

The cellular mechanism by which the dermomyotome contributes to the second wave of myotome development

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SUMMARY

We have shown that a subset of early postmitotic progenitors that originates along the medial part of the epithelial somite gives rise to the primary myotome (Kahane, N., Cinnamon, Y. and Kalcheim, C. (1998). *Mech. Dev.* 74, 59-73). Because of its postmitotic nature, further myotome expansion must be achieved by cell addition from extrinsic sources. Here we investigate the mechanism whereby the dermomyotome contributes to this process. Using several different methods we found that cell addition occurs from both rostral and caudal edges of the dermomyotome, but not directly from its dorsomedial lip (DML). First, labeling of quail embryos with [³H]thymidine revealed a time-dependent entry of radiolabeled nuclei into the myotome from the entire rostral and caudal lips of the dermomyotome, but not from the DML. Second, fluorescent vital dyes were injected at specific sites in the dermomyotome lips and the fate of dye-labeled cells followed by confocal microscopy. Consistent with the nucleotide labeling experiments, dye-labeled myofibers directly emerged from injected epithelial cells from either rostral or caudal lips. In contrast, injected cells from the DML first translocated along the medial boundary, reached the rostral or caudal dermomyotome lips and only then elongated into the myotome. These growing myofibers had always one end attached to either lip from which they elongated in the opposite direction. Third, following

establishment of the primary myotome, cells along the extreme dermomyotome edges, but not the DML, expressed *QmyoD*, supporting the notion that rostral and caudal boundaries generate myofibers. Fourth, ablation of the DML had only a limited effect on myotomal cell number. Thus, cells deriving from the extreme dermomyotome lips contribute to uniform myotome growth in the dorsoventral extent of the myotome. They also account for its expansion in the transverse plane and this is achieved by myoblast addition in a lateral to medial direction (from the dermal to the sclerotomal sides), restricting the pioneer myofibers to the dermal side of the myotome. Taken together, the data suggest that myotome formation is a multistage process. A first wave of pioneers establishes the primary structure. A second wave generated from specific dermomyotome lips contributes to its expansion. Because dermomyotome lip progenitors are mitotically active within the epithelia of origin but exit the cell cycle upon myotome colonization, they can only provide for limited myotome growth and subsequent waves must take over to ensure further muscle development.

Key words: Avian embryo, *QmyoD*, *Qmyogenin*, Myogenesis, Neural tube, Somite, Postmitotic cells, Myoblast proliferation, Satellite cells

INTRODUCTION

Studies addressing the development of the paraxial mesoderm have established that the epithelial somite can be subdivided into distinct domains according to the expression patterns of specific genes and to the fates adopted by the corresponding cells in normal ontogeny. While the *ventral* part of the somite de-epithelializes to give rise to the sclerotome, its *dorsal* half remains transiently epithelial and becomes the dermomyotome which contributes to the dermis, epaxial, hypaxial and limb muscles. The sclerotome can be further subdivided into *rostral* and *caudal* halves, based on its differential ability to support the migration of neural crest cells, the growth of peripheral axons and the formation of distinct vertebral components (Bronner-Fraser, 1986; Goldstein and Kalcheim, 1992;

Kalcheim and Teillet, 1989; Lallier and Bronner-Fraser, 1988; Rickmann et al., 1985; Stern and Keynes, 1987). A further subdivision of the dorsal somitic compartment into *medial* and *lateral* domains has also been suggested. The dorsomedial part of the somite thus develops into epaxial muscles via the intermediate myotome; and the lateral part differentiates into the muscles of the body wall and limbs following migration of a subset of myogenic progenitors from the lateral part of the somite-derived dermomyotome (see Christ and Ordahl, 1995; Cossu et al., 1996; Lassar and Munsterberg, 1996; Yamaguchi, 1997 for reviews).

Initial specification of the somite into its intermediate dermomyotomal and sclerotomal components, was found to be influenced by multiple extracellular signals emanating from the dorsal neural tube and ectoderm and from the notochord/floor

plate, respectively (reviewed by Yamaguchi, 1997, see also Dietrich et al., 1997 and references therein). Furthermore, the expression of regulatory genes that define muscle development can be either activated or repressed in response to varying levels and/or combinations of the above patterning signals (Amthor et al., 1996; Borycki et al., 1997; Dietrich et al., 1997; Goulding et al., 1994; Münsterberg and Lassar, 1995; reviewed in Lassar and Münsterberg, 1996). Interestingly, axial structures and dorsal ectoderm were suggested to induce myogenesis in the mouse through separate pathways involving *Myf5* or *MyoD*, respectively (Cossu et al., 1996).

The molecular nature of these signals has begun to be unraveled in recent studies involving overexpression of locally synthesized factors. While ventral sclerotome patterning is stimulated by Sonic hedgehog (reviewed by Bumcrot and McMahon, 1995), the specification of dorsal somitic structures is positively influenced by coordinate actions of Wnt family members and Sonic hedgehog, and negatively affected by members of the BMP family whose activity appears to be antagonized by noggin (Capdevila et al., 1998; Hirsinger et al., 1997; Marcelle et al., 1997; Münsterberg et al., 1995; Stern et al., 1995).

Surprisingly, while the molecular identity of the signaling factors that induce dermomyotome development and expression of muscle-specific genes is rapidly becoming clarified, the cellular basis of myotome formation and the contribution by the dermomyotome to this process still remain controversial. As the early myotome is composed of postmitotic cells (Langman and Nelson, 1968), its growth can only be accounted for by cell addition from extrinsic sources. Boyd (1960) and Hamilton (1965), suggested that the myotome is colonized by cells that originate only in the dorsomedial lip (DML) of the dermomyotome. In contrast, Langman and Nelson (1968) reported that myotomal precursors delaminate from the entire extent of the overlying dermomyotome, but not from the DML. Other studies claimed that sclerotomal cells are able to reaggregate and contribute to the colonization of the myotome (Mestres and Hinrichsen, 1976). Direct observation of developing somites by electron microscopy, led Christ et al. (1978) to propose that the myotome is composed, to different extents, of cells from all four edges of the overlying dermomyotome. In a later study, Kaehn et al. (1988) proposed that the site of onset of myotome formation is the rostrodorsomedial corner of the dissociating somite, but not the DML. According to this model, myotome formation progresses both in the rostrocaudal and in the mediolateral directions within individual segments. Although this model fits well with the triangular pattern produced by the early growing myotomal cells, it does not explain the observation that the earliest myogenic transcription factors (*myf5*, *QmyoD* and *CMD1*, for mouse, quail and chick embryos, respectively) are localized along the entire medial part of the epithelial somite prior to overt expression of differentiation markers (Borycki et al., 1997; Ott et al., 1991; Piette et al., 1992; Pownall and Emerson, 1992; Pownall et al., 1996).

However, a divergent view was presented more recently (Denetclaw et al., 1997) in a study based on focal dye injections into specific areas of the dermomyotome where the authors showed that myofiber generation is not restricted to the dorsomedial corner of the dermomyotome, as previously proposed (Kaehn et al., 1988), but spread to the entire medial

boundary (the DML) and the medial portion of the rostral boundary of the dermomyotome. The mechanism proposed to account for this contribution inferred the ingression of DML cells underneath the dermomyotome followed by their elongation in rostral, caudal or in both directions simultaneously from their site of origin, with no cell translocation occurring along the longitudinal extent of the somite. Also in contrast to the previous model, the latter proposed a ventrolateral to dorsomedial direction of myofiber addition.

In a recent study, we have re-evaluated the problem of myotome formation (Kahane et al., 1998). Using combinations of DiI labeling and proliferation assays, we have found that the origin of the myotome resides in a group of early postmitotic cells located along the medial aspect of the epithelial somite prior to dermomyotome formation, consistent with the reported expression pattern of the earliest muscle regulatory factors. Upon somite dissociation, these progenitors bend underneath the nascent dermomyotome, migrate in a rostral direction and accumulate transiently in the rostral somitic domain. This progressive rostral migration leads to a triangular shape initially consisting of mesenchymal cells and later of young myofibers elongating in a caudal direction, previously observed upon staining with muscle-specific markers (Kaehn et al., 1988; Borycki et al., 1997). Elongation of these cells that we termed "muscle pioneers" occurs until they all reach the entire rostrocaudal and mediolateral extents of the segment, thereby establishing the primary myotomal structure. Thus, the process of myotome formation begins in the epithelial somite and not in the dermomyotome, i.e. earlier in time and consequently in a different location than previously proposed.

Nevertheless, primary myotome formation by the pioneer cells is only the first wave in a multistage process leading to axial muscle development. In this study, we have characterized the origin and cellular movements that account for the second wave responsible for myotome expansion. We report that this is achieved by cell addition from both rostral and caudal lips of the dermomyotome. Moreover, the DML also contributes to the myotome. Contrary to previous models, we find that cells from the DML relocate longitudinally within the confines of the epithelium to enter the myotome through the medial part of the rostral or caudal dermomyotome edges, but not by way of ingression in the transverse plane. In this plane, the newly added myoblasts localize within the enlarging myotome in a medial position with respect to the pioneer cells (closer to the sclerotome) and along the entire dorsoventral extent of this structure. This topographical segregation shows that the myotome expands in a lateral-to-medial (dermal to sclerotomal) direction.

MATERIALS AND METHODS

Embryos

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

Embryonic microsurgery

After removal of the vitelline and amniotic membranes, the superficial DML was removed unilaterally along the length of somites XIII-XVI in embryos aged between 24-27 somite pairs (numbered according to Ordahl, 1993). Following surgery, embryos were incubated for 1 or 2 additional days.

Detection of dividing and postmitotic progenitor cells

Two protocols were employed:

(1) The first consisted of applying a single pulse of 50 μ l phosphate-buffered saline (PBS, pH 7.3) containing 10 μ Ci of [3 H]thymidine (specific activity 45–47 Ci/mmol; Amersham) onto the blastoderms of 25-somite-stage quail embryos. Embryos were fixed 4, 14 or 24 hours later (at least 5 embryos for each time point). Thirty-somite-stage embryos were similarly treated and fixed after 30 hours ($n=5$). These embryos were analyzed at the level of the rostral somites (Fig 2A,B) where the pattern of cell addition to the already established primary myotome was followed.

(2) The second protocol consisted of applying 2 pulses of [3 H]thymidine at 7-hour intervals, starting at E1. From E2 (average of 20 somite pairs), the embryos were chased with a 100-fold molar excess of cold thymidine until fixation on E4 ($n=8$) or E6.5 ($n=6$). A few embryos that were fixed immediately following the end of pulsing revealed that all cells were labeled (not shown). Therefore, in this protocol, those cells that continued dividing actively during the chase efficiently diluted the radioactive metabolite and became unlabeled. In contrast, the early postmitotic cells retained the label over their nuclei (see Fig. 2D-F).

The above treatments had no significant effects on the normal morphogenesis of embryonic structures, at least until E9, the last age examined (see Kahane and Kalcheim, 1998). Previous studies also reported on normal development upon treatment with similar amounts of the radioactive nucleotide (Lewis, 1977; Summerbell et al., 1986). Moreover, no significant differences were found at E4, when comparing the number of total cells per myotome between untreated embryos, embryos that received only a radioactive pulse, embryos that received both the radioactive pulse and the chase and embryos that received only the chase with unlabeled thymidine (data not shown). Thus, the protocols employed did not adversely affect the proliferation of progenitors that colonize the myotome.

Dil labeling of dermomyotomal sites and fluorescence imaging

Embryo preparation

25- to 30-somite stage embryos were dye-labeled at levels corresponding to somites 12–17, respectively. After removal of the vitelline membrane, a unilateral slit was made in the ectoderm. A small drop of pancreatin (2% w/v) was then added to assist in ectoderm removal and in the separation of the neural tube from the adjacent somites. Enzymatic activity was stopped by newborn calf serum (10% in PBS).

Dye labeling

Borosilicate tubes with filament (OD=1.0 mm, ID=0.5 mm) were

pulled using a vertical puller (Sutter model P-30). Tip diameter of the resulting micropipettes was estimated to be 0.2 μ m. Micropipettes were backfilled either with 1,1', di-octadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) or with 3, 3' di-octadecyl-oxacarbocyanine perchlorate (DiO; Molecular Probes) dissolved in absolute ethanol and further diluted 1:2 in tetraglycol to a final concentration of 0.2% (weight/volume). Micropipettes were then mounted on a Zeiss micromanipulator. Dye injections were performed by iontophoresis. Current was applied through a Ag/AgCl wire placed in a 2 M LiCl solution immediately before somite injections and a 3 second pulse, with 100 nA of current was employed 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 $\times 20$) and epifluorescence. Embryos were viewed with oblique lighting from a fiber optic light source. Several types of focal injections were made: (i) to the center of the DML or to regions adjacent to it; (ii) to the center of the rostral or caudal edges of the dermomyotome; (iii) to the intersomitic region. The accuracy of labeling sites was monitored throughout the procedure by observation under a total magnification of $\times 200$ with combined bright-field and epifluorescence optics. Following dye labeling, embryos were further incubated for various

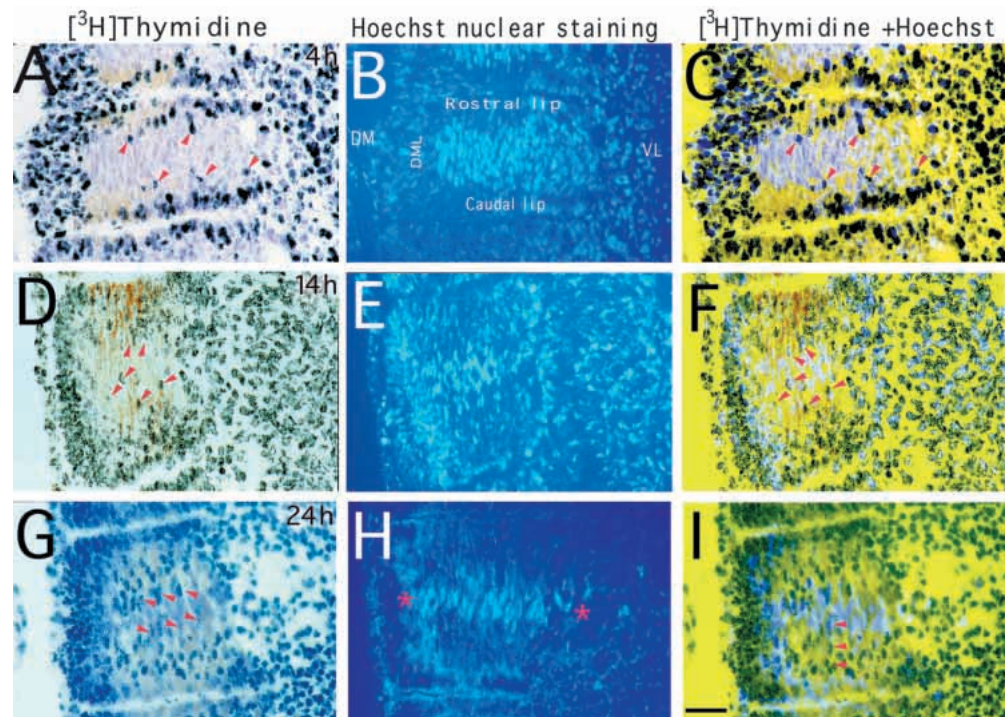


Fig. 1. Growth of the myotome by cell addition from the rostral and caudal lips of the dermomyotome. Sagittal sections at the level of somite XIII of three embryos that were labeled at the 25-somite stage with radioactive thymidine according to protocol I and fixed 4 hours (A-C), 14 hours (D-F) or 24 hours (G-I) following initial pulsing. A,D,G are bright-field images showing autoradiography after thymidine incorporation and 13F4 immunostaining of myotomal fibers (brown color). B,E,H depict the Hoechst-stained nuclei of all cells where it is possible to appreciate the elongated form characteristic of myotomal nuclei, and panels C,F,I are a combination of bright-field and Hoechst staining to show the correspondence between the localization of the thymidine grains and the stained nuclei. Red arrowheads in the different panels point to the time-dependent relocation of labeled nuclei from along the rostral and caudal lips of the dermomyotome toward the center of the myotomes that contain the unlabeled nuclei of the pioneer myofibers (nuclei between asterisks in H). Arrowheads in I point to three radiolabeled nuclei progressing in a row into the myotome, following the rostrocaudal orientation of the preexisting pioneer myofibers. B defines the orientation that was kept in all micrographs: DML (dorsomedial lip of the dermomyotome) is to the left, rostral to the top, caudal to bottom, and VL (ventrolateral part of the myotome) is to the right of the figure. Bar, 35 μ m.

lengths of time ranging from time 0 controls to 24 hours. At the end of incubation, embryos were removed from the eggshell, washed in PBS and fixed in formaldehyde (see below).

Laser scanning confocal microscopy

Fluorescent samples were analyzed using an LSM410 scanning confocal microscope (Zeiss, Jena, Germany) with a He-Ne laser for the excitation wavelength of 543 nm (DiI) and Argon laser for excitation wavelength of 488 (DiO), attached to an Axiovert 135M microscope. Dye-labeled cells were visualized with $\times 10$ or $\times 20$ oil immersion Plan-Neofluar objectives. The samples were optically screened at 2 μm increments through the Z-axis and sequential images were collected using a Pentium 150 personal computer. Confocal images represent cumulative serial sections that include, in each case, all fluorescently stained cells.

In situ hybridization

The *QmyoD* and *Qmyogenin* probes were kindly provided by Charles Emerson (de la Brousse and Emerson, 1990). Probe synthesis and whole mount in situ hybridization were done essentially as described by Borycki et al. (1997). Embryos were then embedded in paraplast and serially sectioned.

Fixation, immunofluorescence and autoradiography

Embryos were fixed either in Bouin's fluid or in 4% formaldehyde and embedded in paraplast. Serial 7 μm sections were mounted on gelatinized slides. Immunostaining with desmin or 13F4 antibodies was performed as described by Rong et al. (1987) to visualize myotomes. If combined with autoradiography, immunostaining was performed before coating the slides with photographic emulsion. Autoradiography, after thymidine labeling, was performed as previously described (Brill et al., 1995). Following development of the photographic emulsion, sections were counterstained with Harris hematoxylin or with the Hoechst nuclear stain.

RESULTS

We have recently shown that a subset of early postmitotic cells originating along the medial part of the epithelial somite constitutes the primary myotome, thus representing the first of several waves of muscle progenitors that colonize the nascent myotome (Kahane et al., 1998). To investigate the mechanism of myotome expansion from this stage onward, we have followed the pattern of cell addition to this growing structure, the localization of newly added myoblasts relative to the pioneer myofibers, and tested the consequences of ablating a putative source of myogenic cells on myotome growth.

Time-dependent addition of myogenic cells from both rostral and caudal lips of the dermomyotome

Previous studies in which short pulses (2-3 hours) of radiolabeled thymidine were delivered to embryos of about 25 somite pairs, have clearly shown that no DNA synthesis takes place within the myotomes at this early time (Langman and Nelson, 1968; Sechrist and Marcelle, 1996; Kahane and Kalcheim, data not shown). In contrast, the overlying dermomyotome previously proposed to generate myoblasts, is composed of actively dividing cells. Therefore, to accurately map the pattern of cell addition from the dermomyotome into the growing myotome, 25-somite-stage embryos were pulsed once with [^3H]thymidine according to protocol I and the distribution of labeled nuclei was observed at the level of somite XIII as a function of time after pulsing. Based on the above, appearance of radiolabeled cells within the myotome must

reflect immigration of cells which took up the nucleotide when still being part of the epithelial dermomyotome, either divided a few times or not at all, and became postmitotic upon myotome colonization thereby retaining the nuclear label over time.

The results presented below are based on analysis of serial sections in the sagittal plane. Similar results were observed in embryos sectioned in both the transverse and frontal planes (data not shown). Fig. 1A-C, represents the situation observed 4 hours after the pulse. At this stage, the majority of labeled cells are confined to the dermomyotome edges while most nuclei of myotomal cells are devoid of any label. These nuclei belong both to the pioneer myofibers and to myoblasts that had colonized the myotome prior to the beginning of the experiment. Only a few labeled cells are apparent at this stage in the extreme part of the myotome in close proximity to the rostral and caudal lips of the dermomyotome (arrowheads in A and C). Fourteen hours following the radioactive pulse, more labeled nuclei are apparent within the 13F4-immunoreactive myotome, few of them approaching its central portion composed primarily of postmitotic (Hoechst-positive/thymidine-negative) nuclei. This front of labeled nuclei is seen close to the rostral and caudal lips of the dermomyotome and along their entire dorsomedial to ventrolateral extent (Fig. 1D-F, arrowheads in D and F). Twenty four hours following the radioactive pulse, many labeled nuclei have already reached the center of the myotome with a few intercalating between the centrally located, unlabeled nuclei of the pioneer myofibers (Fig. 1G-I and arrowheads in G and I). The latter ones remain as a continuous stripe of unlabeled nuclei spanning the entire extent of the myotome reaching on one side the DML and on the other the ventrolateral edge (see Hoechst-positive nuclei in Fig. 1H between asterisks and see also Fig. 2A,B). It follows that no particular concentration of labeled nuclei is apparent in the myotome along the DML (Fig. 1D-I). This picture is complementary to that observed when positively mapping the localization of the pioneer nuclei (Fig. 2D and see Fig. 8 in Kahane et al., 1998).

Furthermore, in many instances, the labeled nuclei were observed to be arranged in rows between the rostral or caudal lips and the myotome center, suggesting that they follow the general longitudinal orientation of the preexisting primary myotome (Fig. 1, arrowheads in I and data not shown). Altogether, these results suggest that addition of newly generated cells to the myotome takes place from both rostral and caudal dermomyotomal boundaries, but not directly from the DML. Knowledge of the presence of a primary myotomal structure connecting between rostral and caudal dermomyotome edges prior to the onset of immigration from these regions raises the hypothesis that myoblasts of the second wave use the primary myofibers as a migratory substrate.

Myotome expansion in the transverse plane takes place in a lateral to medial direction

To investigate whether there exists a patterned addition of cells in the transverse plane of the growing myotomes, quail embryos were pulsed as described under protocol I in the Methods section and further incubated for 30 hours. Transverse sections through the center of the myotome, clearly revealed the presence of 13F4-positive/thymidine-negative nuclei along the lateral extent of the myotome apposed to the dermis (Fig.

2A,B). These nuclei belong to the pioneer myotomal cells that were already postmitotic at the beginning of the pulse (Fig. 2, arrowheads in A). In contrast, the medial part of the myotome that faces the sclerotome contained thymidine-labeled nuclei that represent the newly added generations of myoblasts (arrows in Fig. 2B). At this stage, the two layers of unlabeled and labeled nuclei, respectively, were preferentially segregated. These results suggest that, at least until E4, myotome growth in the transverse plane takes place by the ordered addition of consecutive generations of myoblasts in a lateral to medial direction (Fig. 2C).

To trace the relative localization of the pioneer cells in comparison to younger myoblast generations at later stages of epaxial muscle development, it was necessary to positively mark the pioneer cells. To this end, embryos were pulse-chased with thymidine as described under protocol II. At E4, positively labeled pioneer nuclei are distributed along the entire dorsoventral extent of the myotome and restricted to its dermal side (Fig. 2D, open arrow), similar to the image depicted in panels A and B using the complementary labeling protocol. Consistently, at later stages (E6.5), the pioneer cells retained their relative location in the muscle adjacent to the dermis. At this stage, however, the labeled pioneer cells already constituted a minority within the epaxial muscle as they were intermingled with newly intercalated cells that were devoid of any radioactive labeling. Under this labeling protocol, the significant mass of unlabeled muscle cells had derived from progenitors that were mitotically active during the chase period (Fig. 2E,F arrowheads pointing to the pioneer nuclei) (see Discussion).

Myofiber development following dye labeling of epithelial cells in both rostral and caudal dermatomyotome lips

To substantiate the above finding that epithelial cells of the rostral and caudal dermatomyotome edges contribute to myotome expansion, a group of cells in somites IX–XIV of 25- to 30-somite-stage embryos, was labeled with DiI. Dye injections were directed to cells located in the center of either

the rostral or caudal lips midway between their dorsomedial and ventrolateral extremities (see Fig. 3A,D, which are confocal views of the somites shortly after labeling). Embryos were further incubated for 4 and 18–24 hours. Four hours post-labeling, the fluorescent cells were still confined to the dermatomyotome and no fibers were yet apparent (Fig. 3B,E; $n=23/23$ labeled somites). This result confirms that the dye did not attain any preexisting myofibers. Eighteen to 24 hours after labeling, the presence of fluorescent fibers was revealed in 37 out of the 44 injected somites. These were found to extend either along the entire rostrocaudal extent of the myotome or along a partial length of the myotome anchored to the dermatomyotome lip that was dye-labeled (Fig. 3C,F see also G). In several instances, combinations

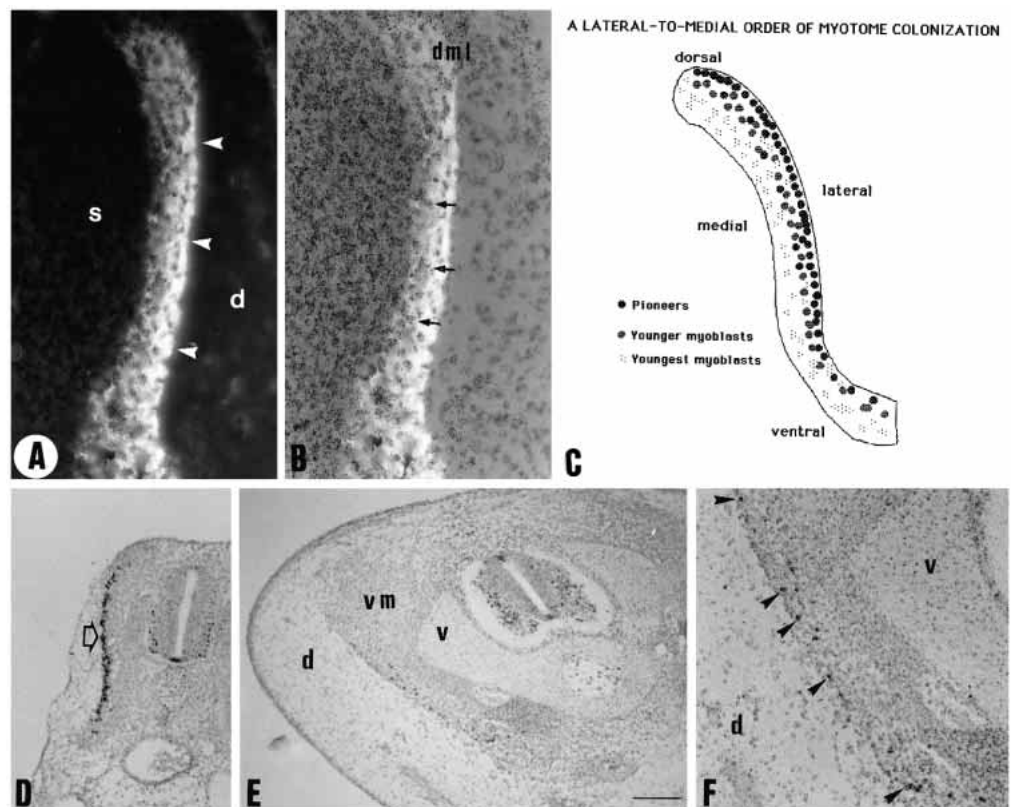
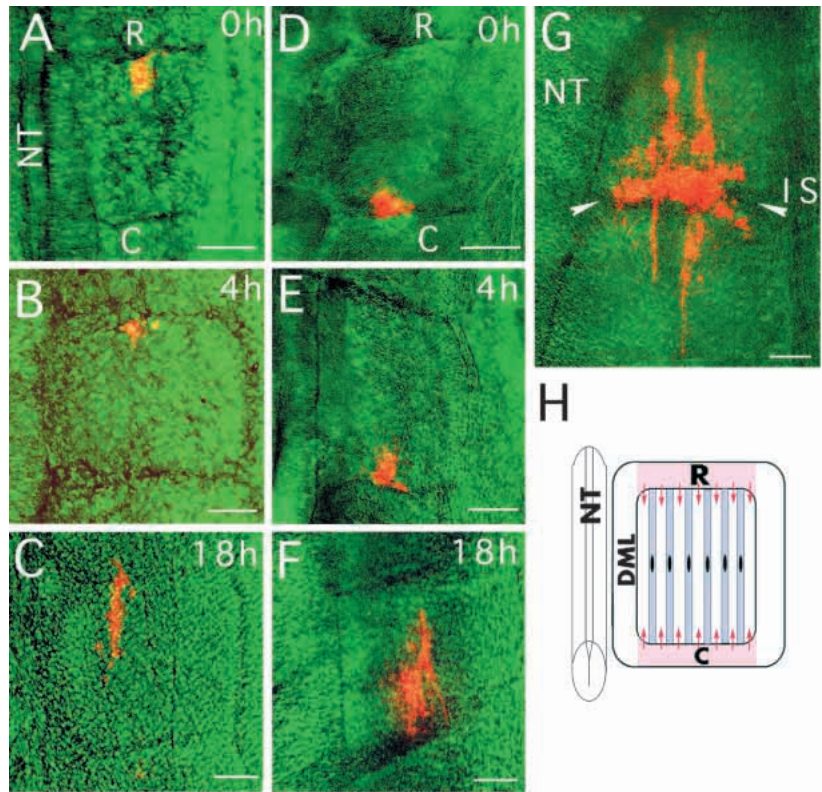


Fig. 2. A lateral to medial order of myotome colonization in the transverse plane. (A,B) Transverse section through the center of a cervical-level somite (13) of an E4 quail embryo that was pulsed with radiolabeled thymidine as described under protocol I. (A) fluorescence; (B) combined fluorescence and bright-field optics. Note in A the presence of 13F4-positive/thymidine-negative nuclei (arrowheads) along the lateral extent of the myotome apposed to the dermis (d). These nuclei belong to the pioneer myotomal cells that were already postmitotic at the beginning of the pulse. In contrast, the medial part of the myotome that faces the sclerotome (s) contains thymidine-labeled nuclei that represent the newly added generation of myoblasts (see small arrows in B). Note that both unlabeled and labeled cells span the entire dorsoventral extent of the myotome. (C) Scheme illustrating a model for myotome expansion in the transverse plane showing three putative generations of myoblasts segregated according to age from lateral (dermal side, older) to medial (sclerotomal side, younger). (D) Transverse section through a cervical somite (15) of an E4 embryo labeled according to protocol II. In this protocol, pioneer cells are positively labeled as they exited the cell cycle early during somitogenesis, complementary to what is shown in panels A and B. The labeled pioneer nuclei, adjacent to the dermis, extend along the entire dorsoventral extent of the segment (open arrow) attaining the dorsomedial lip area. The remaining somite derivatives (dermis and sclerotome) are unlabeled. (E,F) Transverse section through somite 15 of an E6.5 embryo that was labeled according to protocol II. F is a higher magnification of E showing that in the myotome-derived vertebral muscle (vm), the labeled pioneer nuclei remain apposed to the dermal side but, in contrast to E4 (D) the pioneer cells are now scattered between unlabeled cells which at this stage already constitute the prominent muscular mass. Bar, A,B,F, 50 μm ; D, 130 μm ; E, 120 μm .

Fig. 3. DiI labeled cells in the rostral and caudal dermomyotome edges generate myofibers. (A-C) Confocal images after DiI labeling in the center of the rostral lip (R) of the dermomyotome of somite X in 25-somite-stage embryos. Fixation was performed immediately (A), 4 hours (B) or 18 hours (C) after labeling. (D,E,F) Confocal images after DiI labeling in the center of the caudal lip (C) of the dermomyotome of somite XI in 28-somite-stage embryos. Fixation was performed immediately (D), 4 hours (E) or 18 hours (F) after labeling. Note in A and D, the highly localized spots of dye labeling in the dermomyotome lips immediately after injection. Similar localized spots remain 4 hours following labeling showing that no preexisting fibers were stained and no significant dye diffusion takes place (B and E). Note after 18 hours (C), the presence of a fiber in the process of elongating caudal-ward into the myotome. No lateral diffusion of the label along the dermomyotome edge is observed in this somite. In F, a few elongating fibers of various lengths are generated from dye injection in the C edge of the dermomyotome. In this segment, a restricted lateral diffusion of the dye can be observed when compared to similar injections at time 0 and 4 hours. (G) Confocal image after DiI injection in the intersomitic space (IS) between somites XIII and XIV of a 26-somite-stage embryo followed by fixation 20 hours later. Note the homogeneous diffusion of the dye along part of the intersomite (fluorescence between arrowheads). Myofibers are generated from discrete points along the extreme edges and they elongate both rostral-ward and caudal-ward into successive myotomes. (H) Schematic view of the contribution of R and C dermomyotome edges to myotome growth. The neural tube (NT) is to the left in all images; DML, dorsomedial lip. Bars, 50 μ m.



of unit length and partial length fibers were observed. In addition, all somites injected in the extreme boundaries revealed either no or a very limited spreading of labeled cells in the dorsomedial to ventrolateral direction along the epithelium when compared to the initial spot of DiI (Fig. 3 compare panels C with A and F with D).

In another series of embryos, dye injections were directed to the center of the narrow intersomitic space so as to label the epithelial cells pointing toward the outer surface of the corresponding dermomyotome lips. In all those cases ($n=8$) labeled myofibers elongated from the injected area both rostrally and caudally into the two adjacent myotomes (Fig. 3G). It is interesting that in contrast to the spot of DiI which remained highly localized after direct injections into the rostral or caudal lips (Fig. 3C,F), labeling of the intersomitic space resulted in dye diffusion along this cell-free space (Fig. 3G between arrowheads). As a result of this diffusion, myofibers emerged from a rather large area along the lips (Fig. 3G). Taken together, these results show that cells all along the rostral and caudal lips of the dermomyotome have the ability to generate myofibers (Fig. 3H).

DML cells translocate longitudinally until reaching the rostral and caudal lips of the dermomyotome through which they enter into the myotome

Previous studies have suggested that the DML provides the major source of myogenic cells that constitute the myotome by way of ingression underneath the dermomyotomal epithelium

(see Introduction). In contrast, we have shown (Kahane et al., 1998) that the first wave of myogenic cells originates earlier along the medial epithelial somite and gives rise to a primary myotomal structure after several steps of cell migrations that include bending underneath the nascent dermomyotome followed by de-epithelialization, rostral migration and then elongation. In addition, the pioneer fibers attain the DML region throughout development, without being displaced ventrally as inferred from a model implying ingression of DML cells. Moreover, the present results reveal that a major second wave of cells originates along the rostral and caudal edges of the dermomyotome and fibers developing from these epithelial cells follow the same longitudinal direction as their pioneer predecessors while elongating into the myotome. The above data prompted us to clarify the extent and mechanism whereby the DML contributes to myotome development.

To this end, somites IX-XIV in 25- to 30-somite-stage embryos were injected with DiI. Labeling was directed to the center of the DML midway between the rostral and caudal somite extremities (Fig. 4A). Spots had a characteristic diameter that ranged between 16 and 30 μ m. Approximately 10 hours following dye labeling, fluorescent cells were still confined to the epithelium but considerably spread as discrete spots along a distance of up to 170 μ m in the rostrocaudal plane of the DML. This was consistently noticed in all of the 24 labeled somites (Fig. 4, compare cells delimited by dashed line between arrows in B with focal spot in A). This is in striking contrast to the behavior of the dye-labeled cells in the rostral

and caudal extremities which remain localized around the site of injection. It also differs from the diffusion of the dye in the intersomite where it spreads homogeneously in a cell-free space. In a separate time-lapse experiment, injected somites were viewed in living embryos under epifluorescence at various times following initial labeling. Spreading of fluorescent cells along the DML progressed with time and first became apparent about 3 hours following labeling until reaching practically the entire length of the DML after 15 hours of incubation (data not shown). Unfortunately, these embryos did not survive for longer times, perhaps due to the frequent manipulations to which they were subjected for repetitive examination.

In all somites examined 18-24 hours after dye labeling, fluorescent cells were apparent along the rostrocaudal extent of the DML ($n=131$; Fig. 4C,D, delimited by dashed line between arrows). Moreover, myofibers were already visible in the myotomes of 93 out of the 131 injected somites. In 40 out of the 93 cases, many fibers were in the process of elongating into the myotome, thereby extending only along a partial length of the myotome (Fig. 4C,E). Most importantly, these fibers were always anchored either to the rostral or the caudal edge of the dermomyotome and had only one free end (Fig. 4C,E arrowheads pointing to the myofiber extremities). In the remaining 53/93 somites, unit length myofibers were already visible, attached to both rostral and caudal dermomyotome edges (Fig. 4D between arrowheads). These were often combined with partial length fibers. It should be mentioned that in none of the above cases, could we detect fluorescent, growing myofibers bearing two free, unattached processes that could elongate bilaterally, a picture that is predicted from a model involving local ingression of cells following injections made in the center of the DML.

To further explore the contribution of the DML to myotome colonization, DML cells located slightly rostral or caudal to the center of this epithelium (approximately 30-40 μm apart on either side) were labeled either with DiI (red) or DiO (green), respectively ($n=20$). Similar to the situation observed when labeling at the center of the DML, 8 hours following labeling, injected cells initially located away from the center were also found to move along the DML in both directions simultaneously (not shown). Consistent with these observations, these DML cells gave rise to fibers emanating from both extreme edges, although we could see more fibers elongating from the edge that was closer to the labeling site, probably because it took a shorter time for these cells to reach the corresponding lip. Fig. 4E illustrates a set of myofibers that emerged from these labeled cells. While one of the red fibers extends almost all the way from the rostral lip caudally but does not yet reach the caudal edge (see fiber delimited between arrowheads in panel E), an adjacent red fiber extends the whole length

of the myotome. Moreover, two green myofibers are attached to the caudal edge and extend rostrally reaching approximately the middle of the myotome (green fibers delimited by arrowheads in panel E). Note as well the bilateral spreading, along the epithelium, of cells bearing both the dyes. Thus, cells all along the DML are likely to relocate to the extreme rostral and/or caudal lips of the dermomyotome prior to myotome colonization.

Another important feature of the myofibers developing from DML-injected cells is their relative dorsomedial localization in

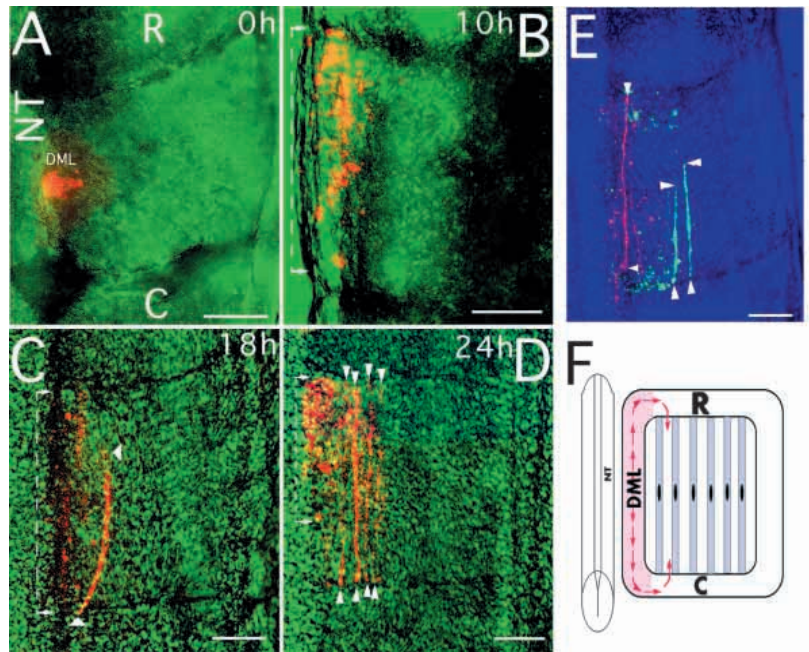


Fig. 4. DiI labeling of the DML generates medially located myofibers following DML cell relocation to the rostral and caudal edges of the dermomyotome. (A-D) Confocal images depicting a time course of the development of myofibers from DML cells labeled with DiI in the center of the epithelium. Injections shown were in somites IX-XIV of 25-27-somite-stage embryos. (A) Localized spot of DiI in the center of the DML followed by immediate fixation. (B) Note the widespread rostrocaudal distribution of distinct dye-labeled cells along the DML epithelium seen 10 hours postlabeling. This distribution is delimited by the dashed line between arrows. (C) 18 hours following dye labeling a similar rostrocaudal distribution of DML cells is apparent (dashed line between arrows) and in addition, an elongating myofiber is seen which has one end attached to the caudal edge of the dermomyotome and one free end in the myotome (marked by arrowheads). (D) 24 hours postlabeling most myofibers have reached both edges of the dermomyotome (marked by arrowheads), while dye-positive epithelial cells are distributed throughout most of the rostrocaudal extent of the DML (dashed line between arrows encompassing most cells). (E) Confocal image representing a double dye injection performed in somite X of a 27-somite-stage embryo. DiI (red) was injected rostral to the center of the DML and DiO (green) caudal to the center of the DML. The embryo was fixed 20 hours later. Note that the red fiber extends caudal-ward almost reaching the caudal edge of the dermomyotome (delimited by arrowheads), while the two green myofibers are attached to the caudal edge and the free ends elongate rostral-ward (delimited by arrowheads). Note as well the presence of distinct fluorescent cells along the DML that have reached both extreme edges. In C,D and E note that DML-derived myofibers are located relatively close to the DML and the axis (the neural tube, NT is to the left in all pictures) occupies approximately the medial quarter of a segment. (F) Schematic view of the movement of DML cells toward the medial part of the rostral (R) or caudal (C) edges of the dermomyotome, followed by entry into the myotome, similar to the fate of cells originating in the R and C lips. Bars, 50 μm .

the myotome remaining close to the DML (Fig. 4C-E). This was measured 24 hours after dye-labeling of the DML in 20 different myotomes. The mean spreading of DML-derived myofibers in the dorsomedial to ventrolateral extent of the myotome was about a quarter of the myotome length ($71 \pm 35 \mu\text{m}$ of a total extent of $268 \pm 35 \mu\text{m}$). This is in striking contrast with the behavior of pioneer myofibers which extend along the entire dorsomedial to ventrolateral aspect of the somite after labeling cells in the center of the epithelial somite (Kahane et al., 1998, and see also Fig. 2A-D).

The above results showing a time-dependent rostrocaudal distribution of fluorescent cells along the DML, their subsequent pattern of elongation into the myotome and their relative restriction to its dorsomedial portion suggest that DML cells first reach the rostral or caudal dermomyotome edges through which they in turn colonize the myotome (Fig. 4F).

The expression of *QmyoD* in the rostral and caudal dermomyotome edge

As previously described (Borycki et al., 1997), the expression of *QmyoD* starts early in the epithelial somite. *QmyoD* transcripts are then expressed in the myotome throughout development. In contrast, *Qmyogenin* is first expressed after initial formation of the myotome and remains restricted to the differentiated cells. In situ hybridization was performed using DIG-labeled *QmyoD* and *Qmyogenin* probes. Analysis of both whole mount and serially sectioned embryos at different stages (16-35 somite stages) revealed that from the 23- to 25-somite stage onward, *QmyoD* transcripts are expressed in epithelial cells of both the rostral and caudal lips of the dermomyotomes, in addition to their known expression in the myotomes (Fig. 5A,D). This additional staining pattern is observed in the 5-7 rostral-most myotomes of the axis in 23-somite embryos and extend further caudal at progressively more advanced stages. This axial region corresponds to a stage when pioneer myotomal cells had reached both the rostral and caudal edges of the segment (Kahane et al., 1998) and new generations of myotome progenitors, originating in the rostral and caudal dermomyotome edges, respectively, begin colonizing the myotome (see above). Consistent with our finding that no cells are directly added from the DML, we find no expression of *QmyoD* in the DML of the appropriate segments (Fig. 5C). In contrast to the expression pattern of *QmyoD* to both myotome and rostral and caudal dermomyotomal boundaries, the distribution of *Qmyogenin* transcripts is restricted to the myotomal compartment (Fig. 5B,E). These results further suggest that the rostral and caudal edges of the dermomyotome contain myogenic cells.

Ablation of the DML

To further assess the relative participation of the DML to myotome growth, direct ablation of the DML along three consecutive somites (XIV-XVI) in embryos with 24-27 somite pairs was performed. At this stage, the primary myotome is already established and cell addition from the dermomyotome lips is underway. Analysis of the embryos 1 or 2 days after surgery revealed the presence of a mesenchyme in the place of the ablated DMLs that failed to regenerate (Fig. 6B,C). When the DML was ablated in younger somites, the removed epithelia were found to regenerate to different extents, thereby precluding

accurate assessment of the contribution of the DML at these stages using microsurgery.

In all operated embryos ($n=11$), the histogenesis of the myotomes was not affected in the operated sides and massive amounts of tissue expressing muscle-specific antigens had developed. Moreover, the morphology of the myotomes was generally normal except for their ventrolateral region which appeared slightly larger and shorter when compared to their intact counterparts on the contralateral side (Fig. 6). Thus, qualitative observation of DML-ablated segments revealed no major deficits in myotome development. This was subsequently confirmed when counting the number of Hoechst-positive nuclei in desmin-immunoreactive myotomes. Cells were counted in every second section of embryos fixed 48 hours after surgery. Under these conditions, the likelihood of counting the same nucleus twice is very small. While all the cells were counted in the operated sides, DML cells negative for the muscle-specific marker, were not considered in the normal side. The number of Hoechst+/desmin+ cells was 1832 ± 192 and 1979 ± 283 cells in the operated as compared to the intact contralateral sides, respectively (mean \pm s.d. of $n=4$ myotomes counted in each side of 4 different embryos). These results further suggest that the DML has a limited contribution to myotome expansion.

DISCUSSION

Most studies dealing with regulation of myogenesis, consider the dorsomedial lip of the dermomyotome (DML) to be the initial and major source of progenitors that populate the myotomes (Christ and Ordahl, 1995, Denetclaw et al., 1997). In a recent work, we begun dissecting out the mechanism of myotome formation and found that this process can be divided into several stages with the onset along the medial part of the epithelial somite prior to somite dissociation and consequent dermomyotome establishment. The first wave of myotome progenitors is thus accounted for by a subset of postmitotic pioneer cells that bend underneath the forming dermomyotome, lose epithelial conformation, migrate rostrally toward the rostral dermomyotome lip area and then elongate to give rise to the primary, longitudinally oriented myotome (Kahane et al., 1998). In the present study, we report that after formation of this primary myotomal structure, addition of subsequent myoblasts to the expanding myotome takes place both from the rostral and caudal dermomyotome edges. The DML also contributes to myotome expansion, however, these cells must first reach the rostral or caudal lips of the dermomyotome through which they enter into the myotome rather than by way of ingression in the transverse plane.

The second wave of myotome development originates from epithelial cells of the rostral and caudal lips of the dermomyotome

The contribution of cells from the rostral and caudal edges of the epithelial dermomyotome is supported by several independent lines of evidence. First, experiments that consisted of pulsing embryos with radioactive thymidine at precise stages reveal the presence of labeled nuclei near the dermomyotome edges shortly after the beginning of the experiment and their time-dependent progression into the

myotome following the same direction as that of the preexisting longitudinal tracks of pioneer myofibers. Second, dye labeling of epithelial cells in either lip or injections in the intersomite that resulted in marking of two adjacent lips simultaneously, clearly show that these edges generate myofibers. Third, *QmyoD* transcripts are expressed along the rostral and caudal edges of the dermomyotomes at the corresponding stages, further suggesting that the QMyoD-positive epithelial cells, similar to the pioneer cells of the medial epithelial somite, might be specified to become myoblasts.

Of note is the fact that cells all along the dorsomedial to ventrolateral extent of the caudal and rostral lips are able to generate myofibers, and this capacity is not limited to the medial portion of the rostral lip of the dermomyotome, as previously suggested (Denetclaw et al., 1997). Several arguments support this notion: the distribution of labeled nuclei entering the myotome (Fig. 1); the elongation of fibers from dye-injected cells from either the center of the lips (this paper) or laterally (our unpublished results) or in the intersomites; and the pattern of QMyoD expression (Fig. 5).

Myoblast addition from the rostral and caudal dermomyotome begins, for instance, in 23- to 25-somite-stage embryos in the rostral-most 5-8 segments and extends further caudal as development proceeds. Therefore, this process succeeds and may even slightly overlap in time the establishment of the primary myotome by the pioneer cells which derive from the medial part of the epithelial somite. Because of this temporal continuity and the different topographical origin of the contributing progenitors, dermomyotome lip cells can be considered to give rise to a second wave of myogenic cells that contributes to myotome expansion.

DML cells relocate to the dorsomedial portion of the rostral and caudal dermomyotome lips through which they contribute myofibers to the dorsomedial part of the myotome

Dye labeling in the center of the DML reveals that its component cells develop into myofibers that are restricted to the dorsomedial portion of the myotome. In striking contrast, similar injections performed at the epithelial somite stage result in the formation of muscle fibers that extend all the way from the dorsomedial to the lateroventral parts of the myotome (Kahane et al., 1998, and see Fig. 2). This implies that while the medial pioneer cells have the capacity to form a full scaffold, distinct parts of the dermomyotome contribute to further growth of this structure in a topographically limited manner.

How does the DML contribute to the dorsomedial myofibers? Our data show that after focal labeling of the DML, a time-dependent redistribution of fluorescent cells along the length of this epithelium is observed that ultimately reaches the rostral and/or caudal dermomyotome extremities. The development of dorsomedially located myofibers follows this longitudinal distribution. The fact that these nascent fibers remain anchored to either the rostral or caudal lips and elongate into the myotome with one free-growing arm, like the cells directly injected in the rostral or caudal lips, suggests that DML cells must first relocate into the dermomyotome edges prior to being able to colonize the muscle. Assuming that the rate of

proliferation of dermomyotome cells is uniform in all lips, the observation that DML cells move to a much larger extent when compared to cells in the rostral and caudal lips, suggests that in addition to proliferation, cell rearrangements such as cell intercalations, must occur along the DML. These findings suggest that at least the DML behaves as a highly dynamic structure and the mechanisms responsible for cell translocation are presently being investigated in our laboratory. Furthermore, the fact that in a period of 24 hours the DML increases in length only by 12% while the dorsomedial to ventrolateral domain of the dermomyotome grows by 38% (our unpublished observations), strongly suggests that DML cells progressively relocate to the rostral and caudal edges of the dermomyotome. This mechanism partly explains the medially directed growth of the dermomyotome (Denetclaw et al., 1997).

Our results on longitudinal relocation of DML cells followed by generation of myofibers through the extreme lips always bearing one side anchored to these edges, conflict with the proposed model of local ingression of DML cells beneath the dermomyotome (Denetclaw et al., 1997). Our data are further substantiated by the observation that pioneer myotomal cells are found all the way from the ventral part of the myotome up to the DML, and that no displacement of the pioneers from the region adjacent to the DML occurs throughout development (Figs 1G-I and 2D-F).

This relocation of DML cells into the dermomyotome extremities followed by the formation of myofibers restricted to the dorsomedial part of the myotome suggests that the DML indirectly contributes to dorsoventral myotome growth to a rather limited extent. For example, we never found DML-derived myofibers located distally in the myotome. Following medial injections, distal fibers could only be found upon labeling of medial epithelial pioneer cells but not DML progenitors. In addition, the consequences of DML ablation on myotome cell number reveal a limited contribution of this epithelium. Although we cannot rule out the possibility that the adjacent epithelia might compensate to a certain extent for the lack of DML, it is clear that the DML as a structure is not the main epithelium contributing to myotome growth. We therefore propose that growth of the myotome in the dorsomedial direction is accounted for by uniform and continuous entry of cells from along the rostral and caudal lips, including DML cells that contribute through the medial-most part of these lips. Cells deriving from these areas progressively intercalate among fibers of the primary myotome and cause its expansion in the dorsal-ventral direction. Our view is consistent with the change in distribution of pioneer cells observed throughout development from an initially uniform population of cells to a scattered population at later stages that nevertheless, still attains the dorsomedial region.

Myotome growth in the transverse plane

In addition to growth in the rostrocaudal and dorsoventral directions, the thickness of the myotome also increases in the transverse plane. Radiolabeling of embryos following establishment of the myotome revealed the localization of new, labeled cells preferentially to areas of the myotome abutting the sclerotome, while the older, unlabeled cells faced the dermal side. This demonstration of topographically segregated cell layers distinctive by age shows that, at least until E4, the

myotome expands in a layered fashion from the lateral-most dermal side to its sclerotomal side (Fig. 2).

It is interesting that this behavior of the avian muscle pioneers is similar to that of their zebrafish adaxial counterparts. Both cell subsets originate from epithelial cells that develop adjacent to the midline and express *MyoD*. They differ, however, in their relative dorsoventral localization. Adaxial cells develop close to the notochord and avian pioneers adjacent to the dorsal half of the neural tube. Moreover, both types of pioneers reorganize later to span the entire extent of individual segments and finally localize in a lateral position within the mature myotomes (Devoto et al., 1996, and this paper).

A model for myotome development in avian embryos

Fig. 7 represents a schematic model for myotome development based on the results summarized in the present study and in Kahane et al. (1998). The myotome originates from a subset

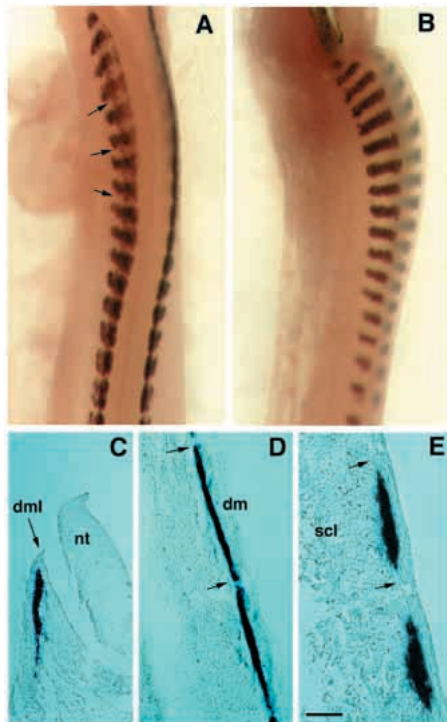


Fig. 5. Expression of *QmyoD* in the rostral and caudal lips of the dermomyotome. (A,B) Whole-mount in situ hybridization of two 32-somite-stage quail embryos hybridized with DIG-labeled *QmyoD* (A) and *Qmyogenin* (B) probes. Arrows in A point to expression of *QmyoD* transcripts in both the rostral and caudal edges of the dermomyotome, in addition to its myotomal expression. In contrast, *Qmyogenin* is restricted to the myotomes (B). (C) Transverse section through somite XVIII of a similarly treated embryo to that in A to show that *QmyoD* is expressed in the myotome but not in the DML at this stage. (D,E) Frontal sections of similar embryos as in A and B, respectively. D shows that *QmyoD* mRNA is expressed not only in the myotomes but also in both the rostral and caudal edges of the overlying epithelial dermomyotome. In contrast, *Qmyogenin* transcripts are restricted to the myotomes (E). The area between the arrows in D and E show the extent of a segment, including the dermomyotome edges. Bar, A, B, 300 µm; C, 50 µm; D, 40 µm; E, 70 µm.

of postmitotic epithelial cells present along the rostrocaudal extent of the medial part of the epithelial somite (A). Formation of the primary myotomal structure takes place first, by a translocation of the medial layer to a ventral position with respect to the dermomyotome. After initial bending, postmitotic cells progressively de-epithelialize and some migrate towards the rostral part of the somite giving rise to the observed triangular pattern that stains for *MyoD* and desmin (blue cells and arrows in B and C). The rostral dermomyotome edge may thus behave as an anchorage site for the local progenitors that begin sending processes in a caudal direction (arrows in C) until formation of the primary myotomal structure that spans the entire dermomyotome underlayer (blue myofibers in D). Upon reaching both segmental extremities, the nuclei of original pioneer cells become concentrated at the middle of the newly formed myofibers (D). Subsequently, the major contribution to myotome expansion derives from both the rostral and caudal dermomyotome lips which also express *QmyoD* at the appropriate stages, and from the DML epithelium whose cells first translocate into the medial-most part of the rostral and caudal lips and then contribute to myofiber generation in a similar way as their rostral and caudal

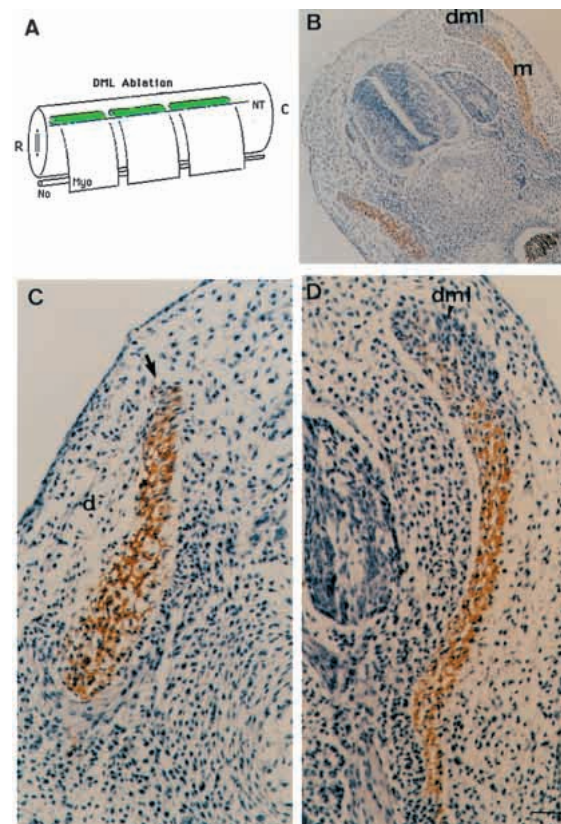


Fig. 6. Ablation of the DML has no significant effect on myotome growth. (A) Removal of three adjacent DMLs at E2.5 (see Methods for details). (B) Transverse section through an E4 embryo showing the normal DML (dml) and 13F4-immunoreactive myotome (m) on the control (right) side. In contrast, note that on the operated side the DML is absent, yet the myotome has a nearly normal appearance and attains the level corresponding to the DML (arrow in C). (C,D) Enlargements of the operated and control sides, respectively. d, dermis; dml, dorsomedial lip; m, myotome. Bar, B, 100 µm; C, D, 35 µm.

counterparts (red arrows in D). These subsequent generations of cells migrate in a longitudinal direction previously established by the myotomal pioneers and progressively intercalate between them in a uniform manner along the dorsoventral extent of the myotome (E and F). In the transverse plane (G), pioneers (blue cells) remain adjacent to the dermal side (L, lateral) whereas younger cells (red cells) initially localize apposed to the sclerotome (medial, M). This age-dependent preferential segregation suggests a lateral to medial order of myotome colonization in the cross-sectional plane. The second wave of myotomal cells thus contributes to myotome expansion in both the dorsoventral direction and the transverse plane.

How are the different muscle progenitor waves established?

The identification of several waves of myotome progenitors that are segregated both in time and space raises critical questions about how each cellular wave is induced in the somite by local environmental signals, what are the signals that affect cells in each of the waves and what molecular markers are expressed in common, or alternatively, differ between them.

Myogenesis in the somite is currently believed to be promoted by combined neural tube/notochord signaling via Wnts and Sonic hedgehog, and inhibited by lateral plate mesoderm signals such as BMP family members. Ectodermal cues were also proposed to positively affect myogenesis (see Introduction). The exact cellular targets for axial signaling remain, however, unknown. Based on their mediadorsal position in the epithelial somite apposed to the neural tube, it is possible to hypothesize that specification of the first wave of pioneer cells depends upon axial cues. This is further supported by the observation that the pioneer cells and *QMyoD* transcripts are colocalized in the medial part of the somite. A complete characterization of this first wave of muscle progenitors should also include the understanding of the factors that cause their migration towards the rostral half of the somite (either stimulatory or inhibitory), their anchorage to the rostral edge and their

subsequent elongation caudal-ward to establish the primary myotome.

The shift in the source of myogenic cells from medial regions apposed to the axis to rostral and caudal lips of the dermomyotome, suggests that further myotome development may be neural tube-independent. This is reinforced by the observation that DML cells, which also participate in the colonization of the myotome through the medial aspect of the rostral and caudal lips, do not express *QmyoD* mRNA while being apposed to the neural tube. This transcription factor is however expressed in the rostral and caudal boundaries during the second wave of myotome growth. Myogenic specification of these cells might thus result from their organization into lip-like structures which, as proposed by Tosney (reviewed in Tajbakhsh and Sporle, 1998), may act as muscle-producing centers. Alternatively, the second myogenic wave might be induced by contact of the pioneer myofibers with cells in the rostral and caudal edges of the dermomyotome, analogous to the recruitment of new muscle cells by founder progenitors that

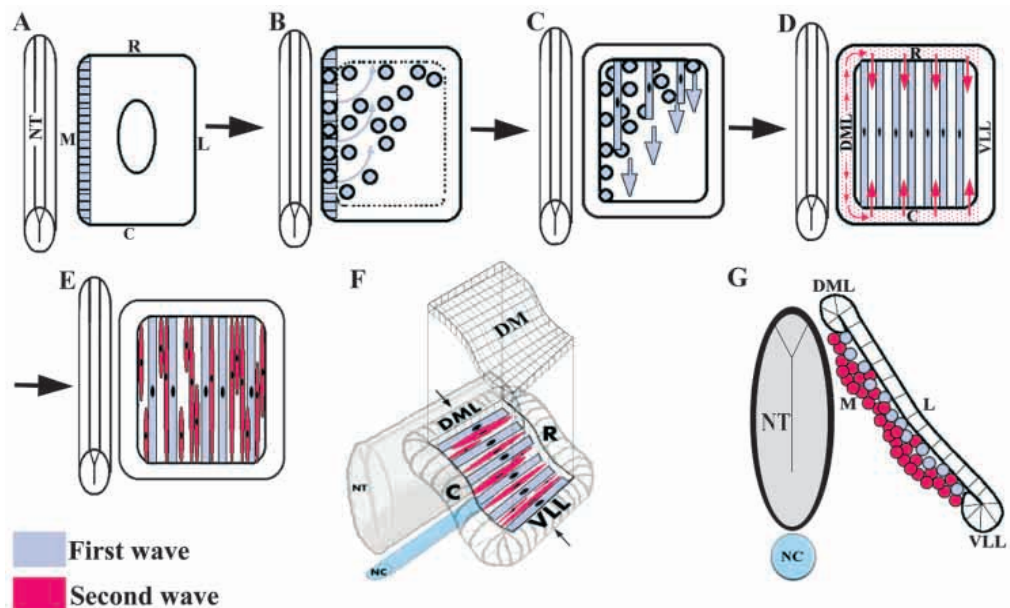


Fig. 7. A model for myotome development. Schematic model representing various stages of myotome development. (A-E) Frontal views; (F) view in depth of a segment from which the dermomyotome epithelium (DM) has been lifted apart leaving the DM edges (DML, VLL, R and C) and the myotome localized ventral to the DM. (G) Cross section of F. (A) The blue squares represent a layer of postmitotic cells present along the medial portion (M) of the epithelial somite. These cells are the pioneer muscle cells. (B) Formation of the primary myotome takes place in several stages, first by a progressive translocation of this medial layer to a ventral position with respect to the dermomyotome followed by progressive de-epithelialization. Pioneer cells then migrate rostral-ward (curved blue arrows). (C) Rostrally located cells gradually send processes that reach the caudal lip of the dermomyotome (blue arrows) giving rise to the triangular shape observed with desmin and *MyoD*. (D) The pioneer myofibers reach both edges of the dermomyotome and their nuclei become restricted to the center of this structure. From this stage, the major contribution to myotome expansion derives from both the rostral and caudal dermomyotome lips that also express *QmyoD* at the appropriate stages, and to a lesser extent from the DML (red stippling to depict cell origins, thin red arrows to represent movement of DML cells to the R and C lips, thick red arrows to depict myofiber elongation into the myotome). This second wave of cells migrates along the primary myotomal fibers. These younger myotomal cells contribute to myotome expansion in the epaxial domain (DML to VLL in F) and also in the transverse plane (G). G represents a cross section through the middle of the myotome, defined by the two arrows in F. In this plane, younger cells localize preferentially apposed to the sclerotome (medial, M) whereas pioneers remain adjacent to the dermal side (lateral, L). This age-dependent segregation suggests a lateral to medial order of myotome colonization in the cross-sectional plane.

was proposed to occur during *Drosophila* myogenesis (Rushton et al., 1995). In such a case, DML progenitors would become specified to a muscle fate only upon arrival of the pioneer myofibers to the rostral or caudal lips, again consistent with the timing of expression of QMyoD in these lips. In addition to an inductive function, or alternative to it, pioneer myofibers could provide the cells of the second wave with a longitudinal, structural scaffold for migration between the rostral and caudal lips. Although direct proof for this model is still missing, cells of the second wave migrate into the myotome in parallel to the preexisting pioneers (Figs. 1, 3 and 4) and progressively intercalate among them (Fig. 2).

At subsequent stages (from E4) significant growth of the myotome takes place, which continues being prominent well after disappearance of the dermomyotome lips (see Fig. 2E-F). Furthermore, in contrast to earlier stages, treatment of embryos from E4 onward with a 2- to 3-hour pulse of thymidine followed by immediate fixation, directly reveals the presence of a population of mitotically active precursors scattered within the myotomes. At this stage, the mitotically active cells were evident only at cervical levels of the axis and progressively at more caudal regions as development proceeded (our unpublished data). Marcelle et al. (1995) have first reported on a similar population of mitotically competent muscle progenitors that coexpresses the fibroblast growth factor receptor FREK. These resident cells endowed with mitotic ability, whose origin is still unclear, are likely to take over myogenesis upon disappearance of the epithelial dermomyotome source and therefore contribute, as a third wave of myoblasts, to the significant mass of unlabeled cells seen at E6.5 (Fig. 2 E,F). The characteristics of this putative third wave of myogenic cells are also unclear. These cells might originate in the dermomyotome lips and migrate into the myotome along with or immediately after the second wave of progenitors while remaining mitotically active in the myotome. Alternatively, they might turn for a while into quiescent cells and then regain the cell cycle. In any of the above cases, it would be important to understand what signals inhibit their transition to the postmitotic state and subsequent differentiation into myofibers. In this respect, it will be meaningful to assess whether these cells observed in the myotomes from E4 onward belong to the population of resident proliferative cells first described by Marcelle et al. (1995) that expresses FREK, a tyrosine kinase receptor which is regulated by FGF2 (Marcelle et al., 1994). In such a case, the FGF2 ligand, found at the appropriate time in skeletal muscles (Kalcheim and Neufeld, 1990) would be an obvious candidate to account for their continuous proliferation.

Altogether, knowledge of the cellular complexity and multistage nature of myotome formation in the avian embryo, should be matched in the future by parallel knowledge of the molecular signaling mechanisms regulating specification, migration and differentiation of cells at each of the stages. Furthermore, gene deletions in mice have revealed the existence of three distinct regulatory programs which correlate temporally and spatially with three waves of recruitment to the expanding myotome (Patapoutian et al., 1995) but an accurate characterization of these waves is still missing. Future studies should clarify whether equivalent cellular and molecular mechanisms regulate myotome formation in both avians and mammals.

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