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The third wave of myotome colonization by mitotically competent progenitors: regulating the balance between differentiation and proliferation during muscle development

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SUMMARY

The myotome is formed by a first wave of pioneer cells originating from the entire dorsomedial region of epithelial somites and a second wave that derives from all four lips of the dermomyotome but generates myofibers from only the rostral and caudal edges. Because the precedent progenitors exit the cell cycle upon myotome colonization, subsequent waves must account for consecutive growth. In this study, double labeling with CM-DiI and BrdU revealed the appearance of a third wave of progenitors that enter the myotome as mitotically active cells from both rostral and caudal dermomyotome edges. These cells express the fibroblast growth factor (FGF) receptor FREK and treatment with FGF4 promotes their proliferation and redistribution towards the center of the myotome. Yet, they are negative for MyoD, Myf5 and FGF4, which are, however, expressed in myofibers.

The proliferating progenitors first appear around the 30-somite stage in cervical-level myotomes and their number continuously increases, making up 85% of total muscle nuclei by embryonic day (E)4. By this stage, generation of second-wave myofibers, which also enter from the extreme lips is still under way. Formation of the latter fibers peaks at 30 somites and progressively decreases with age until E4. Thus, cells in these dermomyotome lips generate simultaneously distinct types of muscle progenitors in changing proportions as a function of age. Consistent with a heterogeneity in the cellular composition of the extreme lips, MyoD is normally expressed in only a subset of these epithelial cells. Treatment with Sonic hedgehog drives most of them to become MyoD positive and then to become myofibers, with a concurrent reduction in the proportion of proliferating muscle precursors.

Key words: Avian embryo, Dermomyotome, FGF, FREK, Muscle satellite cells, MyoD, Myf5, Myogenesis, PAX, Pioneer myotomal cells, Proliferation, Sclerotome, Somite, Sonic hedgehog, Stem cells, Quail

INTRODUCTION

The development of epaxial and body wall (intercostal and abdominal) hypaxial muscles begins with the formation of a transient embryonic structure, the myotome. Different views have been proposed to account for the ontogeny of the early myotome. Using desmin immunostaining to visualize myoblasts, Kaehn et al. (Kaehn et al., 1988) proposed that myotome formation begins at the rostromedial corner of the dissociating somite and then progresses in rostrocaudal and mediolateral directions within individual segments. At variance with this view, recent experiments suggest that myotome fibers are generated from the entire medial boundary (DML) and the medial portion of the rostral boundary of the dermomyotome (Denetclaw et al., 1997), and somewhat later from the ventrolateral lip (VLL; Denetclaw and Ordahl, 2000). In these studies, myoblasts were proposed to ingress from all along the DML and VLL into the myotomal layer and to differentiate into myofibers with no further translocation. Such a view is consistent with an incremental mode of myotome growth in the dorsoventral direction, contributed to by local cell addition at the dorsomedial and ventrolateral extremities (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000).

This view has been challenged by recent findings invoking that development of the post-mitotic myotome is composed of two successive waves that differ in the spatial and temporal origin of their component cells, as well as in the mechanisms by which they colonize the myotome (Cinnamon et al., 1999; Kahane et al., 1998a; Kahane et al., 1998b; Kalcheim et al., 1999). According to this alternative model, a first wave of early post-mitotic progenitors, which expresses the earliest myogenic genes, originates along the entire dorsomedial aspect of the still epithelial somite. This epithelial layer then bends underneath the nascent dermomyotome and its dissociating cells migrate rostrally, leading to a transient triangular pattern that most likely corresponds to the cells described by Kaehn et al. (Kaehn et al., 1988). Elongation of the pioneer myofibers then occurs in a rostrocaudal direction until a primary

myotomal structure is formed that spans the entire mediolateral and rostrocaudal extent of individual segments (Kahane et al., 1998a). Subsequent development of the myotome is accounted for by progressive cell addition from all four dermomyotome lips, where progenitors undergo active proliferation (Kahane et al., 1998a; Kahane et al., 1998b; see also Christ et al., 1978). Myoblast addition from these lips begins, for example, in 23-25-somite old embryos in the rostralmost five to eight segments and extends further caudally as development proceeds. Therefore, this process succeeds and may even slightly overlap in time the establishment of the primary myotome by the pioneer cells. Because of differences in timing and topographical origin of the contributing progenitors, as well as in the mechanisms by which they colonize the myotome, cells arising in the dermomyotome lips can be considered to give rise to a second wave of myotomal cells.

Although second-wave cells originate from all four lips of the dermomyotome, direct elongation of myofibers into the myotome was observed to occur only from along the extreme (rostral and caudal) dermomyotome edges. Thus, cells from the DML and VLL were found first to delaminate, then migrate longitudinally into the extreme lips from which they in turn generate myofibers (Cinnamon et al., 1999; Cinnamon et al., 2001; Kahane et al., 1998b). Thus, actual colonization of the myotome occurs in a direction that is parallel to the pre-existing pioneer myofibers, among which second-wave fibers from all lips progressively intercalate. This suggests that the myotome, comprising all its component waves, expands through the whole mediodorsal to ventrolateral extent, as opposed to expansion just at the extreme VLL and DML lips. This can be compared with the proportional extension of a spring, by progressive intercalation of second-wave cells among myofibers of the primary scaffold (Cinnamon et al., 1999).

Both pioneer cells and muscle progenitors of the second wave withdraw from the cell cycle before generating myofibers (Kahane et al., 1998a; Kahane et al., 1998b; Langman and Nelson, 1968). Thus, the contribution of both first and second waves leads to the development of a myotome expressing MyoD and Myf5 but lacking intrinsic mitotic activity. In view of the transient existence of the dermomyotomal epithelium and the continuous growth of the muscles that derive from the myotomes, mitotically active cells that ensure subsequent growth must arise in the myotomes before dermomyotome disappearance. Mitotically competent muscle progenitors synthesize the fibroblast growth factor (FGF) receptor FREK, whose expression is downregulated upon muscle differentiation (Marcelle et al., 1994; Halevy et al., 1994). A small population of FREK-positive cells is present in cervical myotomes from embryonic day (E)2.5 onwards, suggesting that by this time, the first proliferating muscle progenitors appear in the myotome (Marcelle et al., 1995, Sechrist and Marcelle, 1996). As, at these stages, FREK may not be restricted solely to the mitotically competent muscle sublineage, a direct approach to investigating the ontogeny of these cells is required.

In the present study, we have directly examined the origin and progression of the mitotically active progenitors in relation to the ontogeny of the previous, second wave of myotome colonization. We find that, similar to progenitors of the second wave, the mitotically active cells enter the myotome from both rostral and caudal dermomyotome edges. In addition, these cells express the FGF receptor FREK and respond to FGF4 by increased proliferation and redistribution throughout the myotome. At variance with previous waves, FREK-positive, mitotically competent cells entering the myotome are negative for both MyoD and Myf5. Quantification of the relative contribution of both myogenic cell types revealed that entry of second-wave post-mitotic fibers peaks by 30 somites and progressively decreases with age. In contrast, from 30 somites onwards, the number of proliferating progenitors continuously increases with age, attaining 85% of total muscle nuclei by embryonic day (E)4. These distinctive spatiotemporal and molecular characteristics of the mitotically active cells define a third wave of myotomal colonization. Furthermore, the partial temporal overlap in production of both cellular waves suggests the possibility that the extreme dermomyotome lips contain precursors that are differentially specified to each fate. Consistent with such a possibility, MyoD is normally transcribed in only a subpopulation of epithelial cells within the extreme (rostral and caudal) lips. Furthermore, treatment with Sonic hedgehog (Shh) drives most epithelial cells to express MyoD, followed by a large production of post-mitotic myofibers and a concomitant reduction in the proportion of third-wave progenitors, suggesting that the MyoD-positive epithelial cells are the progenitors of second-wave myofibers.

MATERIALS AND METHODS

Embryos

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

CM-DiI labeling of extreme dermomyotome lip cells and fluorescence imaging

Embryo preparation

Quail embryos were at the 28-32-somite stages at the time of dye labeling. After removal of the vitelline membrane, a unilateral slit was performed in the ectoderm over the intersomitic region at cervical levels of the axis (somites 7-12). A small drop of pancreatin (2% w/v) was then added to assist in the separation of the ectoderm from underlying mesoderm. Enzymatic activity was stopped by newborn calf serum (10% in phosphate-buffered saline (PBS)). The ectoderm completely regenerated over the somites within one hour following the labeling procedure.

Dye labeling

Borosilicate tubes with filament (O.D., 1.0 mm, I.D., 0.5 mm) were pulled using a vertical puller (Sutter model P-30). CM-DiI (C-7000, Molecular Probes) was dissolved in absolute ethanol to a concentration of 1 mg/ml. Just before starting the injections, it was further diluted to a final concentration of 0.1 mg/ml in 10% sucrose in water and micropipettes were backfilled as previously described (Kahane et al., 1998a; Kahane et al., 1998b). 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 2M 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 ×20) and epifluorescence. Embryos were viewed with oblique lighting from a fiber optic light source. Various types of injections were made: (1) to the center of the

rostral dermomyotome lip or at specific sites along its mediolateral extent; (2) to the center of the caudal dermomyotome lip or at specific sites along its mediolateral extent; and (3) to discrete sites along the DML. The accuracy of labeling sites was monitored throughout the procedure by observation under a total magnification of ×200 with combined bright field and epifluorescence optics. After dye labeling, embryos were further grown for 16-20 hours. By this time, BrdU was delivered in ovo, as described below, followed by an additional incubation of 1 hour. At the end of incubation, embryos were removed from the egg, washed in PBS and fixed in formaldehyde.

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 (CM-DiI) and an Argon laser for excitation wavelength of 488 (BrdU immunolabeling, see below) attached to an Axiovert 135M microscope. Analysis was performed on serial transverse sections. The samples were optically screened at 1 μm increments over a total thickness of 7 μm and sequential images were collected and integrated using a Pentium 150 personal computer. Adobe Photoshop was used for image processing.

Grafts of FGF4-coated beads or of Shh-producing cells

Heparin-acrylic beads (Sigma) were cut into quarters and incubated in a solution of FGF4 (R&D Systems, 0.5 mg/ml in PBS) for 2 hours at room temperature. Coated beads were then washed in PBS and grafted. Control QT6 cells or cells expressing Shh (a kind gift from Delphine Duprez) were grown as previously described (Duprez et al., 1998). One day prior to grafting, cells were harvested and seeded onto non-adhesive Petri dishes, where they formed aggregates. Transverse slits were made along the intersomitic regions in embryos aged 28-30 somites at the cervical level (somites 10-15). Either beads or cell aggregates were then grafted into the these slits adjacent to the rostral and caudal extremities. Embryos were further incubated for 10, 24 or 48 hours.

Detection of proliferating cells in vivo by labeling with BrdU or [^{3}H]thymidine

BrdU (50 μl of a 10 mM solution, Sigma) or [^{3}H]thymidine (10 μCi diluted in PBS, specific activity 45-47 Ci/mmol; Amersham) were applied for a total of 1 hour to either CM-DiI-labeled embryos or intact embryos at different stages, respectively. The extent of proliferation at each age was measured as the number of cells with thymidine or BrdU grains ÷ nuclei per desmin-positive myotome. To this end, counts were made of serial frontal sections of three different embryos per embryonic age and expressed as the mean±s.d. of seven to 14 cervical myotomes per experimental point.

Detection of post-mitotic cells of the second wave

Two protocols were used to label second-wave myoblasts that become post-mitotic upon myotome entry (Kahane et al., 1998b).

(1) The first protocol consisted of applying two pulses of [^{3}H]thymidine at 3 hour intervals, beginning at the age of 25 somites, which is when the primary myotome consisting of pioneer myofibers is already established in cervical levels of the axis and the second wave of myotomal cells begins its contribution (Kahane et al., 1998b). Embryos at different ages were pulsed as described above. At the end of a 6 hour exposure to radiolabeled thymidine, they were chased with a 100-fold molar excess of unlabeled thymidine until fixation at E5. In this protocol, cells that were mitotically active during the 6 hour labeling period and were then withdrawn from the cell cycle at or prior to the chase retained the label over their nuclei. In contrast, those cells that continued to divide actively during the chase diluted the radioactive metabolite and became unlabeled.

(2) To measure the number of post-mitotic, second-wave myofibers added to the myotomes between E3 and E4, embryos aged 3 (35 somite pairs) or 4 days received three pulses of [^{3}H]thymidine over 24 hours followed by fixation, sectioning, autoradiography and Hoechst nuclear staining. Myotomal nuclei (Hoechst positive) that remained unlabeled (thymidine-negative) despite a full day of thymidine pulsing were considered to be post-mitotic. The number of such nuclei per myotome was measured at E4 and E5 for cells that exited the cell cycle prior to the onset of labeling at E3 and at E4, respectively.

Fixation, immunocytochemistry and autoradiography

Embryos were fixed in 4% formaldehyde or in Fornoy and embedded in paraplast. Serial 7 μm sections were mounted on gelatinized slides. Immunostaining with desmin or BrdU antibodies was performed as described by Kahane et al. (Kahane et al., 1998a; Kahane et al., 1998b). Secondary antibodies were coupled either to FITC or to horseradish peroxidase. Autoradiography after thymidine labeling was performed as previously described (Brill et al., 1995).

In situ hybridization

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 FGF4 (a gift from Lee Niswander; Niswander et al., 1994) followed by paraffin-wax embedding and serial sectioning at 10 μm. In situ hybridization was also performed on sections and combined with immunohistochemistry after 1 hour pulses with BrdU, as described elsewhere (Sechrist and Marcelle, 1996).

RESULTS

The third wave of mitotically active cells enters the myotome from both rostral and caudal lips of the dermomyotome

Previous studies in which short pulses 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 stage (Langman and Nelson, 1968; Sechrist and Marcelle, 1996). By this time, the primary myotome formed from early post-mitotic pioneers is already established at cervical levels of the axis (Kahane et al., 1998a), and second-wave myoblasts, which are mitotically active in the dermomyotome lips, begin differentiating into myofibers after becoming post-mitotic (Kahane et al., 1998b).

Cells that had incorporated radiolabeled thymidine into their nuclei after a 1 hour pulse appear within myotomes from the 30-35-somite stage onwards at cervical levels of the axis (Figs 1, 5; Marcelle et al., 1995). Their presence must reflect intrinsic mitotic activity, as it takes about 4 to 5 hours for dermomyotome cells to enter the myotome (Langman and Nelson, 1968). Notably, from this stage until about E3.5, the labeled progenitors were localized primarily to both rostral and caudal regions of the myotomes (Figs 1A, 4) whereas similar pulses performed from E4 onwards showed that proliferating cells were scattered throughout the myotomes (Fig. 1B). This age-dependent change in the distribution of precursors with radiolabeled thymidine over their nuclei suggests that mitotically competent cells arise and/or arrive into the extreme lips of the dermomyotomes from which they migrate into the myotomal domain, first localizing to its edges and subsequently colonizing the whole structure.

To examine this possibility directly, a discrete region in the center of rostral or caudal lips of dermomyotomes was

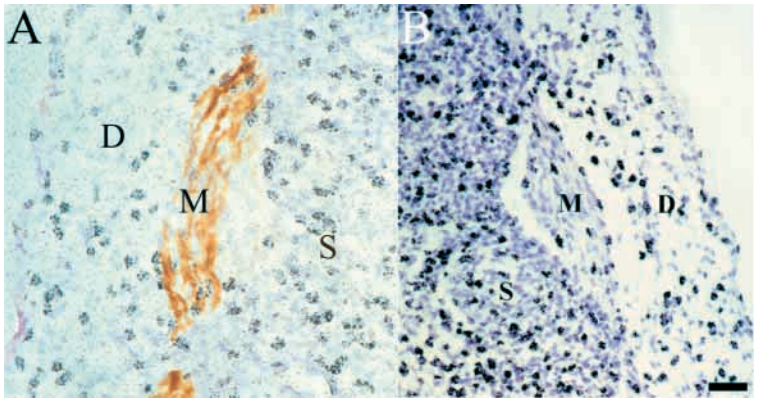

Fig. 1. Mitotically competent cells within the myotome first appear at the extreme edges and later scatter throughout the entire muscle mass. (A) Frontal section through a cervical level of the axis of a 35-somite stage quail embryo showing the presence of thymidine-positive nuclei predominantly at the rostral and caudal extremities of the desmin-positive myotome. (B) Mitotically active cells in the nascent muscle of an E4 embryo are scattered throughout the entire structure. In both cases, short pulses of radiolabeled thymidine were delivered followed by immediate fixation thus reflecting intrinsic cell proliferation. D, dermis; M, myotome, S, sclerotome. Scale bar: 30 μm in A; 70 μm in B.

iontophoretically labeled with CM-DiI, as described under Materials and Methods. Embryos were incubated for additional 20 hours and then pulsed with BrdU for one hour prior to fixation. Double-labeled cells that contained BrdU-immunoreactive nuclei surrounded by CM-DiI-positive membranes were present within the myotomes in both types of epithelial lip injections (Fig. 2A, arrow and data not shown). To assess further whether the whole mediolateral extent of the rostral and caudal lips gives rise to mitotically competent cells, two to three discrete injections were performed along each edge at medial or lateral positions with respect to the center. Mesenchymal cells that revealed both CM-DiI and BrdU labeling were present in nine out of 11 analyzed myotomes (Fig. 2B; data not shown). In all precedent cases, CM-DiI-positive myofibers were also detected in the process of elongating into the myotomes (Fig. 2A, arrowheads delimiting a myofiber; data not shown; Kahane et al., 1998b). These results show that similar to second-wave progenitors, the mitotically competent cells also enter from the extreme rostral and caudal dermomyotome lips. Thus, proliferative cells from dermomyotome edges give rise to two types of myotomal cells: second-wave cells that become post-mitotic and generate fibers and cells that remain mitotically active after myotome colonization.

As the DML and VLL epithelia also generate myofibers of the second wave, we examined whether they produce, in addition, mitotic cells of the third wave. To this end, the length of the DML epithelium was similarly labeled by discrete spots of CM-DiI followed by a 1 hour pulse of BrdU before fixation. Double-labeled CM-DiI/BrdU cells were present in only two out of 22 myotomes examined, whereas CM-DiI-positive myofibers were apparent in 20 out of the 22 myotomes (Fig. 2C). Thus, whereas progenitors that become myofibers are contributed by all epithelial lips, mitotically competent cells of the third wave derive predominantly from the rostral and caudal edges of the dermomyotome (DM).

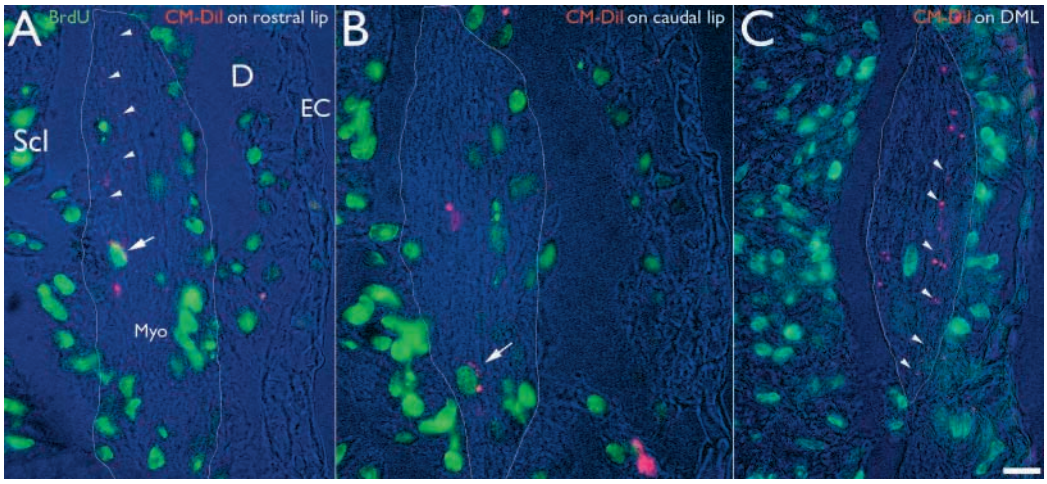

Fig. 2. Mitotically active cells enter the myotome from the extreme rostral and caudal lips of the dermomyotome. Iontophoretic labeling with CM-DiI (red) of (A) the center of the rostral lip, (B) the medial aspect of the caudal lip, (C) the DML of dermomyotomes. Embryos were incubated for additional 20 hours and then pulsed with BrdU (green) for one hour prior to fixation. (A,B) Note the presence within the myotomes of double-labeled cells containing BrdU-immunoreactive nuclei surrounded by CM-DiI-positive material (arrows). The double labeling represents cells that entered from the respective lips and continued proliferating within the myotome. Myofibers spanning part of or the entire rostrocaudal length of the myotome are also apparent (arrowheads in A and C). One day after CM-DiI labeling, dye distribution adopts a dotted appearance in the cell membrane. BrdU-positive/CM-DiI-negative cells were also apparent as only a small number of lip cells were dye labeled. The presence of CM-DiI-positive cells that lack BrdU in their nuclei was evident as well; this finding was expected as a 1 hour pulse enables the detection of only a subset of the mitotic population given a cell cycle length of about 8 hours (Langman and Nelson, 1968; Summerbell et al., 1986). D, dermis; EC, ectoderm; Myo, myotome; Scl, sclerotome. Scale bar: 11 μm.

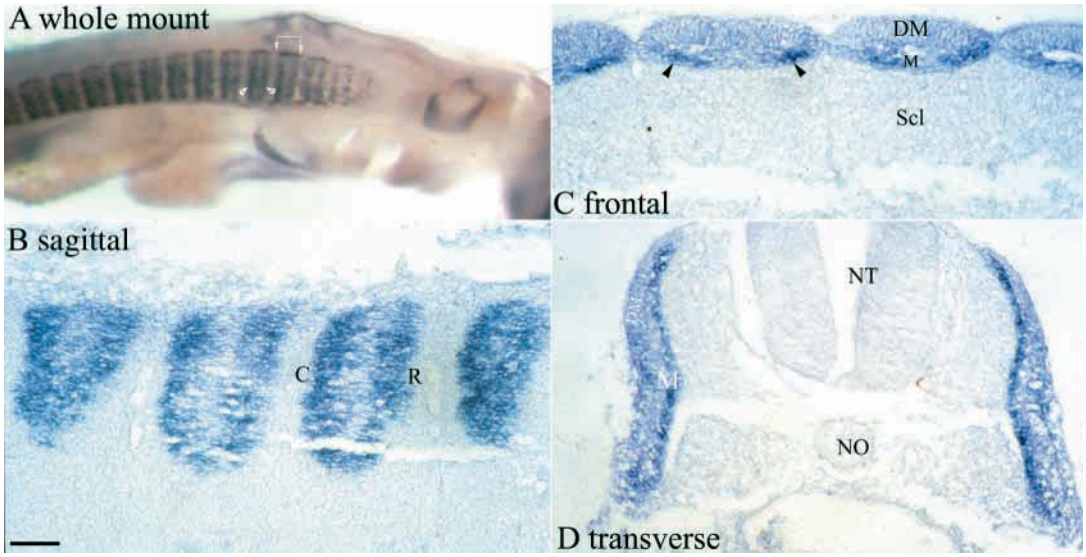

Fig. 3. Expression of FREK mRNA in developing myotomes. (A) Whole-mount in situ hybridization of E3.5 embryo, revealing that expression of FREK is enriched in both rostral and caudal regions of the myotome (arrowheads) as well as in discrete stripes subjacent the DML epithelium (between arrows). (B) Sagittal section of a similar embryo, further showing the distribution to the extreme regions of the myotomes. (C) Frontal section through a cervical region of a 30-somite stage embryo again revealing intense FREK signal at the edges of the myotomes (arrowheads), whereas the rest of the myotome and the overlying dermomyotome show a fainter intensity of labeling (see also D). (D) Transverse section showing that FREK signal is particularly intense in myotomal cells localized towards the sclerotomal portion of the myotome, a position adopted by ingrowing second-wave myofibers relative to pre-existing pioneers that always remain apposed to the dermal side of the myotome. C, caudal; DM, dermomyotome; M, myotome; NT, neural tube; NO, notochord, R, rostral; Scl, sclerotome. Scale bar, 50 μm.

Expression of FREK mRNA by progenitors entering the myotome from the extreme lips

In situ hybridization with a FREK probe of embryos ranging in age from 30-35 somite pairs until E4 revealed that expression of these transcripts is enriched in both rostral and caudal regions of the myotome, whereas the rest of the myotome and the overlying dermomyotome showed a fainter intensity of labeling (Figs 3A-C, 4A). In these regions, FREK mRNA is likely to be expressed by progenitors of the third wave, which enter the myotome from the extreme lips (see Figs 1, 2). Consistent with this possibility, Fig. 4A reveals that FREK-positive cells localized close to the myotome edges have incorporated BrdU, and Marcelle et al. (Marcelle et al., 1995) have shown that FREK is a marker for mitotically competent progenitors both within myotomes and in developing limbs.

In previous studies, we have shown that second-wave cells also enter the myotomes from the extreme rostral and caudal edges of the dermomyotome (Cinnamon et al., 1999, Kahane et al., 1998b). Thus, FREK mRNA could also identify progenitors of the second wave that migrate into the myotome. Confirming this assumption, we observed that in the transverse plane, FREK signal was also intense in myotomal cells localized towards the sclerotomal portion of the myotome (Fig. 3D). This position is adopted by ingrowing second-wave myofibers relative to pre-existing pioneers, which always remain apposed to the superficial, dermal side of the myotome (Kahane et al., 1998b).

In addition, FREK mRNA signal is also apparent in a discrete region subjacent to the DML epithelium (Fig. 3A, area between arrows) and also the VLL epithelium at specific levels of the axis (not shown). FREK signal in these regions spans the entire rostrocaudal extent of individual segments. We have called this area the ‘sub-lip’ domain, as it contains DML- and VLL-derived progenitors of the second wave that have delaminated from the epithelia and undergone a longitudinal migration on their way to the rostral and caudal lips (Cinnamon et al., 2001).

By E3.5, FREK receptor transcripts are still localized to the extreme regions of the myotome in cells that have incorporated BrdU after a 1 hour pulse (Fig. 4A), yet second-wave myofibers are not added any longer to the myotome (see below, Fig. 6 and Table 1). Therefore, at this stage, FREK mRNA is likely to mark predominantly the third wave of mitotically competent cells. Notably, we have occasionally observed the presence of few BrdU-positive/FREK-negative cells within the myotome, suggesting that although most proliferating cells express the receptor, a few may either not express it at all or produce it only transiently.

In contrast to the restricted expression of FREK to the myotome extremities, MyoD and Myf5 mRNAs become preferentially localized at this developmental time to the center of the myotomes, which contain BrdU-negative nuclei of differentiated myofibers (Fig. 4B,C). Thus, under these conditions, the BrdU-positive nuclei are apparent in a MyoD and Myf5-free environment, suggesting that most proliferating progenitors do not express the above factors (arrows in Fig. 4B,C). Notably, some mRNA signal is occasionally detected towards the rostral and caudal edges of the myotomes (see Fig. 4B and data not shown). In such areas, it is not possible to assess whether the BrdU-positive cells co-express MyoD, but this possibility is highly unlikely, as MyoD is apparent within the myotomal fibers whose nuclei are located in the center of the myotome, whereas BrdU is in nuclei of local mesenchymal cells.

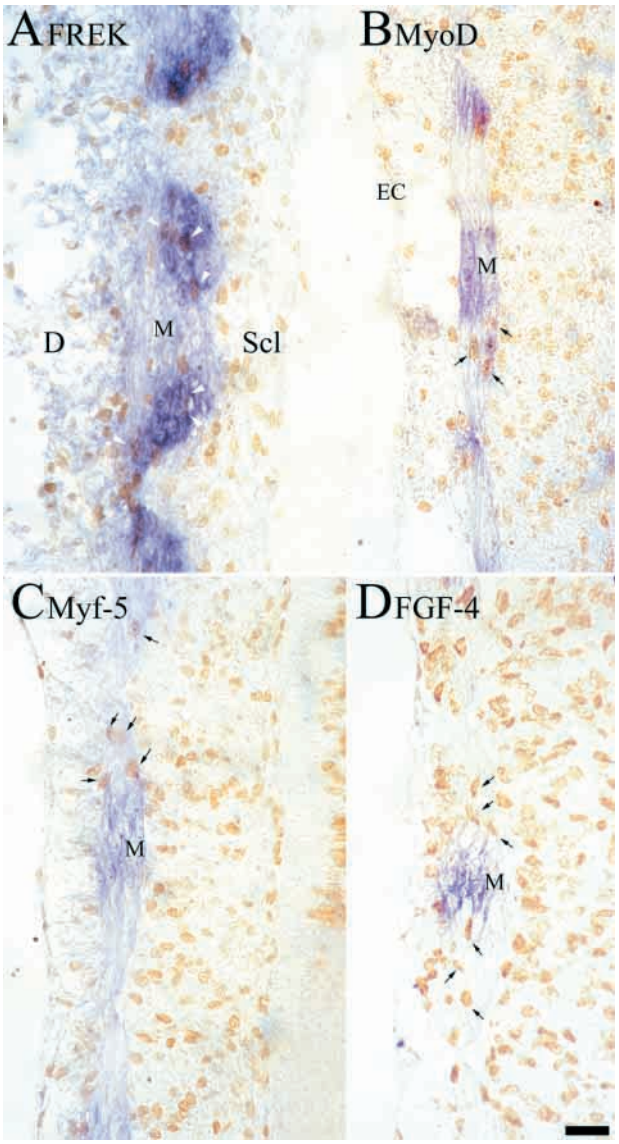

Fig. 4. FREK (but not MyoD, Myf5 or FGF4) is expressed by proliferating cells of the third wave that enter the myotome. E3.5 quail embryos (45 somites approximately) were labeled with BrdU for 1 hour followed by fixation and in situ hybridization. Frontal sections were hybridized with probes to (A) FREK, (B) MyoD, (C) Myf5 and (D) FGF4, and then subjected to immunodetection of BrdU. Note that in all cases, BrdU-labeled nuclei are preferentially localized towards the rostral and caudal regions of the myotomes (small arrows in B-D; white arrowheads in A). In A, the BrdU-positive nuclei are a subset of FREK-containing cells (white arrowheads) that are localized in the myotome extremities. MyoD, Myf5 and FGF4 are enriched at this developmental time in the center of myotomes (central region marked by M). These contain the nuclei of differentiated myofibers that are BrdU negative. Rostral is towards the top. D, dermis; EC, ectoderm; M, center of myotome; Scl, sclerotome. Scale bar, 25 μm.

Also reciprocal to the expression pattern of FREK, we detected FGF4 mRNA in the cytoplasm around the centrally located post-mitotic myofiber nuclei but not elsewhere in the myotome in regions that contain BrdU-positive/FREK-positive precursors (Fig. 4D, compare with 4A). In addition, FGF4 protein is distributed throughout the entire length of differentiated myofibers but not in mesenchymal myotomal cells (data not shown). These results suggest that most mitotically active progenitors express within the myotome FREK mRNA but are negative for MyoD, Myf5 and FGF4.

Table 1. Addition of second-wave myofibers between E3 and E4

Embryonic age	Total cells/ myotome* (Hoechst-positive nuclei/myotome)	Post-mitotic cells/myotome (Hoechst-positive/ [^{3}H]thymidine-negative nuclei)	Myotomes counted
E3 to E4	5230±60	651±51	12
E4 to E5	10220±275	786±19	8

E3 and E4 embryos received three pulses of [^{3}H]thymidine over 24 hours, followed by fixation, sectioning, autoradiography and Hoechst nuclear staining. The number of thymidine-negative nuclei/myotome (mean ± s.d.) represents the total post-mitotic cells present in the myotome from the onset of labeling. These values were measured at E4 for cells that exited the cell cycle at E3, and at E5 for the corresponding ones at E4.

*Values represent the mean ± s.d. of the total number of cells/myotome counted at E4 and E5. Age differences were significant (P<0.0001; ANOVA) for both parameters measured.

The percentage of total post-mitotic myotomal cells at E4 is 15%. Note that it was calculated as the ratio between the number of post-mitotic cells at E4 (786 cells/myotome) to the total cell number present at the same age (5230 cells/myotome).

FGF4 promotes the proliferation and redistribution of third-wave progenitors within the myotome

As FREK has been shown to act as a receptor for FGF (Marcelle et al., 1994) and FGF4 is expressed in myotomal fibers, we examined the possibility that myofibers influence, via FGF4, the development of the proliferating FREK-positive progenitors. FGF4-coated beads were grafted in the intersomitic spaces of cervical-level segments in 30-somite stage embryos, a time that corresponds to the onset of immigration of the third wave of proliferating precursors. At E3.5, the distribution of FREK mRNA signal was predominant in the rostral and caudal portions of control myotomes and of myotomes that were exposed to control beads, as previously described (Figs 5B-D, 3A-C). In striking contrast, the distribution pattern of FREK mRNA became uniform along the rostrocaudal aspect of the myotome in FGF-treated segments (Fig. 5A,D). We next examined whether this change in FREK distribution reflects an altered pattern and/or amount of proliferating cells within the myotomes. To this end, embryos were similarly treated with FGF4 and pulsed for 1 hour with BrdU prior to fixation. The number of BrdU-positive nuclei was counted in serial transverse sections at both treated and contralateral sides. As depicted in Fig. 5E, the distribution of BrdU-positive cells along the rostrocaudal extent of control myotomes shows that the majority of mitotic nuclei are still localized towards the extremities of the myotome with few cells in the center. This bimodal distribution pattern was totally lost upon exposure to FGF, which instead revealed a homogeneous dispersion of the cells along the myotomes (n=9), a feature normally observed only at later stages. This change in distribution of proliferating cells corresponds to the uniform pattern of FREK mRNA expression observed in the treated embryos.

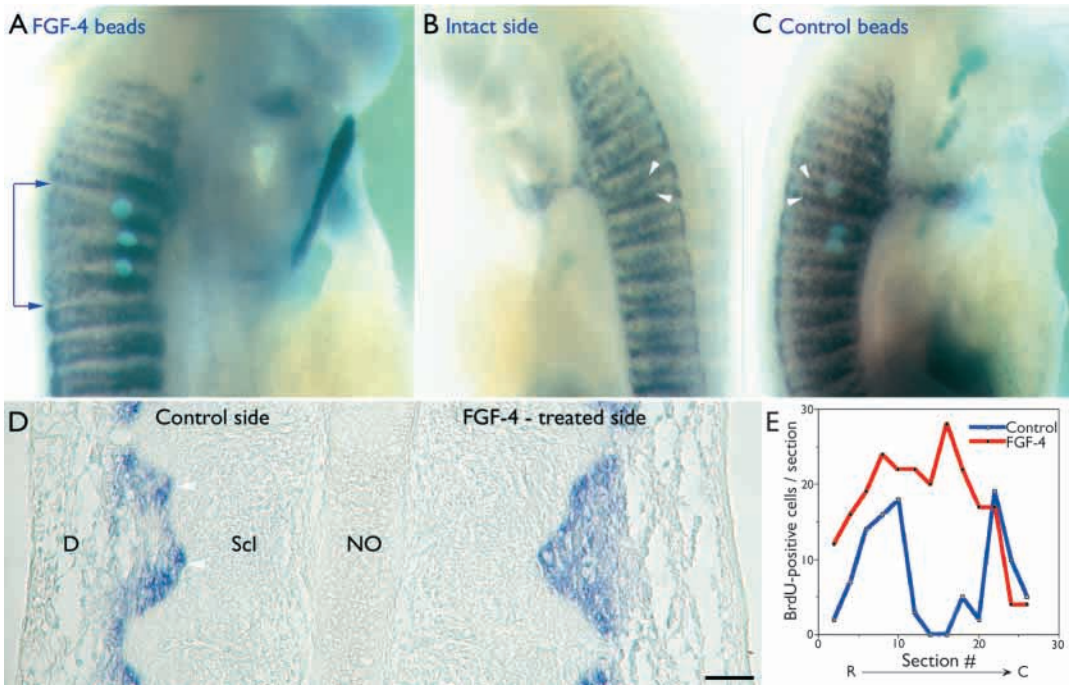

Fig. 5. Effect of added FGF4 on the amount and topographical distribution of third-wave progenitors. (A-D) In situ hybridization for FREK of E3.5 quail embryos that received an implant of FGF4-coated beads at the 28-30-somite stage (A, treated side; B, contralateral side) or control beads (C). Note the preferential distribution of FREK signal to the rostral and caudal portions of the myotomes in B and C (arrowheads); in A, FREK transcripts are homogeneously dispersed in the vicinity of the implanted beads (area between arrows). (D) Frontal section at the level of the notochord (NO) of a FGF-treated embryo, showing the preferential distribution of cells to the extreme regions of the myotome (arrowheads) in the control side when compared with the uniform distribution of FREK mRNA in the treated side. (E) Thirty-somite stage embryos received FGF4-soaked beads as depicted above. One hour before fixation at E3.5 they were pulsed with BrdU. The number of BrdU-positive cells was counted in serial transverse sections starting from the rostral end to the caudal extreme of each myotome. Note that in the control side (blue), the majority of labeled cells are found close to the extreme edges of the myotome with very few in the center, whereas in FGF-treated embryos (red), their distribution is uniform along the myotome. Note also that the number of labeled cells is higher in most sections comprising the treated side (see Results for quantification). C, caudal; D, dermis; Scl, sclerotome; R, rostral. Scale bar: in D, 22 μm for D.

Notably, an average increase of 2.1-fold in the number of BrdU-positive cells per myotome was also observed in FGF-treated myotomes when compared with the contralateral ones (2.25±0.5, *n*=4 and 1.98±0.29, *n*=5 myotomes in two different embryos, respectively; see also Fig. 5E) with no apparent change in the overall size of the myotomes and staining for desmin protein (data not shown; see Fig. 5D). Thus, exogenous FGF promotes both the topographical redistribution and the proliferation of third-wave progenitors. Taken together, these data suggest that FGF/FREK may be involved in the redistribution of third-wave progenitors from the extreme lips of the DM (where they originate) towards the center of the myotome and in regulating their proliferation.

Progressive expansion of the proliferating population of myotomal progenitors is concomitant with a time-dependent decrease in the generation of post-mitotic myofibers of the second wave

We next quantified the age-dependent development of progenitors of the third wave. To this end, single, 1 hour pulses with radiolabeled thymidine were delivered to embryos at different stages followed by immediate fixation. The number of cells with thymidine-positive grains over nuclei was scored per myotome as described under Materials and Methods. At 35 somites, cervical-level myotomes contained an average of 53±14 cells in the S phase of the cell cycle. The number of such cells progressively increased and reached almost four times (185±11) the initially counted value at the age of 44 somites (E3.5) (Fig. 6). A separate experiment in which the proportion of post-mitotic cells per myotome was directly counted (Table 1 and see below) revealed that by E4, mitotically active progenitors within myotomes already attained about 85% of the total population. Thus, within two days from their first appearance, the mitotically competent progenitors became the predominant population of myotomal cells.

As both second and third waves enter the myotome from the extreme edges of the dermomyotomes, it was important to compare the time-dependent behavior of both cell types. To this end, we examined the temporal dynamics of second-wave progenitors that were previously shown to enter the myotomes as emerging post-mitotic fibers (Kahane et al., 1998b). In this protocol, embryos at different ages were pulsed for 6 hours with radiolabeled thymidine starting after establishment of the pioneer myotome and then chased with unlabeled metabolite until fixation at E5, as described in Materials and Methods (protocol 1). Cells that continued dividing during the chase diluted the radioactive metabolite and became unlabeled. In contrast, cells that were mitotically active during the 6 hour labeling period and then exited the cell cycle remained labeled

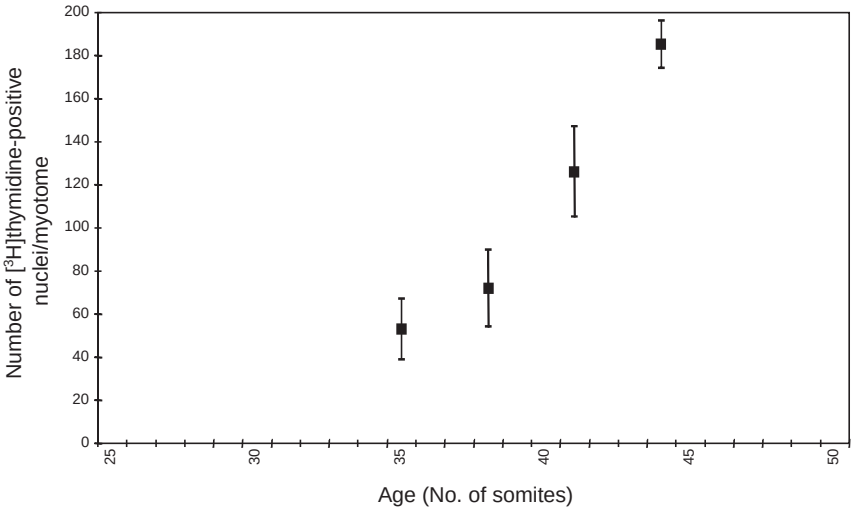

Fig. 6. Age-dependent increase in the number of mitotically-active progenitors of the third wave. Single pulses of radiolabeled thymidine, 1 hour long, were delivered to embryos at different stages followed by immediate fixation. The number of cells with thymidine-positive grains over nuclei was scored per myotome, as described in Materials and Methods. Results represent the mean±s.d. of seven to 14 cervical myotomes per experimental point.

and represent a subpopulation of the second wave. Fig. 7 illustrates the number of such post-mitotic cells present per myotome at various stages. Generation of second-wave fibers was maximal at the 30-32 somite-stage in cervical regions of the axis and then progressively decreased until E3.5 (46 somite pairs, approximately).

To measure directly the total number of myofibers still added between E3 and E4, embryos aged 3 (35-somite pairs) or 4 days were pulsed with [^{3}H]thymidine over 24 hours, followed by fixation (Materials and Methods, protocol 2). Myotomal nuclei (Hoechst positive) that remained unlabeled (thymidine negative) despite a full day of thymidine pulsing were considered to be post-mitotic. The number of such nuclei per myotome was measured at E4 and E5 for cells that exited the cell cycle prior to the onset of labeling at E3 and at E4, respectively. Table 1 shows that the total number of post-mitotic cells at E3 was 651±51 and it increased to 786±19 within a day. This represents a 20% addition of myofibers to the myotome during the measured time interval. Notably, in the same time frame, the total number of cells in the myotomes doubled and the post-mitotic fibers of both first and second waves remained a minority of the myotomal population. So, the process of primary myofiber differentiation in cervical myotomes begins around the 25-somite stage and is completed by E4, whereas addition of proliferating progenitors starts by 30 somites and progresses from then on. Although in the present measurements of proliferating cells it was not possible to discriminate between the relative contribution of cell entry and intrinsic cell proliferation, our dye labeling experiments showed that during the early stages considered (28-35 somite pairs), dermomyotome lips generate both post-mitotic fibers as well as mitotically active progenitors that enter into the myotome (Fig. 2, data not shown; Kahane et al., 1998b). This finding could indicate that rostral and caudal lips of the dermomyotome contain cells that are differentially specified to generate the two types of myogenic precursors.

Expression of MyoD by a subset of dermomyotomal progenitors

Results of dye labeling of dermomyotome lips, together with quantitative measurements of each cell population, have clearly shown that there is a temporal overlap in the generation of the two cell types from the rostral and caudal lips of the overlying dermomyotome. To begin investigating whether these lips might contain distinct progenitors for each cell type, we first examined the possibility that they display a molecular heterogeneity. In a previous study (Kahane et al., 1998b), we have reported that the rostral and caudal lips of the dermomyotome contain MyoD-positive cells. Here, we show that in 35-somite stage embryos, MyoD is expressed in only a subset of cells in both the rostral and the caudal dermomyotome lips (Fig. 8). Since progenitors of the third

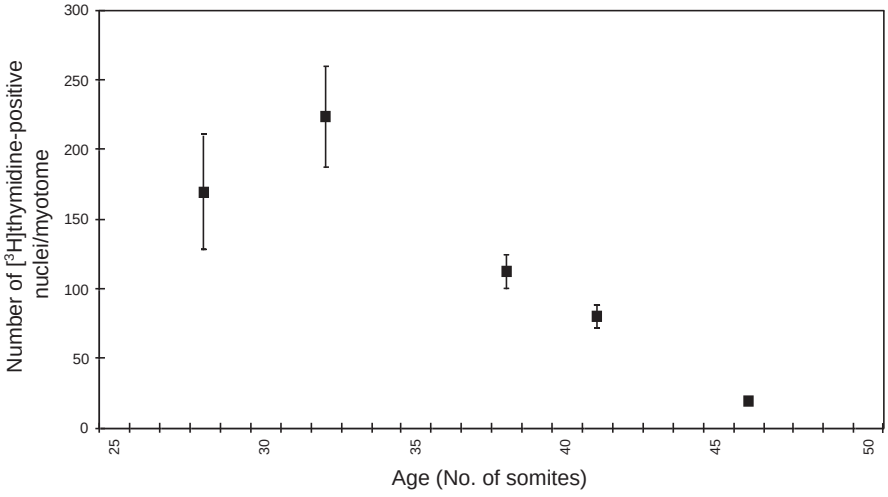

Fig. 7. Age-dependent decrease in generation of post-mitotic, second-wave myofibers. Embryos at different ages starting after establishment of the pioneer myotome were pulsed for 6 hours with radiolabeled thymidine and then chased with unlabeled metabolite until fixation at E5 as described in Materials and Methods (protocol 1). Cells that continued dividing during the chase diluted the radioactive metabolite and became unlabeled. In contrast, cells that were mitotically active during the 6 hour labeling period and then withdraw from the cell cycle remained labeled and represent a subpopulation of the second wave. Note that the number of post-mitotic cells is maximal at the 30-32-somite stage in cervical regions of the axis and then progressively decreases until E3.5 (approximately 46 somite pairs). Results represent the mean±s.d. of 15 myotomes per experimental point.

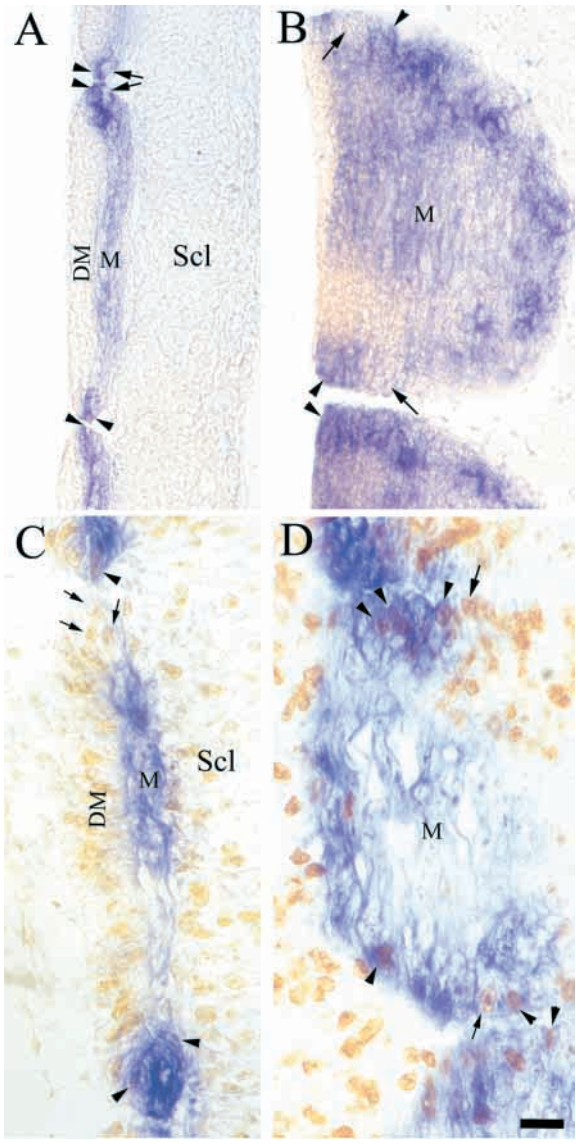

Fig. 8. MyoD is expressed in subsets of epithelial progenitors within rostral and caudal dermomyotome lips. (A,B) Quail embryos (35-somite stage) were hybridized with a MyoD probe. Some embryos also received a single, 1 hour pulse with BrdU after fixation, in situ hybridization and then immunodetection of BrdU (C,D). Note in A (frontal section) and B (sagittal section) that only some of the epithelial cells in both rostral and caudal dermomyotome lips contain MyoD-positive cells (arrowheads), while other cells are negative for the marker (arrows). In addition, C (frontal section) and D (sagittal section) reveal the presence of Myo-D-positive/BrdU-positive precursors (arrowheads, putative progenitors of the second wave) and also of Myo-D-negative/BrdU-positive epithelial cells (arrows, presumed third-wave cells). Rostral is towards the top. DM, dermomyotome; M, myotome; Scl, sclerotome. Scale bar: 25 μm in A,B; 18 μm in C,D.

wave are negative for MyoD (see Fig. 4), it is unlikely that they originate from MyoD-positive epithelial precursors. Thus, we propose that MyoD-positive cells in the lips are the direct progenitors of the post-mitotic myofibers of the second wave. Notably, we observed that many MyoD-positive cells residing in the epithelial lips did incorporate BrdU after a 1 hour pulse (Fig. 8C,D), further substantiating the notion that expression of myogenic genes precedes the transition to the post-mitotic state and suggesting that the latter progenitors exit the cell cycle only by the time they colonize the myotome as differentiating myofibers.

Shh-induced expression of MyoD in the dermomyotome epithelium is followed by enhanced myofiber differentiation

To assess further whether the subset of MyoD-positive cells in the DML lips represent the progenitors of second-wave myofibers, we reasoned that any procedure that induces expression of MyoD in otherwise negative cells of the DML epithelium should result in enhanced myofiber formation. Shh was shown to induce MyoD expression and myogenesis at early somitic stages (see, for example, Borycki et al., 1998; Borycki et al., 1999; Johnson et al., 1994; Marcelle et al., 1997). We therefore grafted Shh-producing cells at a stage corresponding to the onset of the third wave (30 somites at cervical level). Within 10 hours, Shh-producing cells stimulated expression of MyoD in many epithelial cells residing in the dermomyotome when compared with fewer progenitors expressing the gene within the rostral and caudal edges of the control side or of QT6-grafted embryos (Fig. 9A-C; data not shown). Notably, many of the epithelial MyoD-positive cells were also BrdU positive (Fig. 9A). One and 2 days later, a dramatic increase in the size of the myotome composed of well developed post-mitotic myofibers that expressed MyoD, Myf5 and FREK was observed in Shh-treated segments. In addition, these myotomes also contained mitotically active cells of the third wave that were detected to a similar extent and distribution in both operated and control segments despite a dramatic increase in myotome size (Fig. 9; data not shown) caused by a reduced proportion of third-wave cells per myotome under experimental conditions. Taken together, these data show that expression of MyoD in the dermomyotome lips is functionally correlated with the subsequent generation of post-mitotic fibers (second wave), suggesting that the MyoD-positive cells in the lips are their respective progenitors.

DISCUSSION

The early myotome is a post-mitotic structure formed by two successive waves of muscle progenitors that give rise to mononucleated fibers spanning the entire rostrocaudal and dorsoventral extents of individual segments (Cinnamon et al., 1999; Kahane et al., 1998a; Kahane et al., 1998b; Kaehn et al., 1988). Subsequent development of the myotomes on their way to become distinct muscles requires the advent of a proliferative phase that accounts for an active, yet highly regulated growth. In the present study, we provide evidence for the origin, molecular properties and factor responsiveness of the mitotically competent population of myotomal progenitors and compare them with the dynamics of the second wave of myotome colonization.

We have found that mitotically active cells enter the myotome from the entire mediolateral aspect of both rostral and caudal dermomyotome edges. This is consistent with expression of FREK mRNA and BrdU labeling all along the

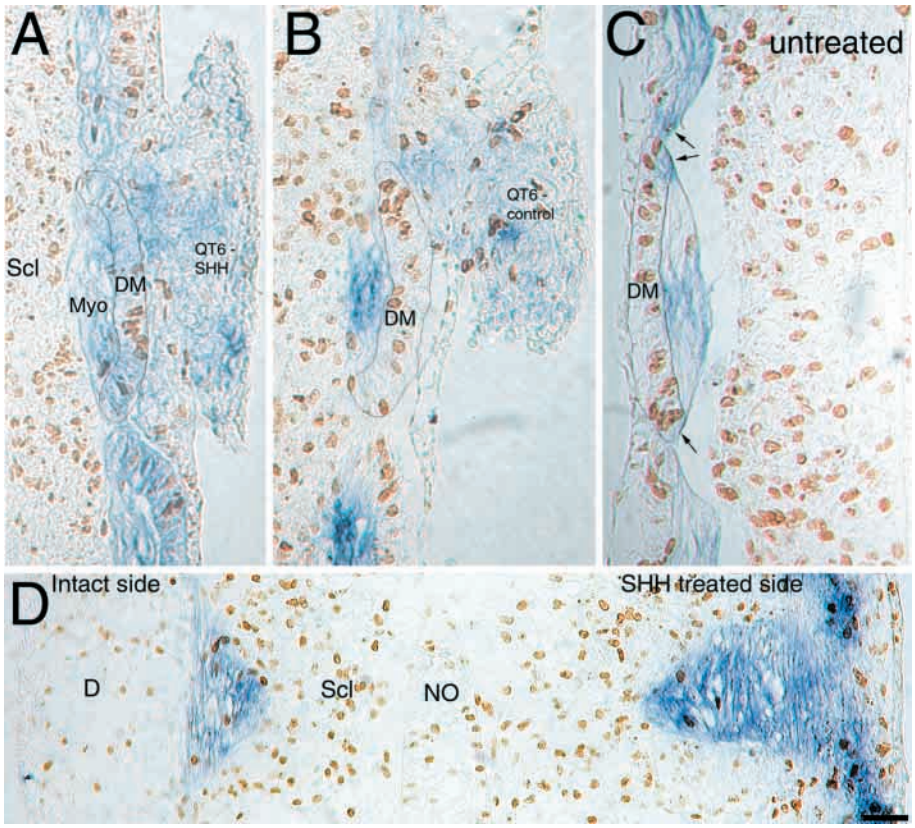

Fig. 9. Shh promotes MyoD expression in epithelial DML lips followed by enhanced myofiber differentiation. Control QT6 or Shh-expressing QT6 cells were grafted at the cervical level of 30-somite-stage embryos. The latter were further incubated for 10 hours (A-C) or 48 hours (D), pulsed with BrdU for 1 hour and in situ hybridized for MyoD or Myf5 in combination with immunodetection of proliferating cells. In control segments (B,C), MyoD is expressed in only a few cells of the extreme dermomyotome (DML) lips (see, for example, arrows in C). In contrast, most epithelial DML progenitors, which are adjacent to the Shh-expressing cells, express MyoD in A. The DML was highlighted by a thin line. Note that grafted control (B) as well as the Shh-expressing QT6 cells (A) produce MyoD. (D) Two days after grafting, the myotomes are much bigger in the operated side that received Shh when compared with the contralateral intact side or to QT6-grafted myotomes (not shown). These myotomes contain well-developed and organized myofibers expressing Myf5 (and also MyoD, not shown), and also reveal the presence of BrdU-positive cells. Note also the absence of dermis (D) in the operated side. Myo, myotome; NO, notochord; Scl, sclerotome. Scale bar: 15 μm in A-C; 40 μm in D.

myotomal edges (Figs 3B, 5; N. K. and C. K., unpublished). Notably, cells of the second wave also arise in the DML and VLL epithelia, delaminate into a sub-lip domain and re-migrate to the extreme edges prior to generating myofibers (Cinnamon et al., 1999; Cinnamon et al., 2001; Kahane et al., 1998b). The possibility was then examined that the DML might also contribute to the mitotically competent population. Whereas the presence of labeled DML-derived fibers was detected in almost all myotomes, mitotically active cells (CM-DiI positive/BrdU positive) were apparent in fewer than 10% of the myotomes examined. This is in strong contrast with the presence of double-labeled mitotic cells in 90% of the myotomes upon injection of the extreme edges. Thus, we conclude that proliferating progenitors of the third wave predominantly derive from the rostral and caudal edges of the DM. This inference is also consistent with our observation that the latter cells express FREK but are devoid of MyoD or Myf5 (this paper). At variance, DML- and VLL-derived cells co-express FREK, MyoD and Myf5, while migrating along the sub-lip domain to reach the extreme edges of each segment (Cinnamon et al., 2001), suggesting they may be already specified to become myofibers rather than to contribute to a population that continues proliferating within the myotome anlage.

Short labeling experiments with thymidine or BrdU revealed that the mitotically active cells localize first at the extreme ends of the myotomes close to their origins and later scatter throughout their entire mass. This time-dependent shift in relative localization is accompanied by an increase in the number of proliferating cells, suggesting that two simultaneous events take place: continuous proliferation of the progenitor pool that enters the myotomal environment, and cell migration towards the center of the myotome. We report that exogenously added FGF4 stimulates both processes. Consistent with our observations, several studies have implicated FGF family members and their receptors in regulating both myoblast proliferation and/or differentiation (see, for example, Flanagan-Steet et al., 2000; Kudla et al., 1998; Olson, 1992; Olson and Hauschka, 1986; Olwin et al., 1994a; Olwin et al., 1994b; Templeton and Hauschka, 1992).

Furthermore, FGF receptor 1 activity has been reported to be necessary for myoblast migration into the forelimb bud (Itoh et al., 1996). Within the myotome, it is possible that the migrating mitotic precursors of the third wave use the surface of pre-existing myofibers that express FGFs as a substrate for movement. We have observed that myotomes exposed to FGF4 also reveal a rather loose arrangement of myofibers when compared with the tightly packed fibers of control embryos (N. K. and C. K., unpublished). This might be related to the fact that the BrdU and FREK-labeled progenitors redistributed faster along the myotome under experimental conditions when compared with controls. In such an event, FGF4 might alter adhesive properties of the myofiber surface, in turn affecting the movement of the mesenchymal population. Whether FGF4 is the endogenous factor that acts upon the mitotic FREK-positive progenitors remains to be elucidated. Local activity of FREK could be driven by several FGFs present in differentiated myofibers at the relevant stages, such as FGF2, FGF4 or FGF8 (this paper; Joseph-Silverstein et al., 1989; Kalcheim and Neufeld, 1990; Niswander et al., 1994).

As previously discussed (Amthor et al., 1999; Brand-Saberi and Christ, 1999), it is necessary that the organism produces the appropriate number of muscle cells, as any deficiencies during early development are unlikely to be compensated for during later stages (Tajbakhsh et al., 1997). It is noteworthy that after an initial phase in which there is simultaneous entry of progenitors that become readily post-mitotic (second wave) and others that remain mitotically competent (third wave), the production of the post-mitotic fibers decreases with age and is practically accomplished by E3.5. In contrast, the number of mitotically active cells within the myotome constantly increases. This is reflected by a doubling in cell number measured between E4 and E5 with no significant change in the number of post-mitotic cells. These results suggest that there are distinct phases of muscle development in which proliferation and differentiation can be separated despite an early temporal overlap. A first phase in which the primary myotome forms by a process of rapid withdrawal from the cell cycle and differentiation into mononucleated fibers (first and second waves), a second phase of cell proliferation with no significant muscle differentiation (third wave, between E3 and E5), and a third phase that begins by E6 in avian embryos in which the mitotically competent progenitors begin differentiating into multinucleated myofibers. Correspondingly, it is interesting to point out that in E8-E9 embryos, the multinucleated myofibers in both epaxial and hypaxial muscles of the body wall contain a contribution that derives from the primary myotome, in addition to a major component that stems from third-wave progenitors (N. K. and C. K., unpublished). Thus, the early post-mitotic myotome composed of mononucleated myofibers would serve as a scaffold for patterning subsequent muscle morphogenesis.

To understand the development of the third wave of muscle progenitors further, it is important that specific markers for this population be available. In the avian embryo, FREK is present in replicating cells in both myotomes and limb muscles (Marcelle et al., 1994; Marcelle et al., 1995). Expression of the gene encoding FREK remains on satellite cells of adult muscles and is downregulated upon differentiation. In vitro, FREK appears to be regulated in a biphasic manner by different concentrations of FGF ligands (Halevy et al., 1994). Consistent with published results, we find that FREK mRNA signal is particularly intense in mitotically active mesenchymal progenitors. Moreover, we further extend these data to show that these cells represent a third wave of myotomal progenitors that originates in the extreme dermomyotome lips and are therefore initially present in the extreme edges of the myotomes. However, we find that within myotomes, FREK is not an exclusive marker of the mitotic population as it is also expressed earlier by pioneer myofibers and then by second-wave progenitors, including cells that migrate longitudinally subjacent to the DML and VLL epithelia (Cinnamon et al., 2001; Fig. 3A), and cells that exited the cell cycle and localized in a medial position with respect to the pioneers (see Fig. 3D; Kahane et al., 1998b). In fact, treatment with Shh, which triggers myofiber differentiation, also stimulates FREK expression initially in the dermomyotome epithelia and later in myofibers (N. K., unpublished). Thus, we predict that FREK may potentially transduce distinct kinds of signals in the various muscle cell types. While our initial results stemming from changes in ligand activity suggest a possible involvement in proliferation and adhesion/migration processes, direct manipulation of the gene encoding FREK should provide a more conclusive answer.

Within the myotome, third-wave progenitors express FREK, but are negative for MyoD, Myf5 or FGF4. This pattern resembles that expressed by myogenic progenitors on their way to the limb which produce FREK before MyoD or Myf5 (Marcelle et al., 1995). It is, however, at variance with progenitors of the second wave, which express FREK, MyoD and Myf5 (Kahane et al., 1998b; Cinnamon et al., 2001). These differences might be related to a different status of progenitor proliferation. Consistent with such a possibility, we find that second-wave myoblasts that express MyoD are mitotically active within the epithelia or the sub-lip domain but enter the myotome as post-mitotic cells. This is in line with the finding that MyoD activity is necessary for subsequent withdrawal from the cell cycle (Crescenzi et al., 1990; Halevy et al., 1995; Sorrentino et al., 1990; Yablonka-Reuveni et al., 1999). In contrast, cells of the third wave continue proliferating within the myotome. Thus, this latter population might derive from the BrdU-positive/MyoD-negative cells that were revealed in the extreme epithelial lips (Fig. 8).

It is interesting to point out in this respect that between 30 somites and E3.5, the epithelial dermomyotome lips generate simultaneously cells of the two types, suggesting that a heterogeneity between second- and third-wave progenitors already arises within their sites of origin. Consistent with this notion, MyoD mRNA signal is detected in only a subpopulation of dermomyotome lip cells. In addition, treatment with Shh, a floor plate and notochord-derived factor with effects on specification, survival and proliferation of distinct somite-derived lineages (Borycki et al., 1998; Duprez et al., 1998; Johnson et al., 1994; Munsterberg et al., 1995; Marcelle et al., 1997; Marcelle et al., 1999; Teillet et al., 1998), promotes MyoD expression in otherwise negative epithelial cells. This initial expansion of MyoD is followed by the formation of enlarged myotomes composed of post-mitotic myofibers accompanied by a reduced proportion of proliferating precursors of the third wave. Thus, we propose that epithelial cells of the rostral and caudal lips that acquire MyoD, exit the cell cycle upon myotome colonization and differentiate as second-wave myofibers. Epithelial progenitors within the lips that normally lack MyoD would adopt alternative fates: becoming the third-wave population if entering the growing myotome (this paper) or behaving as a stem cell population if remaining within the dermomyotomes (Denetclaw and Ordahl, 2000). The mechanisms that account for this early fate segregation within an apparently homogeneous epithelial environment remain an unanswered question.

Among the many unsolved issues that concern the development of the mitotically active phase of the myotome, a relevant one refers to the developmental potentials of third-wave progenitors. The possibility remains open that the mitotically active population gives rise to both muscle and non-muscular lineages present in vertebral muscles. Whether the mitotically competent cells of the third wave that originate in the dermomyotome edges are the direct progenitors of all fetal and adult satellite cells also remains to be clarified, particularly in light of recent studies showing that a population of muscle

satellite cells is derived from the embryonic vasculature (reviewed by Seale and Rudnicki, 2000).

This study is dedicated to the memory of Lea Averbuch-Heller; physician, scientist and dear friend. We extend our thanks to all members of our laboratory for helpful discussions. This study was supported by a grant from the Israel Research Foundation to C. K.

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