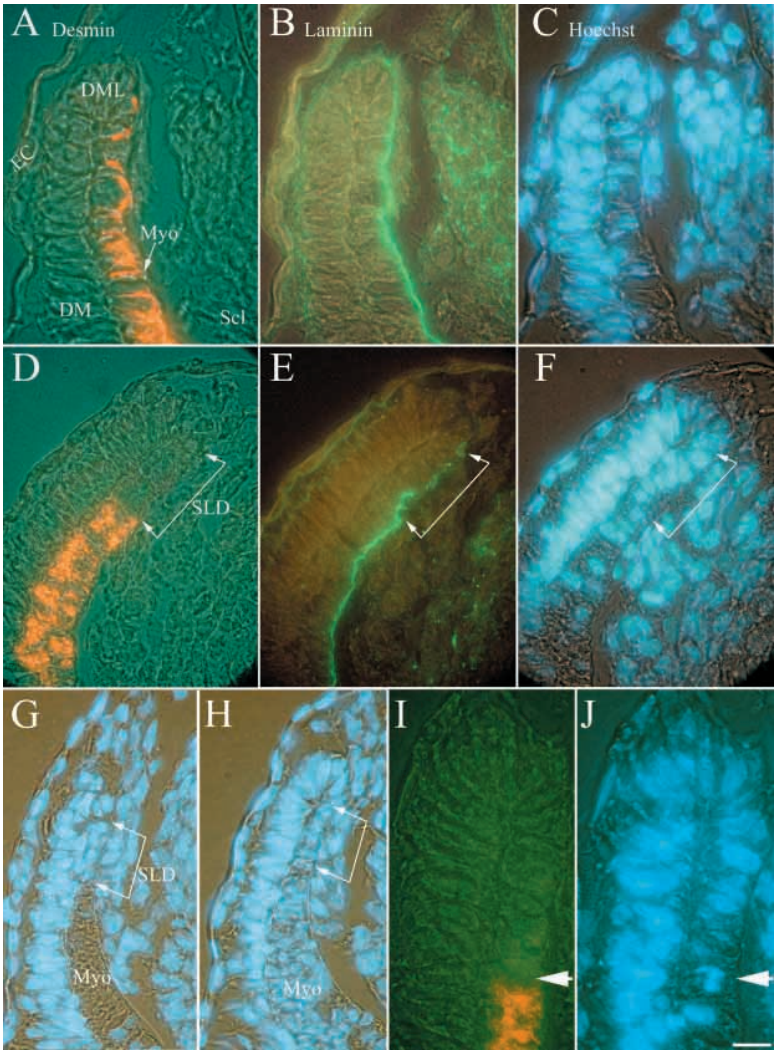

Fig. 5. Features of the sub-lip domain as revealed by co-immunostaining with antibodies against desmin and laminin, combined with Hoechst nuclear staining. (A-C) Transverse section from cervical somite 13 of a 22-somite quail embryo showing that desmin-positive myotomal cells are in continuity with the epithelium of the DML, representing pioneer myofibers, see text (A). (B) Laminin immunoreactivity is revealed as a continuous lamina underlying both the DML and the myotome. (C) Note the compact arrangement of nuclei in the overlying epithelial sheath when compared with the more scattered appearance of nuclei in the underlying pioneer myofibers. (D-H) Transverse section from cervical somite 13 of a 35-somite quail embryo showing a desmin-negative region, the SLD, between the desmin-positive myotome and the DML (D, between arrows). (E) Laminin immunostaining underneath the SLD is incomplete and much weaker than in corresponding areas of younger embryos (between arrows, compare with panel B). (F) The nuclei in the SLD are round in shape when compared with the overlying epithelium, see also H. (G,H) Sections from two different regions of a segment showing that distribution of nuclei in SLD is homogeneous along the rostrocaudal plane, while myotomal nuclei are predominant in the center of the myofiber (H) but very few when approaching the edges (G); note as well that towards the segment edge (G), the epithelial conformation of the dermomyotome becomes slightly distorted, yet the SLD nuclei can be clearly distinguished based on serial section analysis. In G,H, the myotome was delineated based on the corresponding image stained for desmin (not shown). (I,J) Transverse section showing in the SLD the presence of a mitotically active cell (Hoechst-positive nucleus in metaphase, arrow in J) localized dorsal to the desmin-positive myotome (arrow in I). DM, dermomyotome; EC, ectoderm; Myo, myotome; Scl, sclerotome; SLD, sub-lip domain. Scale bar, 12 μm in A-F; 16 μm in G,H; 4 μm in I,J.

those described in the previous section, was detected in the three rostralmost somites of 25-somite embryos. In 30-somite embryos, the SLD was apparent in all segments rostral to the 15th somitic pair, and in embryos aged 35-40 somites, the SLD was found in all somites rostral to the 23rd-28th somites. Thus, the SLD that defines a longitudinal pathway for migrating myoblasts of the second wave, develops progressively in a rostral to caudal sequence and is eventually present at all axial levels. Furthermore, the local change detected in structure of the basal lamina marks the transition between the first wave and the onset of the second wave of myotome colonization. These results are fully consistent with previous data in which the initial time of addition from dermomyotome lips was determined using thymidine and DiI-labeling. In those studies, we showed that myoblast addition begins, for instance, in the rostralmost 5-8 segments of embryos aged 23-25 somites and extends more caudally as development proceeds (Kahane et al., 1998b).

Cells in the SLD express MyoD, Myf5 and FREK mRNAs

DML cells that delaminate into the SLD, downregulate expression of *Pax3* and do not express desmin protein during longitudinal migration through this path. To begin identifying the markers expressed by SLD cells, in situ hybridization was performed for MyoD, Myf5 and FREK. Observation of whole-mount preparations revealed in all cases the presence of two parallel stripes of mRNA signal that spanned the entire rostrocaudal extent of individual segments. These stripes were apparent in regions corresponding to the DML and VLL (Fig. 7A,D,F, delimited between arrows). Although at the level of the DML, expression of all markers was observed throughout the axis following the temporal sequence discussed above, expression in VLL regions was primarily restricted to nonlimb and noncervical areas of the axis (Fig. 7 and data not shown).

Transverse sectioning and reconstruction of the images from serial observation further revealed that the two dorsomedial and ventrolateral parallel stripes seen in the whole mounts correspond to the SLD underlying the DML and VLL epithelia, respectively (Fig. 7B,C,E,G, arrowheads). In addition to expression in the SLDs, all markers were also found in the myotome. For instance, Myf5 mRNA was, at this stage, primarily concentrated in the cytoplasm around the centrally located nuclei of the myofibers, so that the expression pattern in the whole mounts looked like a single stripe crossing the center of somites between their dorsomedial and ventrolateral edges (Fig. 7A). Hence, a transverse section taken from the

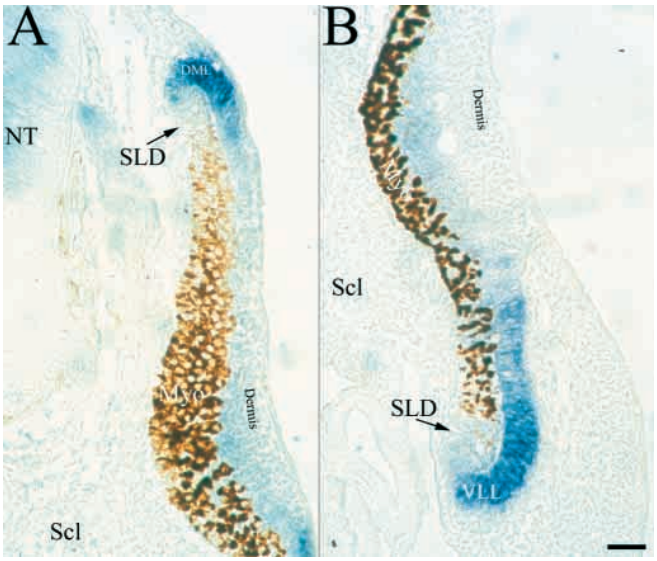

Fig. 6. Cells in the sub-lip domain are negative for both desmin and Pax3. (A) Transverse section through a cervical somite of a 38-somite quail embryo hybridized with a probe for Pax3 (blue) and labeled with desmin antibodies (brown color). (B) Flank level of the same embryo depicting the ventrolateral lips (VLL). Note the presence of sub-lip domains (SLD, arrows) subjacent to both the DML (A) and VLL (B), which contain cells negative for Pax3. Pax3 instead strongly stains the DML and VLL epithelia. The SLD is also negative for desmin, which is present in differentiated myofibers. NT, neural tube; Scl, sclerotome. Scale bar, 23 μm.

middle of a somite revealed a continuity between staining of the SLDs and the myotome (Fig. 7B). However, in sections taken rostral or caudal to the center of the somite, the presence of Myf5 in the myotome decreased dramatically, while cells in the SLD retained a high level of expression (Fig. 7C). Likewise, expression of MyoD was also apparent along the SLD (Fig. 7D,E) which is negative for desmin (Fig. 7E). Within the myotome, MyoD mRNA signal, similar to that of Myf5, is weaker towards its extreme rostral and caudal edges (yet still more prominent than Myf-5), highlighting the signal in the SLD (Fig. 7E; compare Fig. 7D with 7A).

FREK has previously been shown to be expressed in mitotically active muscle precursor cells (Marcelle et al., 1994; Marcelle et al., 1995). Recent results have shown that mitotically active FREK-expressing myoblasts enter the myotome from along both rostral and caudal edges of the dermomyotome as part of a third wave of myotome colonization that partially overlaps in time the contribution by the second wave (N. K. and C. K., unpublished observations). Consistent with these observations, FREK mRNA appears as two parallel stripes along the rostral and the caudal regions of the myotome. Thus, the expression pattern of FREK in the myotome differs and is in general reciprocal to that of MyoD and Myf5. Yet in the SLDs subjacent to the DML and VLL epithelia, FREK is expressed in a similar pattern to that observed for MyoD and Myf5 (Fig. 7). Altogether, these results suggest that cells migrating through the SLD that are negative for Pax3 and desmin represent an intermediate stage between dermomyotome progenitors and differentiated myofibers, being a subset of proliferating progenitors specified to the myogenic lineage.

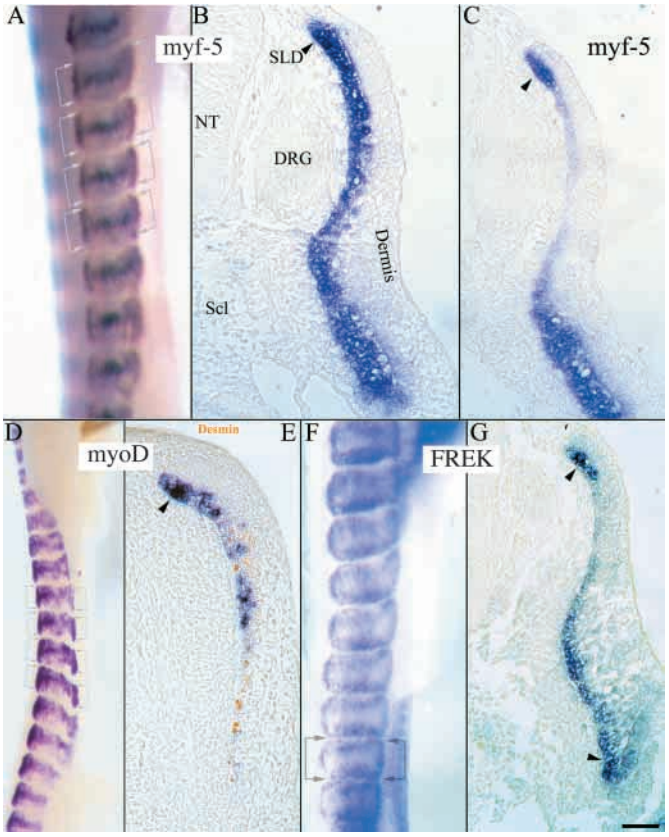

Fig. 7. Cells in the sub-lip domain express MyoD, Myf5 and FREK mRNAs. Whole-mount in situ hybridization followed by transverse sectioning of 35-38 somite quail embryos with probes for Myf5 (A-C), MyoD (D,E) and FREK (F,G). Areas shown correspond to the flank level of the axis. Besides the known expression patterns of Myf5, MyoD and FREK in distinct myotomal cells (see text), all three markers are synthesized by migrating cells in the sub-lip domains (SLD). Note in the whole-mount in situ hybridized embryos (A,D), the presence of two stripes corresponding to the sub-lips in the DML and VLL regions that extend throughout the length of the segment (delineated between arrows); in the transverse sections, it is apparent that the overlying epithelia of the lips are negative for these markers. (B) A section taken from the middle of the somite where Myf5 mRNA is expressed both in the myotome as well as in the SLD. Slightly away from the middle (C), Myf5 expression in the myotome decreases while the pattern in the SLD remains very similar and does so along the entire length of the somite (not shown). (E) A transverse section hybridized with MyoD and then stained for desmin protein, further revealing that the SLD is MyoD positive but lacks desmin expression. In contrast, the more ventrally localized myotome contains both MyoD- (blue) and desmin-positive cells (brown). (F) FREK expression is enriched in the extreme R and C regions of the myotome. (G) In the transverse plane, FREK is evident in both the SLDs and myotome. Arrowheads indicate the dorsal and ventral sub-lip domains. DRG, dorsal root ganglion; NT, neural tube; Scl, sclerotome. Scale bar, 30 μm in B,C,E,G.

DISCUSSION

In previous studies, we have reported that all four lips of the dermomyotome contribute to generation of myofibers that join by intercalation the pre-existing pioneer myotomal structure. Of these lips, the rostral and caudal ones contribute by direct

elongation, whereas cells from the DML and VLL have first to reach the extreme edges before generating myotomal fibers (see Introduction). These studies left unanswered the question of the mechanism of cell translocation into the extreme edges. Given the nature of the DML and VLL, epithelial structures with limited cell motility, it was necessary to clarify the cellular mechanism whereby such dramatic translocations take place along individual segments. In the present study, we report on the development and features of a new migratory pathway for translocation of muscle progenitors that derive from the DML and VLL and that contribute to the second wave of myotome growth. We show for the first time that these cells do not translocate along their respective epithelia. Instead, they first delaminate into a sub-lip domain (SLD), then migrate longitudinally through this pathway to reach the rostral or caudal edges of each segment from which myofibers are generated into the growing myotome. Furthermore, we demonstrate that SLD progenitors are mesenchymal cells that express MyoD, Myf5 and FREK but are negative for Pax3 and desmin. Moreover, we find that the histological and molecular features of this migratory pathway develop in a rostrocaudal sequence along the axis that precisely corresponds to the onset of the second wave of myotomal colonization, as previously assessed by thymidine-labeling experiments (Kahane et al., 1998b).

Longitudinal migration and the mechanism of myotome formation

Recent studies of myotome development suggested that the myotome expands in a medial direction by incremental ingression of DML cells (Denetclaw et al., 1997 – but see Christ et al., 1978) and in a lateral direction by addition of VLL-derived progenitors (Denetclaw and Ordahl, 2000). We also find that the DML contributes to a medial expansion of the myotome (Kahane et al., 1998b) and the VLL to lateral growth (Cinnamon et al., 1999). However, two essential issues still differ between the two proposed models. The first concerns the mechanism by which the DML and VLL give rise to myofibers. Ordahl and colleagues have reported that cells first ingress from along these edges and then begin differentiating into myofibers in situ with no apparent longitudinal translocation (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000). At variance with this result, we provide evidence based on lineage tracing and molecular characterization, that cells from along these edges first delaminate into an area subjacent to the lips, which we termed the SLD, and that this domain in fact defines a migratory pathway that supports the longitudinal translocation of mesenchymal progenitors. Moreover, based on the longitudinal movement of the mesenchymal cells (this paper) and on the observation of many labeled somites in which partial-length fibers were always anchored to one of the extreme edges and never had two free ends in the somite, we conclude that it is only upon reaching the rostral or caudal edges of the dermomyotome that DML and VLL-derived cells generate myofibers. Thus, although the four lips of the dermomyotome are the source of second-wave fibers, actual differentiation and elongation takes place exclusively from the rostral/caudal edges in a direction that is parallel to the pre-existing pioneer myofibers (Cinnamon et al., 1999; Kahane et al., 1998b).

The second problem concerns the overall mechanism of myotome growth. If cells of the DML and VLL were the only source of the myotome, then a model of incremental growth in the medial and lateral directions would be correct (Denetclaw and Ordahl, 2000). However, DML and VLL cells are neither the only nor the earliest cells to form the myotome. In contrast to the previous authors, we have also found that the entire rostral and caudal dermomyotome lips contribute significantly to the expansion of the myotome (Cinnamon et al., 1999; Kahane et al., 1998b). Moreover, we have shown that progenitors of all four lips progressively integrate by intercalation among a pre-existing scaffold formed by the pioneer myofibers (Kahane et al., 1998a; Cinnamon et al., 1999). Thus, given the multiple sources of myotomal cells and the regulated temporal contributions of the first (pioneer) and second waves, and based on the progressive intercalation of second-wave myoblasts among myofibers of the first wave all along the muscle including its extremities, we propose that overall myotome growth occurs in a uniform manner along the dorsomedial to ventrolateral extent rather than by local increments. This uniform expansion corresponds to the sum of cell additions from several intermixing sources: first, the rostral and caudal lips, which are progressively added all along the growing structure, and second, local contributions by DML cells, which account for expansion in the medial direction (in agreement with Denetclaw et al., 1997; see Kahane et al., 1998b), and by VLL cells, that participate in growth in the lateral direction (Cinnamon et al., 1999; Denetclaw and Ordahl, 2000).

Delamination of progenitors from the dorsomedial and ventrolateral dermomyotome lips

Similar to muscle progenitors that engage in long-range migration, epithelial cells from the DML along the entire axis and from the VLL at specific axial levels, have to undergo a process of epithelial-to-mesenchymal conversion prior to engaging in short-range longitudinal movement. Although the mechanisms responsible for delamination of these progenitors remain unknown at present, several insights can be gained from the development of other dermomyotome derivatives. For instance, mesenchymalization of the epithelial dermomyotome into dermis was found to involve the interaction between neurotrophin 3 derived from the neural tube and its high affinity receptor TrkC present in a mediolateral gradient along the dermomyotome (Brill et al., 1995). Whether neurotrophin 3 is operates at earlier stages to influence delamination of myogenic progenitors remains to be tested. Furthermore, delamination of limb progenitors residing in the VLL was found to be triggered by SF/HGF derived from the limb mesenchyme (see Introduction). However, this factor seems unlikely to act upon VLL cells at flank levels or on DML progenitors because, at least in mice, it is absent adjacent to these regions and despite the fact that the Met receptor is present in both the medial and lateral lips, inactivation of the genes for SF/HGF or Met produces no apparent defects in myotome-derived muscles, while severely affecting myoblast subsets that migrate into the limb, diaphragm, tongue and shoulder (Bladt et al., 1995; Dietrich et al., 1999).

An interesting parallel can be drawn between delamination of dermomyotome lip progenitors and that of pre-migratory neural crest cells localized in the dorsal domain of the neural tube. The onset of migration of neural crest cells depends upon

a successful transition of the epithelial pre-migratory cells into mesenchyme. In their epithelial conformation, neural crest cells like other neuroepithelial cells, are closely associated polarized progenitors. When converting into mesenchyme, they become only loosely associated or fully individualize, acquire motile properties and invade initially the extracellular matrix surrounding the dorsolateral neural tube (reviewed in Le Douarin and Kalcheim, 2000). Electron microscopic analysis has shown that a basal lamina is present around the neural tube except in the region that overlies the neural crest cells where it is actually incomplete (Erickson, 1987). Notably, reconstitution of a continuous basal lamina does not take place until emigration of neural crest cells is completed (see, for example, Martins-Green and Erickson, 1987). A somewhat comparable situation is observed in the SLD, despite the fact that these cells do not traverse the basal lamina in the same way as delaminating neural crest cells. Whereas the young dermomyotome and myotome rest on a continuous basal lamina, the later developing SLD exhibits a discontinuous staining for laminin and sometimes even a complete lack of the laminin-containing basal lamina. It is possible that the delaminating cells in both cases participate in proteolytic breakdown of the basal lamina that might otherwise act as a barrier for mesenchymalization and/or movement or serve other, yet unknown functions.

The mechanism that accounts for delamination of neural crest cells has begun to be elucidated. A balance between the activities of bone morphogenic protein 4 (BMP4) and noggin in the dorsal neural tube was found to trigger the emergence of the pre-migratory progenitors from the neural epithelium (Sela-Donenfeld and Kalcheim, 1999). BMP4 is, however, not likely to exert a similar direct effect on the DML epithelium because at stages when the SLD becomes functional, the DML epithelium expresses high levels of noggin that could potentially inactivate any BMP signaling (Marcelle et al., 1997; Sela-Donenfeld and Kalcheim, 1999). Yet, in the dorsal neural tube of avian embryos, BMP4 regulates the expression of Wnt1 and Wnt3a (Marcelle et al., 1997; Burstyn-Cohen and C. K., unpublished observations) and these factors might in turn affect delamination of lip progenitors, either directly or through the induction of secondary Wnts in the DML, like Wnt11 (Marcelle et al., 1997).

Longitudinal migration of myotomal progenitors follows delamination and precedes differentiation

Whereas limb muscle progenitors are considered as bona fide migratory cells, the significance of cell migration in the formation of the myotome remains virtually unknown. For instance, adaxial cells in zebrafish embryos arise adjacent to the notochord and give rise to slow muscles localized in the superficial part of the myotome (Devoto et al., 1996; Felsenfeld et al., 1991). To reach this position, adaxial cells migrate radially away from the notochord by mechanisms that remain unexplored. Avian pioneer muscle cells arise along the entire dorsomedial extent of epithelial somites. Upon somite dissociation, they bend as an epithelial sheath underneath the forming dermomyotome, and delaminate and migrate as mesenchymal cells towards the rostral edge of each segment from which they generate the earliest myotomal myofibers that extend in a caudal direction, until forming the primary myotome (Kahane et al., 1998a). This polarized longitudinal migration could be led by attractive cues originating in the rostral part of the segments or, alternatively, by repellent signals that develop in the caudal somitic moieties. The latter mechanism could be analogous to that involved in controlling the segmental migration of neural crest cells through the somites (reviewed by Kalcheim, 2001; Krull, 1998; Debby-Brafman et al., 1999).

Notably, migratory movements are also part of the second wave of myotome colonization. In this study we have directly shown that myoblasts originating in the DML after the establishment of the primary myotome, migrate longitudinally along the length of one segment through the SLD until reaching the extreme edges of the dermomyotome where they join cells from the rostral and caudal lips and generate myofibers (see also Kahane et al., 1998b). A transverse section analysis of the behavior of CM-DiI-labeled cells was not performed for the VLL. Nevertheless, a time-dependent longitudinal redistribution of DiI-labeled cells along the VLL was previously shown by whole-mount analysis of flank levels of the axis (Cinnamon et al., 1999), that mirrored the behavior of DML-derived cells. Furthermore, similar molecular properties are displayed by the SLD at ventrolateral and dorsomedial regions (this paper). Hence, we conclude that analogous processes operate along both medial and lateral edges of the dermomyotome.

In this case, an analogy can also be drawn with the behavior of neural crest cells. A short-range longitudinal migration, taking place between dorsal neural tube and adjacent somites along a length of one and a half segments in either direction, was found to precede transverse movement into the rostral domain of each somite (Teillet et al., 1987). Regardless of the exact trigger, such a longitudinal movement of neural or mesodermal progenitors might serve cells that originate from all the length of a segment to sense local cues until reaching target areas where appropriate signals for homing are encountered.

An important question in this regard concerns the mechanism by which the longitudinal migration here described occurs. Since cells in both the lips and the SLD proliferate, the possibility had to be considered that continuous cell division displaces the progenitors within the SLD towards the extreme dermomyotome edges. This possibility was already considered to be highly unlikely, based on the measured rates of cell movement compared with the length of the cell cycle (Kahane et al., 1998b; Cinnamon et al., 1999). In further support of this content, this study clearly illustrates that 10 hours after dye labeling, cells spread along 60% of somitic length (about 90 μm) when compared with only 20% of somitic length (30 μm approximately) by 6 hours, a time point at which they became apparent in the SLD (see Fig. 3). Thus, a net distance of about 60 μm (30 μm in either direction or about three cell diameters) was traversed by the labeled cells within 4 hours, a time corresponding to less than half the length of the cell cycle of somite progenitors (Langman and Nelson, 1968; Primmett et al., 1989). Therefore, passive displacement due to mere proliferation cannot account for cell migration along the SLD. We thus favor the alternative mechanism by which the mesenchymalization of lip progenitors taking place during delamination from the medial and lateral lips generates cell motility that is required for longitudinal migration.

The state of specification of cells in the SLD

Migratory progenitors that give rise to limb muscles express Pax3, Met, Lbx1 and FREK but do not transcribe myogenic genes until later stages (Bladt et al., 1995; Bober et al., 1991; Dietrich et al., 1998; Marcelle et al., 1994; Marcelle et al., 1995; Goulding et al., 1994; Pownall and Emerson, 1992; Smith et al., 1994; Williams and Ordahl, 1994; Yang et al., 1996). In contrast, myotomal myoblasts, long considered to be a nonmigratory subset, begin expressing muscle-determining genes even prior to the formation of myofibers (Pownall and Emerson, 1992, Kahane et al., 1998a). In light of the finding that DML and VLL cells undergo migration through the SLD prior to differentiation, it was relevant to ask whether the SLD subpopulation expresses myogenic factors. Our finding that these mesenchymal progenitors express both MyoD and Myf5 strongly suggest that these cells undergo migration as specified myoblasts. A similar situation holds for the pioneer myotomal progenitors, which, in the avian embryo, express MyoD and then Myf5 when still confined to the somitic epithelium and later in migration express them towards the rostral edge of the dermomyotome (Kahane et al., 1998a; Kalcheim et al., 1999). Thus, myotomal progenitors of the first and second waves are likely to migrate as specified myoblasts. In this context, it is important to notice that in the absence of Myf5 gene activity, muscle progenitor cells migrate aberrantly and gave rise to dermal and sclerotomal cells instead of muscle (Tajbakhsh et al., 1996). Thus, expression of myogenic genes and, therefore, specification to the myogenic lineage, is important for the acquisition of reponsiveness to local cues that ensure patterned cell migration.

Furthermore, cells in the SLD also express the FGF-like receptor FREK. Although the precise role of FREK signaling in muscle development remains unknown, it has been found to be associated with migrating and proliferating myoblasts in both limb buds and myotomes (Marcelle et al., 1994; Marcelle et al., 1995; N. K. and C. K., unpublished observations). Thus, expression of FREK in migrating SLD progenitors could be linked to the observation that some are mitotically active. It is noteworthy that migratory cells in the SLD are both mitotically active and express myogenic genes (this paper). Furthermore, a subset of muscle progenitors in the rostral and caudal lips of the dermomyotome was also found to express MyoD (Kahane et al., 1998b) and retain proliferative capacity while being within the epithelia (N. K. and C. K., unpublished observations). At variance, a subset of pioneer myotomal progenitors expresses MyoD in the dorsomedial region of the still epithelial somite, yet most of these cells have already withdrawn from the cell cycle (Kahane et al., 1998a). Hence, within the myotome, both MyoD and Myf5 are expressed in postmitotic myofibers, but prior to myofiber differentiation these factors are transcribed by various types of myogenic progenitors whether postmitotic or still mitotically active cells.

In contrast to expression of MyoD, Myf5 and FREK, cells in the SLD have downregulated Pax3, which is expressed by their epithelial ancestors, and do not yet express desmin, that reflects overt muscle differentiation. Thus, SLD cells exhibit intermediate properties between dermomyotome and myotome. Absence of Pax3 from these migratory cells is at variance with the continuous expression of this transcription factor in long-range migratory precursors. Yet, Pax3 expression in the DML and VLL epithelia may be required for delamination of lip progenitors into the SLD and/or for triggering expression of secondary genes that affect subsequent longitudinal cell movement along the medial and lateral SLDs. Consistent with these possibilities, it was recently reported that in Splotch mutant mice that lack *Pax3* gene activity, the dermomyotome lips are severely disorganized, and both dermomyotomes and myotomes fail to elongate medially and laterally, leading to malformations in the entire trunk musculature (Tremblay et al., 1998).

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Note added in proof

Hirsinger et al. (2000) have recently reported on the expression of Notch1 and Delta1 in 'immature myotomal cells located between the dorsal lip and more differentiated myotubes expressing Mf20'. This expression pattern precisely corresponds to cells in the sub-lip domain (SLD) described here. As shown in the present study, the SLD is not part of the myotome; instead, it represents a distinct migratory pathway for myogenic progenitors.

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