Lineage analysis of the avian dermomyotome sheet reveals the existence of single cells with both dermal and muscle progenitor fates

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Summary

The dermomyotome develops into myotome and dermis. We previously showed that overall growth of the dermomyotome and myotome in the mediolateral direction occurs in a uniform pattern. While myofibers arise from all four dermomyotome lips, the dermis derives from both medial and lateral halves of the dermomyotome sheet. Here we mapped the fate of this epithelial sheet by analyzing cell types that arise from its central region. We found that these precursors give rise not only to dermis, as expected, but also to a population of proliferating progenitors in the myotome that maintain expression of PAX7, PAX3 and FREK. Given this dual fate, we asked whether single dermomyotome precursors generate both dermal and mitotic myoblast precursors, or alternatively, whether these cell types derive from distinct epithelial founders. In-ovo clonal analysis revealed that single dermomyotome progenitors give rise to both derivatives. This is associated with a sharp change in the plane of cell division from the young epithelium, in which symmetrical divisions occur parallel to the mediolateral plane of the dermomyotome, to the dissociating dermomyotome, in which cell divisions become mostly perpendicular. Taken together with clonal analysis of the dermomyotome sheet, this suggests that a first stage of progenitor self-renewal, accounting for dermomyotomal expansion, is followed by fate segregation, which correlates with the observed shift in mitotic spindle orientation.

Key words: Avian embryo, Cell dissociation, Desmin, Epithelial-mesenchymal conversion, Myotome, PAX3, PAX7, Satellite cells, Somite

Introduction

The somites are segmented structures that develop stepwise to give rise to the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome (DM) (Christ and Ordahl, 1995). In addition, the medial portion of the epithelial somite is composed of a specialized subset of progenitors that differentiate into the earliest myofibers, extending across the entire mediolateral domain of each segment (Kahane et al., 1998a; Kahane et al., 2002). Upon dissociation, sclerotomal cells give rise to vertebrae, ribs and tendons (Brand-Saberi and Christ, 2000; Huang et al., 2000a; Brent et al., 2003). DM precursors give rise to the epaxial muscles of the back, and to the hypaxial muscles of the body wall (abdominal and intercostal) and limbs, to the dorsal dermis, to the blood vessels and to the scapula blade at thoracic levels of the axis (Ben-Yair et al., 2003; Brent and Tabin, 2002; Buckingham, 2001; Huang and Christ, 2000; Huang et al., 2000b; Kalcheim et al., 1999).

The DM consists of a central epithelial sheet and four contiguous curved edges: the dorsomedial and ventrolateral lips (DML and VLL), which are in apposition to the neural tube and intermediate/lateral plate mesoderm, respectively; and the rostral and caudal lips, abutting successive intersomitic spaces. Lineage tracing of distinct regions within the DM revealed that, following establishment of the initial myotomal scaffold formed of pioneer cells (Kahane et al., 1998a; Kahane et al., 2002), subsequent myofibers arise from progenitors residing in all four lips of the DM (Kahane et al., 1998b; Kahane et al., 2002; Cinnamon et al., 1999; Cinnamon et al., 2001; Huang and Christ, 2000; Venters et al., 1999; Gros et al., 2004) [see other references for a primary contribution by the DML and VLL only (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Ordahl et al., 2001; Venters and Ordahl, 2002)].

Because the precedent progenitors exit the cell cycle upon myotome colonization, continuous growth and muscle differentiation are ensured by subsequent cellular contributions. Starting at embryonic day (E) 2.5, mitotically active progenitors enter the myotome while expressing the FGF receptor FREK but not MyoD, Myf5 or FGF4 (Marcelle et al., 1995; Sechrist and Marcelle, 1996; Kahane et al., 2001), yet they do not undergo muscle differentiation until E6 (Kahane et al., 2001). Using a combination of iontophoretic injections of CM-DiI with BrdU labeling, we found that they originate from both rostral and caudal DM edges at a time when these lips still supply myofibers to the myotome and the DM is still fully epithelial (Kahane et al., 2001). Notably, within 2 days from

their first appearance, these mitotically competent progenitors become the predominant myotomal population (85% of total nuclei approximately), suggesting that they might also arise from additional, yet unexplored, sources.

The central portion of the DM in between the marginal lips has been classically referred to as dermatome, as it contributes to the dermis of the back (Brent and Tabin, 2002, Scaal and Christ, 2004). By E3.5 in the avian embryo, this central sheet begins dissociating, leaving the epithelial lips intact for a few days yet. We found that the resulting dorsal dermis derives from dissociating progenitors residing along the entire medial to lateral extent of epithelial somites. As these project onto corresponding regions of the DM sheet, the developing dermis is generated in a regionally restricted fashion (Ben-Yair et al., 2003). These results contrast with previous findings suggesting that the entire dorsal dermis derives from medial somitic halves exclusively (Olivera-Martinez et al., 2000). Altogether, lineage tracing studies and direct measurements of growth patterns led us to conclude that the dorsal somite, the DM and the resulting myotome and dermis expand by a coherent and proportional mechanism whereby all derivatives exhibit a direct topographical relationship with their predecessors (Kahane et al., 2002; Ben-Yair et al., 2003). A direct spatial relationship between myocytes in the myotome and their progenitors in the DM was also observed using the LaacZ method of clonal analysis in mouse embryos (Eloy-Trinquet and Nicolas, 2002).

In addition, available fate mapping results suggest that dermal and muscle fates are spatially segregated to distinct regions of the DM, with muscle progenitors arising from the DM edges and dermis from the central DM sheet. In the present study we analyzed the fate of the central DM and found that, in addition to dermis, it also furnished cells that colonized the underlying myotome. Further characterization revealed that these cells are proliferative myotome progenitors (PMPs) that maintain expression of PAX7 and PAX3, two DM markers that are downregulated in the nascent dermis. These cells also express FREK, similar to previously characterized proliferative progenitors emanating from the extreme DM lips. Hence, the mitotically competent wave of myotome colonization arises sequentially from two sources, first from the rostral and caudal DM lips (Kahane et al., 2001) and slightly later from the dissociating DM sheet.

Our finding that the central DM develops into PMPs as well as dermis raised the question whether this epithelium is composed of differentially specified progenitors, or alternatively, whether both cell types arise from single DM cells. To resolve this question, we performed clonal analysis in ovo. We found that single DM precursors give rise to both derivatives. We also identified the presence of intermediate progenitor types, suggesting that fate segregation occurs progressively in association with a sequential conversion of the DM from epithelium into mesenchyme. Notably, these events are associated with a change in the plane of cell division, which, in the young epithelium, is parallel to the apico-basal orientation of the cells and then, starting prior to dissociation, progressively shifts to become mostly perpendicular to their apico-basal axis. Our results support the notion that the DM sheet is composed of (at least) bipotent progenitors able to give rise both to myogenic and dermogenic lineages.

Materials and methods

Embryos

Fertile quail (*Coturnix coturnix Japonica*) and chick (*Gallus gallus*) eggs from commercial sources were used in this study. Analysis was restricted to interlimb levels of the axis (somites 21-26). Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

CM-DiI labeling combined with Pax7 immunostaining

The central region of flank-level DMs of stage 16/17 embryos was labeled midway between adjacent intersomitic clefts with CM-DiI as previously described (Kahane et al., 1998a; Cinnamon et al., 2001). Embryos were re-incubated until E4 (stage 20), fixed in 4% formaldehyde and processed for cryostat sectioning. Sections were then counterstained with PAX7 antibodies as described below.

Electroporation

An expression construct encoding an enhanced version of GFP, the pCAGGS-AFP (4 μg/μl) (Momose et al., 1999) was microinjected into flank-level DMs of HH16/17 quail embryos. The central sheet of a single DM was microinjected with a minimal amount of DNA using a micropipette mounted on an FST MM-33 micromanipulator and positioned approximately 45° to the longitudinal axis of the embryo. For electroporation, the negative L-shape tungsten electrode was placed underneath the blastoderm with the tip just ventral to the medial part of the DM and the positive electrode was located in a dorsal position with respect to the DM. A square wave electroporator (BTX, San Diego, CA) was used to deliver a single pulse of current at 20 volts, 10 milliseconds long. Embryos were re-incubated for 6 hours to monitor localization of fluorescent protein following initial expression of the transgene and then re-incubated until E5. Embryos were fixed in 4% formaldehyde, processed for paraffin wax embedding and sectioning and then immunolabeled with antibodies to GFP and desmin in combination with in-situ hybridization for chick-specific *Dermo1* (Scaal et al., 2001).

Transfection of GFP-encoding DNA into single DM progenitors

The GFP-encoding construct was microinjected unilaterally into the central DM sheet of stage 18 embryos. Six consecutive flank-level DMs were injected per embryo. Injection was carried out using a micromanipulator-mounted micropipette as described above. Micropipettes had a tip diameter ranging between 8 and 10 μm. The epithelium of each DM was pierced once through the ectoderm with the micropipette and a minimal volume of DNA mixed with Fast Green was pressure injected. GFP fluorescence became evident as early as 2 hours after transfection. However, in order to ensure robust and uniform levels of transgene expression, the re-incubation period for the calibration\control series was 6 hours. Additional embryos were grown for 24 or 48 hours, fixed in 4% formaldehyde, processed for paraffin-wax embedding and immunolabeled with antibodies to GFP and desmin.

Calibration series

To achieve transfections of single DM cells, different construct concentrations were examined by counting the number of GFP-expressing cells in serial sections of treated embryos 6 hours following transfection. An optimal DNA concentration ranging between 0.05 and 0.1 μg/μl was determined, which produced an acceptable success rate (see below).

Control series

Once an optimal DNA concentration was established, 46 embryos were injected as described above. In these experiments, six consecutive DMs were injected on both sides of each embryo. Serial section analysis revealed the presence of 33 GFP-expressing cells per

552 injected DMs. In 29/31 successful injections, a single GFP-positive cell was identified per DM 6 hours after injection. In 2/31 such injections, two GFP-expressing cells per DM were detected. The percent of successful injections resulting in a single GFP-expressing cell is, therefore, assessed to be higher than 90% at the specified DNA concentration.

Experimental series

Thirty eight and 41 successful transfections resulting from injection of 600 and 498 DMs in 100 and 83 embryos, were analyzed in serial transverse sections 24 and 48 hours after treatment, respectively. The clonal progeny of single transfected cells was classified according to location in either the myotome, the intermediate domain (ID) and/or the dermal layer, based on position relative to the desmin-immunostained myotome and on stage-specific landmarks. The latter included the compact PAX7-positive ID at 24 hours, and the PAX7-negative loose dermal mesenchyme at 48 hours (see Results). Successful cases were classified as producing exclusively dermal cells, myotomal precursors, ID cells or any combination of the three derivatives. Segments containing labeled cells in either sclerotome or ectoderm were not considered.

BrdU incorporation

BrdU (50 μl of a 10 mmol/l solution; Sigma) was applied for 1 hour to E4 embryos, followed by fixation in 4% formaldehyde and processing for cryostat sectioning. Embryos were sectioned at 10 μm, and immunolabeled with anti-BrdU and anti-PAX7 antibodies as described below.

Embryo processing and sectioning

Embryos were fixed with either 4% formaldehyde or Fornoy (the latter for in-situ hybridization on sections) and processed for paraffin-wax embedding as previously described (Cinnamon et al., 1999). Serial 10 μm transverse sections were mounted on Superfrost/Plus slides. For immunohistochemistry with PAX7 and PAX3 antibodies, embryos were processed for cryosectioning as previously described (Ben-Yair et al., 2003).

In-situ hybridization

Embryos were subjected to in-situ hybridization on sections (Kahane et al., 2001) with avian-specific probes for *Frek* (Marcelle et al., 1994), *Dermo1* (Scaal et al., 2001), *Pax7* and *Pax3* (gifts from Peter Gruss). While the original antisense PAX3 probe was prepared by *Bam*H1 digestion at the 5′ end of the insert, a new, shorter probe containing 100 bp upstream of the original sequence was prepared by *Pvu*II digestion, ensuring no PAX7 was recognized. Hybridized sections were further subjected to immunohistochemistry with anti-desmin, anti-PAX 7 or anti-GFP antibodies.

Immunohistochemistry

Immunostaining was carried out with polyclonal and monoclonal antibodies to desmin (ICN, 1:100 and Sigma, 1:20, respectively), to GFP (Molecular Probes, 1:200), polyclonal anti-BrdU antibodies (Abcam, 1:150), and monoclonal antibodies to PAX3 and PAX7 (Hybridoma Bank, 1:10). Specificity of the latter antibodies to their respective antigens was confirmed by Western blot analysis (not shown). Secondary antibodies coupled either to FITC, Rhodamine or horseradish peroxidase were used.

Determination of the plane of cell division

Embryos at HH16/17, 18 and 20 were fixed in 4% formaldehyde. Whole embryos were immunolabeled with γ-tubulin antibodies (Sigma, T3559, diluted 1:500) to label the centrosomes and further processed for paraffin-wax embedding. Serial 10 μm transverse sections were then subjected to Hoechst nuclear staining. Individual mitoses were identified and respective γ-tubulin and Hoechst stainings were separately photographed with a magnification objective of ×100 using an Olympus DP70 digital camera. Since the two centrosomes belonging to individual mitoses were sometimes found at slightly different focal depths, each mitosis was documented by capturing 2-3 consecutive images. The angles of the mitotic spindles relative to the mediolateral plane of the DM epithelium were measured from the combined (γ-tubulin +Hoechst) images using Adobe Photoshop 7.

Statistical analysis

Significance of the results was determined by the $\chi 2$ test ($P<0.0001$). The power of the test was calculated using the SAS Macro for sample size analysis.

Results

The central DM contributes cells that colonize both dermis and myotome

To investigate the fates of epithelial cells of the central DM region, we performed focal electroporations with a GFP-encoding vector directed to the center of the epithelial sheet and incubated the embryos for an additional 48 hours when a

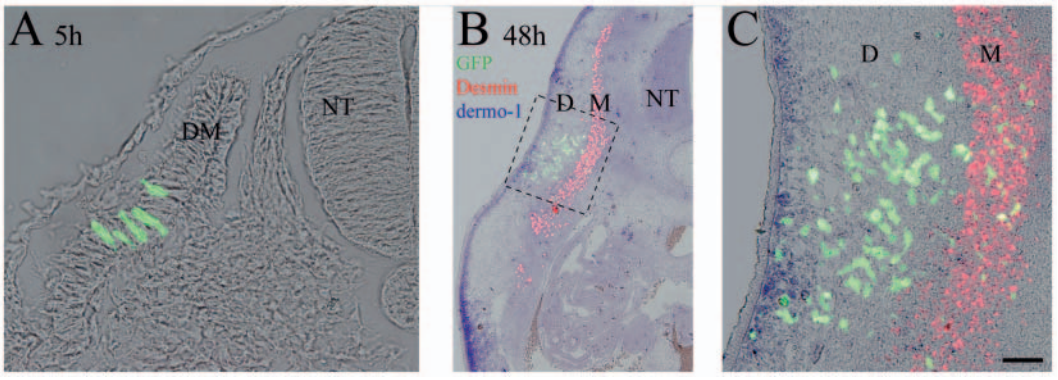


Fig. 1. The DM sheet gives rise to derivatives that colonize both the dermis and the myotome. (A) Five hours after electroporation, GFP-expressing cells (green) are located in the epithelial DM sheet. (B,C) At E5, GFP-labeled cells are present in the desmin-positive myotome (red) intermingled among desmin+ fibers. Labeled cells are also present in the dermis. At this stage, the *dermo-1* mRNA signal (blue reaction product) is predominantly expressed in the superficial domain of the young dermis close to the ectoderm, whereas GFP-expressing cells are widespread throughout the entire dermis. (C) High magnification of the insert in B. Note that the GFP-labeled cells in myotome and dermis are located at corresponding mediolateral regions, further substantiating the proposed coherent mode of DM development (Ben-Yair et al., 2003). D, dermis; DM, dermomyotome; M, myotome; NT, neural tube. Scale bar: 20 μm in A; 180 μm in B; 50 μm in C.

mesenchymal dermis was already apparent. Five hours after electroporation, GFP-positive cells were visible as epithelial cells in the central region of the DM, thus confirming the localization of transfections (Fig. 1A). Two days later, GFP-positive cells were found in both the dermis, the lateralmost region of which expresses *Dermo-1* mRNA, and in the desmin-positive myotome, respectively (Fig. 1B,C). These results demonstrate that DM progenitors of the central epithelium dissociate to populate not only the dermis, as originally described, but also the myotome.

Central DM-derived myotomal cells are mitotically active and express PAX7, PAX3 and FREK

Next, we characterized the phenotype of the DM-derived cells that entered the myotome. At E4-5, two main cell populations constitute the myotome, postmitotic mononucleated myofibers and mitotically active progenitors (Kahane et al., 2001). Analysis of whole embryos revealed that the DM-derived labeled cells within the myotomes were not myofibers (not shown), and this was further confirmed in serial transverse sections, which showed GFP signal and desmin immunoreactivity in non-overlapping cells (Fig. 1B,C). Therefore, we asked whether they are mitotically competent and what markers characterize them. Since PAX7 is a recognized somitic marker, we first examined its expression at progressive stages of development. PAX7 was expressed in virtually all somitic cells at the epithelial stage, both in DM as well as in prospective sclerotome progenitors (Fig. 2A). Upon dissociation, sclerotomal cells downregulated PAX7, which remained in the nascent DM, including its curved lips, but not in the myotome (Fig. 2B). DM dissociation began at its center and the process then spread both medially and laterally (Fig. 2C) (Ben-Yair et al., 2003). Notably, at this stage, few PAX7-positive cells were already detected in the underlying myotome (arrowhead in C) as well as in the dissociating DM (arrow in C and see next section). At E5, a dermal anlage was already

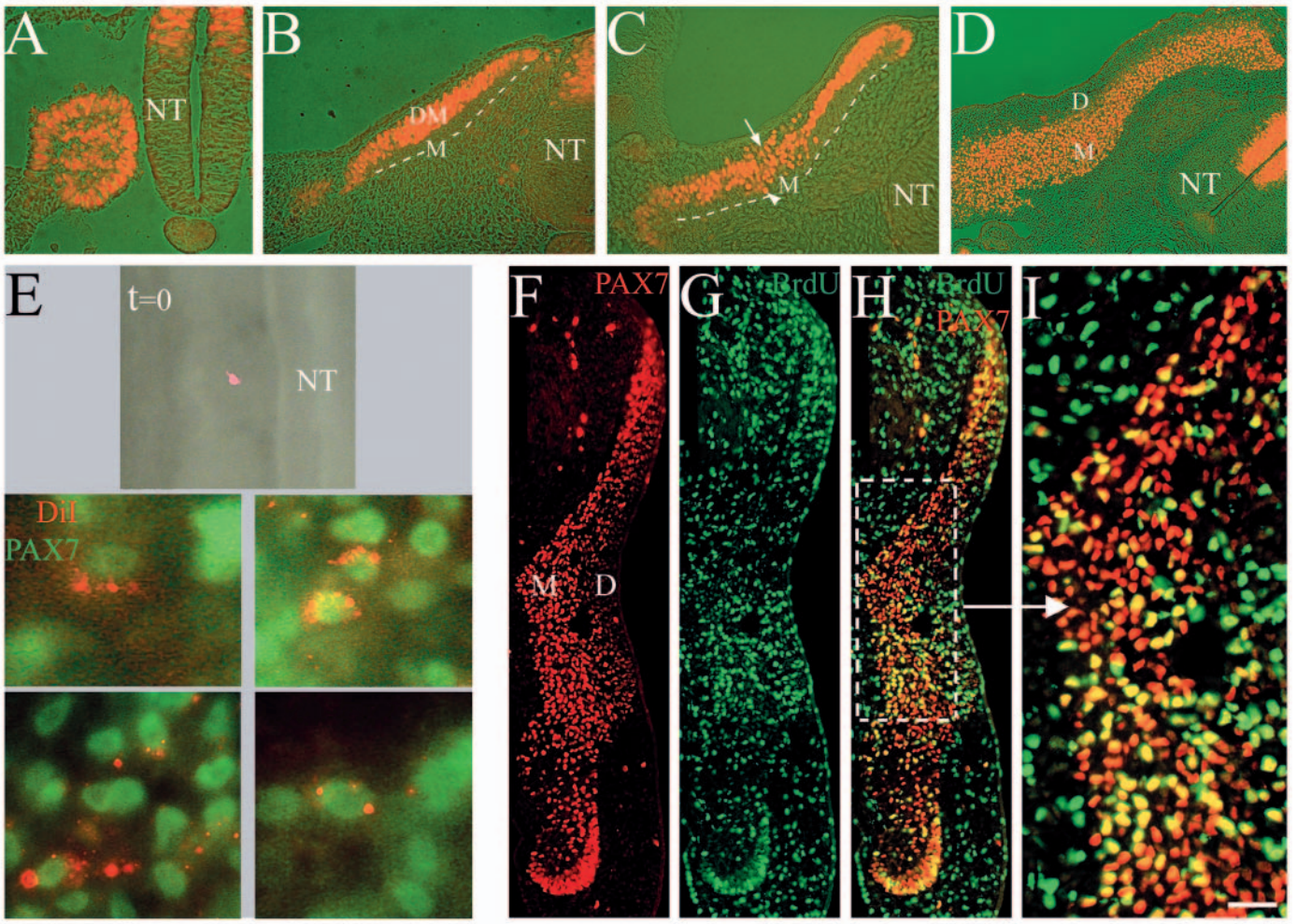


Fig. 2. DM-sheet-derived cells that enter the myotome are mitotically competent progenitors that retain PAX7 expression. (A-D) Sequential expression of PAX7 immunoreactive protein to developing somites. (A) E2; (B) E3; (C) E3.5; (D) E5. See text for details. (E) DM-derived cells traced by labeling with DiI co-express PAX7 in the myotome. Time zero (t=0) image, showing a dorsal view of a somite at E2.5 immediately following labeling with DiI to the central DM sheet. The four panels below illustrate sections through the myotome of E4 embryos expressing nuclear PAX7 (green) surrounded by membrane-associated DiI (red). (F-I) PAX7-positive cells in the myotome are mitotically active. E4 embryos were pulsed for 1 hour with BrdU, fixed and processed for PAX7 and BrdU immunolabeling. (F) PAX7. (G) BrdU. (H,I) Overlay between the images in F and G. Yellow cells co-express both PAX7 and BrdU. See text for details. D, dermis; DM, dermomyotome; M, myotome; NT, neural tube. Scale bar: 25 μm in A,I; 44 μm in B,C; 165 μm in D; 5 μm in E (sections); 37 μm in F-H.

present and its component cells had downregulated PAX7, whereas abundant cells that retained strong expression were present in the myotome (Fig. 2D).

To directly assess whether the PAX7-positive cells in the myotomes originate from the overlying DM sheet, focal labeling of the DM with CM-DiI was performed (Fig. 2E, time=0), and embryos were further incubated for 2 days and subjected to PAX7 immunostaining. Double-labeled cells containing PAX7-immunoreactive nuclei surrounded by CM-DiI-positive membranes were present within the myotomes (based on serial section analysis of three embryos; Fig. 2E, four lower images), demonstrating that myotomal progenitors of central DM origin retain PAX7 expression.

Next, we asked whether these PAX7-positive cells are mitotically active. To this end, E4 embryos were pulsed with Brdu for 1 hour, and fixed and processed for immunostaining with antibodies to Brdu and PAX7. As shown in Fig. 2F-I, double-labeled cells were found throughout the myotome (n=4). As expected, only a subset of PAX7-expressing cells co-expressed BrdU, as only a fraction of the proliferative cells in the myotome are encountered in the S-phase of the cycle during a 1 hour period. These results demonstrate that the myotomal progenitors of central DM origin that express PAX7 are mitotically active.

Since the DM is characterized by expression of both PAX7 and PAX3, we determined whether these myotomal progenitors also retained PAX3. Fig. 3A shows the presence of PAX3-positive cells in the myotome (white arrowheads). These cells do not overlap with the desmin-expressing myofibers, suggesting they belong to the mitotically active category. In previous studies, proliferative progenitors in the myotome were shown to express FREK (Kahane et al., 2001; Marcelle et al., 1995; Sechrist and Marcelle, 1996). We then examined whether PAX7-expressing myotomal progenitors co-express FREK. Virtually all PAX7-positive cells also expressed *frek* mRNA (Fig. 3B). Such double-labeled cells were detected throughout the myotome, suggesting that all proliferative progenitors, whether derived from the rostral and caudal lips as originally demonstrated (Kahane et al., 2001) or later from the DM sheet (Fig. 2), express both markers. Hence, the central DM epithelium produces mitotic cells that colonize the myotome and co-express PAX7 and FREK. Because the desmin-negative myotomal cells also express PAX3, the data suggest that the PMPs co-express all three markers.

Sequential stages of DM dissociation

The finding that PAX7 is a marker for DM-derived mitotic cells in the myotome and that it disappears from the forming dermis (Fig. 2D), enabled us to follow the relative onset of these two cell types. At flank regions of the axis, the epithelial DM of stage 18 embryos expressed PAX7 but the underlying desmin-immunoreactive myotome was still devoid of PAX7 immigrants (Fig. 4A; see also Fig. 2B). By stage 19/20 the central DM lost epithelial conformation and formed a compact cell aggregate (Brill et al., 1995) that retained PAX7 expression; we termed this DM-derived transient structure 'intermediate domain-ID'. At these stages the first PAX7-expressing cells had already invaded the myotome (Fig. 4B; see also Fig. 2C) but no dermis was yet apparent. By E4 (stage 23), three distinct domains were clearly visible: the desmin-immunoreactive myotome containing PAX7-positive cells; the compact ID, which expresses PAX7; and the beginning of a PAX7-negative dermis composed of scattered mesenchymal cells (Fig. 4C). A similar picture was observed when monitoring PAX3 expression with the PAX3-positive ID located between the myotome and the nascent dermis (Fig. 3A). A day later (E5, stage 26) the ID domain had almost disappeared. Instead, most PAX7+ cells were in the myotome intermingled among desmin-positive fibers and also located medial to them, and a well-developed dermis was present (Fig. 4D). Hence, the DM epithelium undergoes sequential dissociation into PMPs and dermis through a transient ID configuration. Close inspection of the dynamics of PAX3 and PAX7 expression also suggests that the initial contribution of

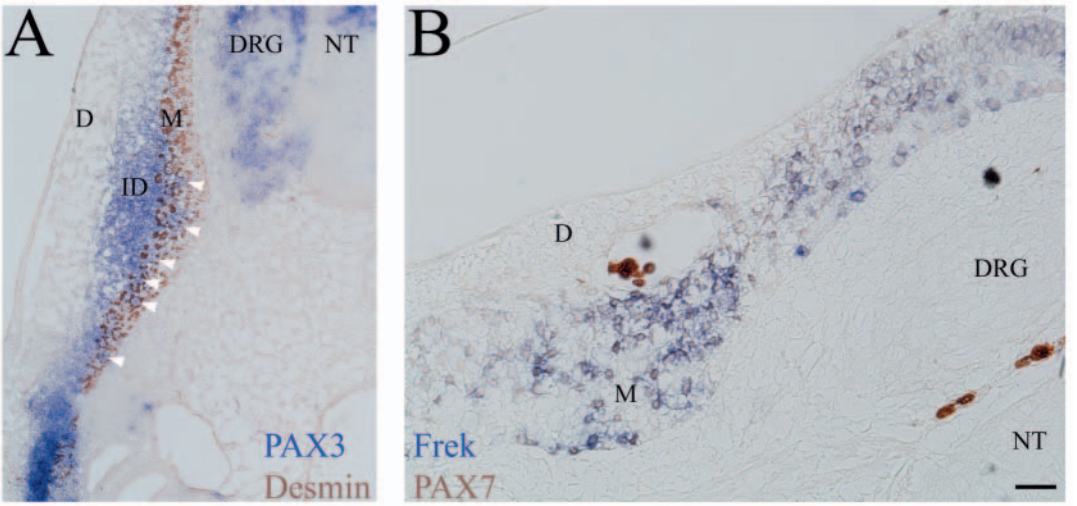


Fig. 3. Expression of *Pax3* and *Frek* mRNAs by mesenchymal cells in the myotome. (A) Cells expressing *Pax3* mRNA (blue, arrowheads) intermingle but do not overlap with desmin-positive fibers (brown) in the E4 myotome. At this stage, *Pax3* is also transcribed in a DM-derived intermediate domain located between myotome and forming dermis (see Fig. 4 and corresponding text for details). (B) Co-expression of PAX7 immunoreactivity (brown) and *Frek* mRNA (blue) in mesenchymal cells within the myotome. D, dermis; ID, intermediate domain; M, myotome; NT, neural tube. Scale bar: 30 μm in A; 23 μm in B.

the central DM to the myotome slightly precedes that of dermis.

Single DM progenitors produce both mitotic myoblasts and dermis

Our finding that populations of central DM progenitors give rise both to dermis and to PMPs, raised the question whether the DM sheet is composed already of distinct progenitors differentially specified to each fate, or alternatively, whether a single cell can give rise to both fates. To analyze this question, we traced the fate of individual DM cells for 1 and 2 days after labeling, and considered the distribution of labeled clones among the three identified domains, the ID, the myotome (as PMPs), and the dermis.

GFP-encoding DNA was introduced into single DM progenitors as described in the Materials and methods. Of 276 somites similarly injected (time zero controls), labeled cells were found in only 31 cases (11%). Of these 31 somites, in 29 cases a single GFP-expressing epithelial progenitor was detected 6 hours following transfection, the earliest time point in which a strong and unambiguous GFP signal became evident (Fig. 5A). In the two remaining somites, two adjacent epithelial cells were found that might have resulted from a double transfection event, or alternatively from a single transfected cell that divided within the 6 hour interval until fixation. These results show that, although being highly ineffective, the conditions we used are suitable for labeling single progenitor cells.

Serial section analysis of embryos re-incubated for 24 hours following transfection revealed a similar proportion of positive cases as the time zero controls (38 somites with labeled cells out of 600 transfected somites; 6.3%). At this stage, the existing anlage included the myotome and the ID, with only a rudimentary dermis (see Fig. 4C). Accordingly, 63% of transfected cells were found in the ID and 25% in the myotome, while only 10% were located in the nascent dermal primordium (Fig. 6A; Fig. 5B-D; and data not shown). As expected from the transient lifespan of the ID, at 48 hours post-transfection, the proportion of labeled cells in the ID significantly decreased to 8.2% ($P<0.0001$), while rising instead in the definitive myotomal and dermal compartments to 52.6 and 39.1%, respectively (Fig. 6B; $n=41$ somites with labeled cells out of 498 total transfected cases; 8.2% efficiency). Notably, no significant difference was observed when comparing the relative proportions of labeled PMPs with dermis between 24 and 48 hours ($P=0.13$). Together with the observations that a higher percentage of PMPs than dermis was present 1 day after transfection (Fig. 6A) and that PAX7-positive PMPs had already appeared in the myotome prior to initial dermis formation (Fig. 2C), these data show that initial colonization of the myotomal anlage by DM-derived cells precedes and is more significant than that of the dermis.

The same embryos were then analyzed to determine the progeny derived from each clone. Each injected cell could give rise to one of six possible outcomes (PMP, PMP+ID, D, D+ID, ID or PMP+D; Fig. 6C-E). The number of clones in each category was determined at 24 and 48 hours. At 24 hours, typical clones consisted of an average of 2-3.25 cells in the various categories (ranging between 2-5 cells/clone; Fig. 6E). This corresponds to a maximum of two successive cell divisions from the time of transfection. Although within 24 hours clones containing up to eight cells/clone would still be acceptable, given a somitic cell cycle length of about 9 hours (Primmett et al., 1989), to further ensure analysis of the progeny of single transfected cells clones containing more than five cells were discarded. The number of such clones was only four out of 38 total cases. At 48 hours, characteristic clones contained an average of 9-16 cells per clone (range of 4-35 cells in the different categories; Fig. 6E), corresponding to 3-5 cell divisions. Altogether, these values and the time zero calibrations/controls further confirm the clonal origin of transfected DM progenitors.

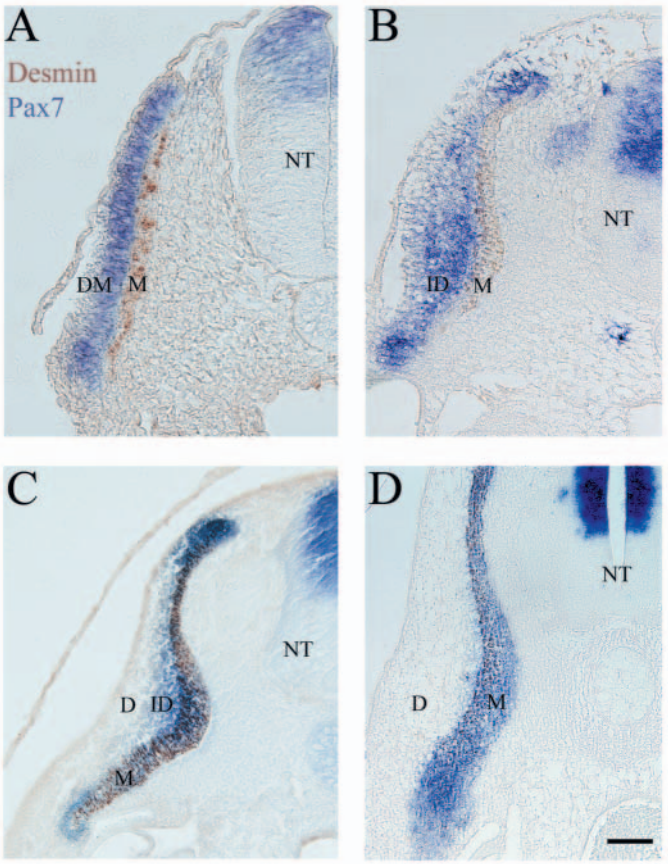


Fig. 4. Sequential dissociation of the epithelial DM and formation of its derivatives as revealed by morphology and *Pax7* mRNA expression. Transverse sections in-situ hybridized with PAX7 (blue) and immunolabeled with desmin (brown). (A) The epithelial DM at E3 expresses PAX7, but the underlying myotome is devoid of PAX7+ cells. (B) At E3.5 the central DM partially dissociates into a compact cell aggregate (ID) that retains PAX7 expression. Few PAX7+ cells become apparent in the myotome but no dermis is yet apparent. (C) By E4 three distinct domains are clearly visible, the desmin+ myotome containing PAX7+ cells, the compact ID, which expresses PAX7, and the beginning of a PAX7-negative dermis composed of scattered mesenchymal cells. See also a similar expression pattern of PAX3 between the myotome and dermis in Fig. 3A. (D) At E5 the ID domain has almost disappeared and most PAX7+ cells are in the myotome intermingled among desmin+ fibers and also located medial to them. A well developed dermis is present. D, dermis; DM, dermomyotome; ID, intermediate domain; M, myotome; NT, neural tube. Scale bar: 40 μm in A; 55 μm in B; 60 μm in C; 120 μm in D.

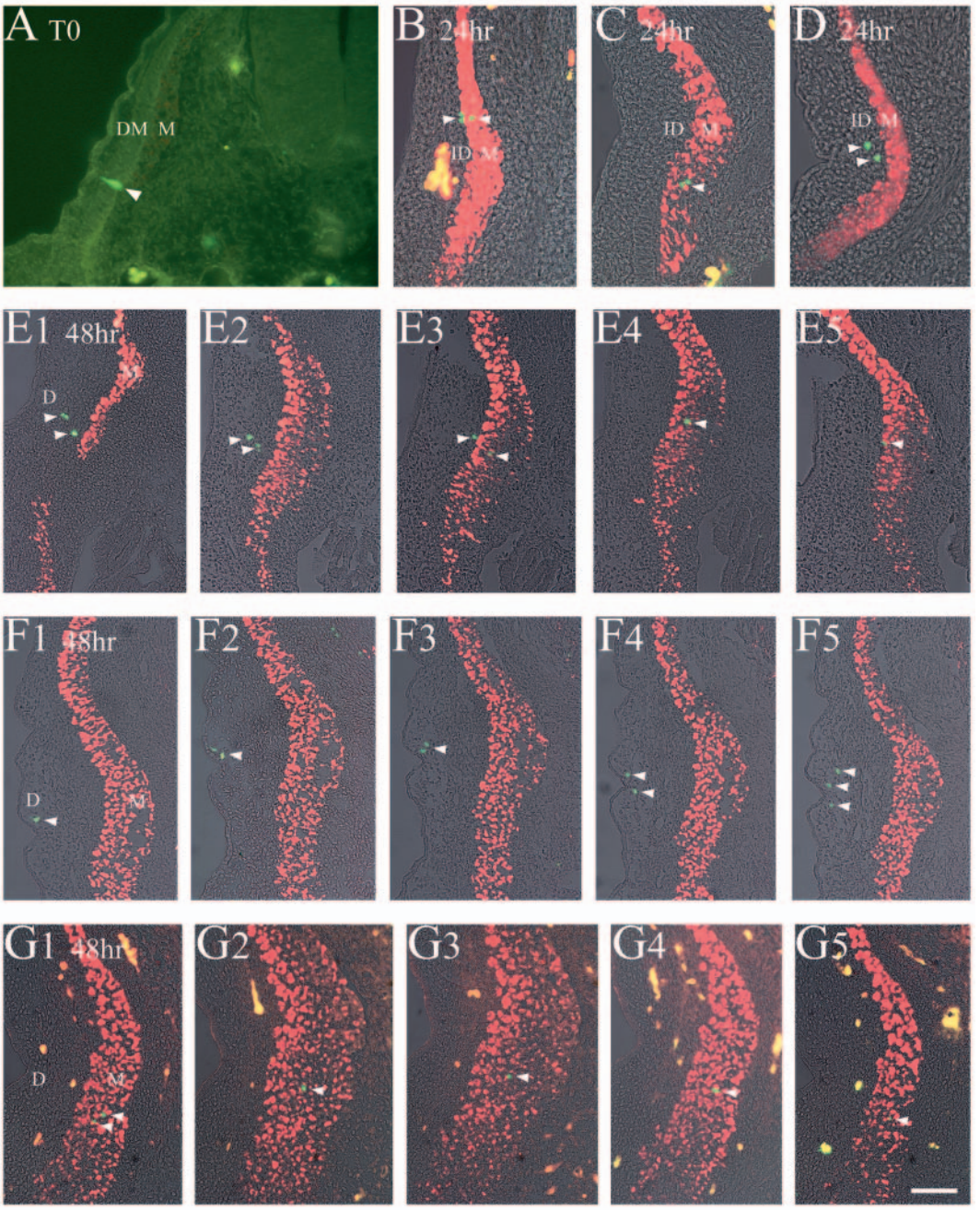


Fig. 5. The distribution of clonal progeny from the central DM sheet. Transverse sections of segments transfected with a GFP-encoding DNA into single cells followed by double desmin (red) and GFP (green) immunolabelings. (A) Time zero control. Six hours after transfection, a single cell is apparent spanning the apico-basal thickness of the epithelium. (B-D) Twenty-four hours after transfection, labeled progeny is localized to the ID and myotome (B), to the myotome only (C) or to the ID only (D). Each panel represents the overlay of two to three serial sections. (E-G) Sequences of five serial sections, each showing the distribution of clonal progeny 48 hours after transfection. (E1-E5) Labeled cells in both myotome and dermis. (F1-F5) Progeny restricted to dermis. (G1-G5) GFP+ descendants localized to the myotome. In all cases, GFP+ cells in myotome are desmin-negative. GFP+ cells are marked by white arrowheads. Yellow labeling represents blood cell autofluorescence. D, dermis; DM, dermomyotome; ID, intermediate domain; M, myotome; NT, neural tube. Scale bar: 40 μm in A; 50 μm in B-D; 70 μm in E-F; 60 μm in G.

Analysis of the composition of single clones at 24 hours post-transfection revealed that in 38.2% of cases, the progeny of a single injected cell were still confined to the DM-derived ID (Fig. 6C,E; see Table 1 in the supplementary material). One day later, no labeled cells could be detected any longer in only the ID. Instead, in 48.8% of injected somites, the progeny of labeled founders was found in both the PMP and the dermis (Fig. 5E1-E5; Fig. 6D,E; see Table 1 in the supplementary material). In 50% of the latter clones, labeled progeny were also detected in the residual ID, but for simplicity this triple clonal combination was pooled along with the PMP+D category. These results demonstrate that the DM contains single cells that give rise to both myogenic as well as dermogenic lineages. Since the proportion of clones containing labeled progeny in the ID (ID alone, PMP+ID and dermis+ID) at 24 hours is significantly reduced a day later (79.3% compared with 19.5%, respectively; Fig. 6E, $P<0.0001$) we infer that the mixed clones containing both PMP and dermis are likely to derive from the disappearing ID population between 24 and 48 hours post-transfection (E4 and E5, respectively).

Along with mixed PMP+dermis clones, others containing labeled cells in the myotome or in dermis exclusively (21.9 and 9.7%, respectively at E5) (Fig. 5F,G; and Fig. 6E) were also observed. These cells might have originated from a population of early-specified progenitors that coexist along with at least 'bipotent precursors' in the DM. Alternatively, they might have derived from similar bipotent progenitors that were

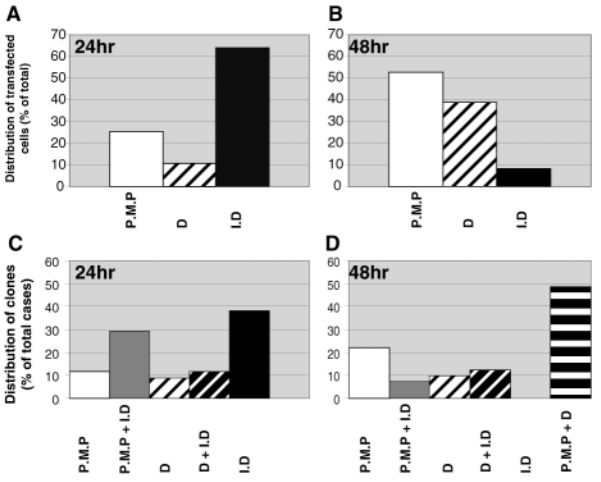
nevertheless stimulated by local cues to express only one of their potential fates (see Discussion). In 19.5% of total cases (14% approximately of total cells at E5), we detected the presence of mixed clones containing cells in either the PMP+ID or dermis+ID (Fig. 6E). Whereas cells in PMP and dermis are likely to further propagate their own lineages, cells in the ID could give rise either to PMPs, to dermis or to both phenotypes. The latter possibility seems the most likely one based on the observation that the disappearance of the ID is compensated for by appearance of mixed PMP+dermis clones between E4 and E5. Analysis for longer time periods would be required to follow the final fates of ID progenitors still remaining at E5. This is, however, complicated by the transient lifespan of the GFP transgene, which we found to be strongly transcribed for 48 hours and then to progressively decline.

Clonal analysis clearly reveals the existence of bipotent DM cells fated to become both PMPs and dermis. Furthermore, it also uncovers the presence and dynamics of intermediate progenitors along these lineages (PMP+ID, dermis+ID, ID only), and of clones with single phenotypes (see Fig. 9).

A shift in the plane of cell division occurs in the DM before complete dissociation

To begin exploring the mechanism/s that account for partitioning of the progeny of the central DM sheet into myotomal and dermal primordia, we investigated the planes of cell division taking place in the apical aspect of the DM epithelium as a function of development. To this end, embryos were stained with γ-tubulin antibodies to label the centrosomes and Hoechst to label the DNA (Fig. 7E-G). The precise angles of mitoses were measured with reference to the mediolateral plane of the DM epithelium, as described in Materials and methods. Similar to the classification used by Cayouette et al. (Cayouette et al., 2001), we arbitrarily adopted a spindle angle of <45° relative to the mediolateral DM plane to define cell divisions as 'parallel' and ⩾45° to define mitoses as 'perpendicular'. While parallel cell divisions would result in two daughter cells positioned side by side along the mediolateral direction of the epithelium similar to their ascendants, perpendicular divisions would generate two daughter cells localized in apical and basal positions, relative to each other. The majority of mitoses (95.6%) in the young DM at stage 16/17 were of the parallel type, with only 4.3% perpendicular mitoses (Fig. 7A,D,E; *n*=115 mitoses counted in four embryos). With ongoing development, a progressive shift in the orientation of cell divisions was monitored; at stage 18 the number of perpendicular divisions in the mature DM epithelium increased to 39.8%, with a corresponding decrease in that of parallel divisions (60.15%; *n*=133 mitoses counted in four embryos) (Fig. 7B,D,F). Notably, by stage 20, corresponding to the dissociation of the central DM sheet and formation instead of the compact ID, the proportion of perpendicular cell divisions further increased to 71.7% of the total (*n*=145 mitoses counted in four embryos) (Fig. 7C,D,G).

These results suggest that initial cell divisions that occur in the parallel plane serve for growth of the DM epithelium in the mediolateral direction, the main direction of DM elongation (Ben-Yair et al., 2003). With progressive development, the remarkable shift observed in the plane of mitosis of mature DM and nascent ID cells, which precedes the appearance of mixed clones with PMP and dermal cells, suggests that this shift may be of functional significance for lineage segregation of ID precursors into PMP and dermal fates.



E

Distribution of clonal progeny			P.M.P	P.M.P +I.D	Dermis	Dermis +I.D	I.D	P.M.P +Dermis	Total
Time following injection	24hr	%Clones	11.7%	29.4%	8.8%	11.7%	38.2%	0%	
		n	4	10	3	4	13		
		Average	2.25	3.1	2	3.25	2.8		n=34
		Range	2-3	2-4	2	2-4	2-5		
	48hr	%Clones	21.9%	7.3%	9.7%	12.2%	0%	48.8%	
		n	9	3	4	5		20	
		Average	9.6	10.3	13.25	9		16	n=41
		Range	4-16	8-14	7-18	5-13		4-35	

Fig. 6. Quantification of the sequential distribution of clonal progeny from the central DM sheet. (A,B) The proportion of total transfected cells localized in the myotome as PMPs, in dermis and in the ID at 24 (A) and 48 (B) hours after transfection. (C,D) Composition of the clones deriving from single DM progenitors at 24 and 48 hours after transfection, respectively. (E) Detailed distribution and size of clonal progeny (see text for details).

Discussion

In the present study we have demonstrated that the central DM sheet produces progenitors that colonize both the dermis and the myotome. In the latter, they behave as mitotically competent progenitors that maintain expression of PAX7, PAX3 and FREK. The finding that the central DM develops into mitotic PMPs as well as dermis raised the question of whether this epithelium is composed of distinct progenitors specified to each lineage, or alternatively, whether both cell types arise from single DM cells. Lineage tracing of single DM cells shows that DM derivatives appear sequentially. In the first stage, a non-epithelial compact ID arises, the progeny of which is found either in the ID or in the myotome and to a lesser extent in the dermis. At a second stage, complete dissociation is achieved, with daughter cells in both the myotome and the dermis. These results demonstrate that single DM founders give rise to both derivatives. This event is preceded by a sharp change in the orientation of cell divisions that, in the young epithelium, are parallel to the mediolateral direction of the DM and then, in the ID, shifts to become mostly perpendicular. Taken together, our results support the notion that the DM sheet

contains bipotent progenitors able to give rise to both myogenic and dermogenic lineages.

Regional fate map of DM derivatives

Three main derivatives directly arise from the DM: postmitotic mononucleated myofibers, mitotically competent PMPs and the dorsal dermis. The present findings show that the central portion of the DM gives rise both to PMPs and to dermis, further complementing previous data concerning fates of the DM lips. An outline of derivatives of the various DM regions is illustrated in Fig. 8. Using DiI labeling and pulse-chase experiments with thymidine, we reported that myofibers originate from all four lips of the overlying DM (Kahane et al., 1998b; Kahane et al., 2002; Cinnamon et al., 1999; Cinnamon et al., 2001). The origin of myofibers from the four edges was further confirmed using the quail marker technique and GFP electroporation (Huang and Christ, 2000; Gros et al., 2004).

In addition to producing postmitotic myofibers, the rostral and caudal edges of the DM also generate mitotically active PMPs, a process that begins by E2.5 at cervical levels of the axis (Kahane et al., 2001) (Fig. 8). Notably, both myofiber and PMP generation temporally overlap, suggesting the existence of an early heterogeneity among cells in these particular lips. Consistently, MyoD is expressed in only a subset of extreme lip precursors and its expression was found to be correlated with generation of postmitotic fibers (Kahane et al., 2001).

The question remained of the fate of the central DM sheet. Contrary to previous findings (Olivera-Martinez et al., 2000), we observed that the entire mediolateral aspect of the DM (and of the earlier epithelial somite), contributed to the dorsal dermis, with dermal cells demonstrating a direct spatial relationship vis-à-vis their epithelial predecessors (Ben-Yair et al., 2003). This is consistent with the dorsal dermis developing when the whole DM is still epaxially located before its hypaxial domain expands into the somatopleura (Christ et al., 1983). Surprisingly, in initial studies we observed that labeling the central domain of the DM with CM-DiI also produced cells that colonize the myotome but are mesenchymal rather than fibers (Ben-Yair et al., 2003). The timing of myotome colonization by these progenitors (from E3.5) and their characterization as mitotically active, PAX3/7/FREK-positive cells (see Results), led us to conclude that epithelial progenitors from the non-lip regions of the DM are late contributors of the third wave category of mitotically active progenitors, which initially invade the myotome from the rostral and caudal lips (Kahane et al., 2001). Hence, by the time of dissociation, the DM epithelium produces mesenchymal

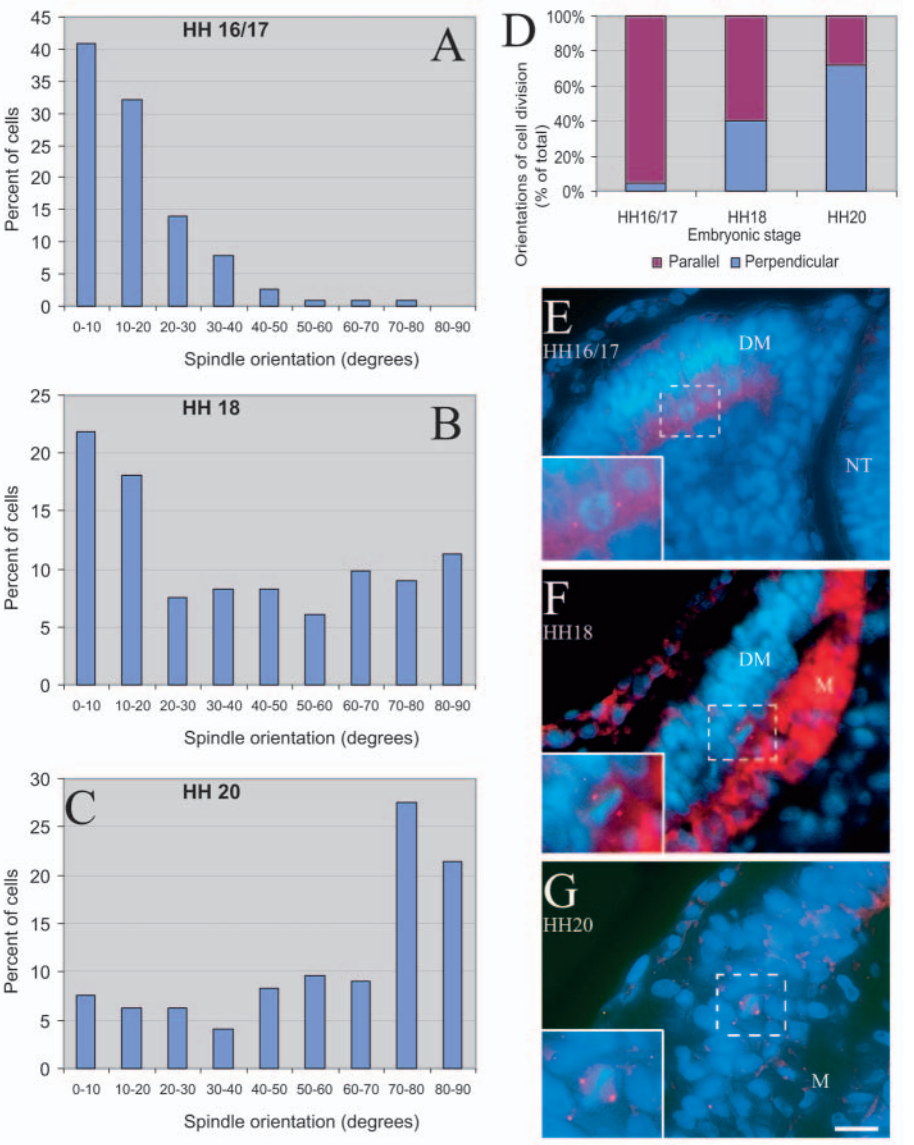


Fig. 7. A shift in the plane of cell divisions occurs in the DM prior to complete dissociation. (A-C) Frequency histograms of the orientation of mitotic spindles in the DM at stages HH16-20 of development. An angle of 0° represents a fully parallel orientation of mitosis vis-à-vis the mediolateral plane of the DM epithelium, whereas an angle of 90° reflects perpendicular cell divisions. (D) Quantification of the orientations of cell divisions as a function of embryonic stage. All measurements concerned the flank level of the axis. (E-G) Transverse sections of embryos aged E2.5, E3 and E3.5, respectively, double-labeled with γ-tubulin (red dots) and Hoechst nuclear stain (blue). Note in E and F the epithelial structure of the DM. In G the central portion of the epithelium is dissociating. Dividing cells are enclosed in inserts and high magnifications of the inserts highlight the planes of cell division. Note that in addition to centrosome-specific staining, γ-tubulin immunolabeling is also apparent throughout the cytoplasm of tissues such as myotome and ectoderm. DM, dermomyotome; M, myotome, NT, neural tube. Scale bar: 12 μm in E,F; 10 μm in G; 6 μm in E,G (inserts); 7 μm in F.

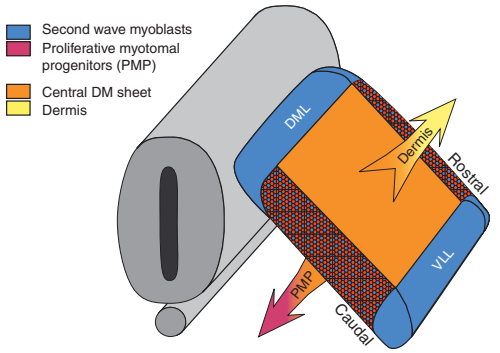


Fig. 8. Fate map of the DM. Schematic representation of the derivatives emanating from the different regions of the DM epithelium. All four lips (blue) give rise to myofibers as part of the second wave of myotome colonization that follows establishment of the early pioneer fibers. In addition, the rostral and caudal lips also generate the first mitotically active muscle progenitors that we termed 'third wave' (Kahane et al., 2001) (PMP, red). The DM sheet (orange) dissociates and its progeny colonizes the two opposite anlage, the subectodermal space to give rise to dermis (yellow arrow) and the myotome to give rise to mitotic muscle progenitors (red arrow). Hence, the proliferating PMPs derive from two distinct sources: first from the rostral and caudal DM lips (Kahane et al., 2001) and later from the DM sheet (this paper).

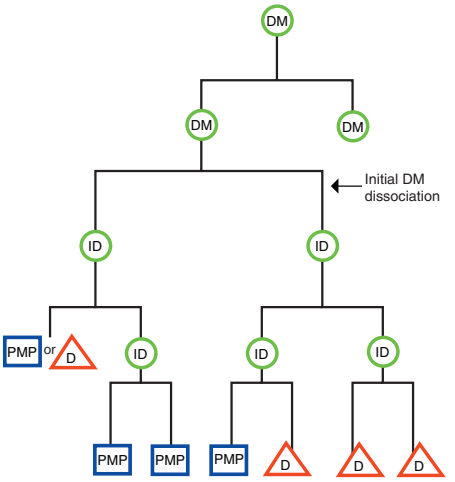


Fig. 9. Sequential segregation of lineages from founder cells in the DM sheet. Schematic representation of the stage-dependent generation of restricted progeny from the DM epithelium. DM precursors act as stem cells with self-renewal ability in the epithelium. They express homogeneously both PAX3 and PAX7, divide with an orientation parallel to the apico-basal thickness of the epithelium, and give rise to progeny that remain confined to the DM and contribute to its mediolateral growth as long as the epithelium lasts. The DM epithelium begins its dissociation into the ID, which reveals an aggregated conformation. Most ID precursors either self-renew for one day and/or segregate asymmetrically into ID and PMPs or ID and dermis (intermediate restricted progeny). Finally, a significant proportion of ID cells generate both PMP and dermis at E5, suggesting that these still keep a stem-like character (see text for details).

cells that sort out in two opposed directions; both superficially to colonize the subectodermal space and give rise to dermis, and also toward the myotome to further contribute to its growth (Fig. 8). Thus, the immediate fates of DM cells appear to be regionalized, with myofiber generation limited to the DM lips, PMP production to the rostral and caudal lips followed by the DM sheet, and dermis exclusively from the DM sheet. Although dermis is likely to be a major derivative, a subset of DM progenitors at flank levels of the axis also contributes to the scapula blade (Huang et al., 2000a), though the precise location of these precursors in the DM remains to be mapped, and to endothelial cells (Huang et al., 2000b). Likewise, the final fate of the PMPs may be varied. Although most progenitors are likely to withdraw from the cell cycle at progressive stages to undergo myogenesis, some may also contribute to muscle fibroblasts and others may remain as undifferentiated muscle satellite cells. A limitation of the present study is that due to the transient lifespan of transfected GFP, daughter cells cannot be followed for long enough to determine their final fates.

Our study showing a unique behavior of the DM sheet would suggest that this area follows a developmental molecular program that is, at least partly, different from that of the DM lips. Consistent with such a possibility is the expression of several transcription factors such as *EN1* and *SIM1* to different mediolateral subdomains of the central DM sheet in both avian and mouse embryos (Cheng et al., 2004; Sporle, 2001). Notably, following DM dissociation, these markers remain expressed in both myotomal and dermal domains at corresponding mediolateral locations (Cheng et al., 2004), consistent with results of our lineage studies (this paper) (see also Ben-Yair et al., 2003). Another gene that characterizes the central DM region is *Alx4*, which later becomes restricted to the dermis (Cheng et al., 2004). Reciprocally, we show here that PAX 3 and 7, initially widespread throughout the entire avian DM, get restricted to the PMPs in the myotome and disappear from the nascent dermis. Several studies also identified genes specifically localized to a central region of the myotome. In mice, *Myf5*, *Fzd9* and connexin 40 are transiently expressed in this subdomain [Sporle (Sporle, 2001) and references therein]. Regulatory sequences of the *Myf5* gene directing expression of *nlacZ* were detected in an intercalated subregion of the myotome in transgenic mice (Hadchouel et al., 2000; Hadchouel et al., 2003). *Xmyf5* was detected in the presumptive DM of *Xenopus* somites (Grimaldi et al., 2004). Altogether, these results would suggest the existence of myogenic regions in the DM proper other than the lips. It remains to be clarified whether the various myotomal markers are expressed in myofibers, in PMPs or indistinguishably in both cell types.

Lineage segregation of the DM

As the DM is a structure that produces several derivatives, one of the critical questions related its development concerns the timing and mechanisms of segregation of the different lineages from the primary pseudostratified epithelium. In-vivo lineage tracing permits the fate of individual progenitors to be assessed under normal conditions because the labeled cells remain subjected to the instructive/selective environments of the embryo. Given that DM fates are regionally restricted (see previous section), and that specific DM domains generate more than one cell type, the question of the state of specification of individual progenitors is an important one. For instance, rostral and caudal lips of the DM contain heterogeneous subsets of

MyoD-positive and -negative cells, which correlate with their outcome as myofibers versus PMPs, respectively (Kahane et al., 2001). Although not yet formally demonstrated, these data would suggest an early fate restriction among epithelial precursors residing in the extreme DM lips.

In this study, we focused our initial analysis on the central sheet of the DM, which produces both PMPs and dermis. We uncovered the existence of individual DM progenitors that generate both cell types, others that produce exclusively PMPs or dermis, and intermediate stages containing combinations of ID cells and PMPs or ID cells and dermis. Clearly, the different outcomes could be temporally separated, with 80% of clones 1 day after labeling still containing cells in the ID, with PMPs segregated slightly before dermis, and clones containing both PMPs and dermis appearing between 1 and 2 days post-transfection. These results stress a time-dependent generation of restricted progeny from the early DM (Fig. 9). Such an outcome could either be explained as stochastic differentiation of a homogenous population of initially multipotent cells or by the presence of both multipotent and fate-restricted precursor cells in the early DM. Our data generally favor the first mechanism, for several reasons. First, expression of PAX3 and PAX7, as well as the weak transcription of *frek* mRNA, are homogeneous in the DM sheet and DM-derived ID with no apparent segregation to cell subsets, unlike that observed for MyoD in the extreme DM lips (Kahane et al., 2001). Second, all mixed clones containing both PMPs and dermis become apparent only 48 hours post-transfection (Fig. 9) but not a day earlier. This strengthens the notion that even in the ID, a significant proportion of progenitors still remain bipotent. Third, 12 hours after transfection we already observed clones containing cells in ID+PMPs at a time that precedes formation of a mesenchymal dermis (R.B.-Y. and C.K., unpublished); the time gap between these two cell types is further emphasized by the higher proportion of clones containing PMPs compared with dermis at 24 hours. This earlier segregation of PMPs does not necessarily need to result from the existence in the DM epithelium of distinct progenitors. Instead, it is likely to occur because the well-developed myotome, which exists prior to the initial dermis, stimulates or at least permits the entry of stem-like cells that differentiate further to myotome colonization. Fourth, the orientation of cell divisions in the DM is mostly parallel to the mediolateral plane of the epithelium, consistent with a distribution of the resulting progeny along the direction of DM growth. This favors the notion that the central DM is composed of a homogeneous population of self-renewing epithelial progenitors (see next section) rather than a mixture of cells with different states of commitment.

The existence of single progenitors that generate more than one cell type has recently been documented in a specific somite domain using a retroviral approach (Kardon et al., 2002). In this study, 26% of clones derived from the lateral portion of hindlimb-level somites of chick embryos were shown to contain both muscle cells and endothelial cells that colonized the limb. Furthermore, within the muscle lineage, they were not restricted in their ability to develop into fast versus slow fibers, or to populate specific muscles in the limb. Likewise, the mitotically active PAX7/3-positive PMPs that we identified in the myotome might not necessarily be restricted to give rise to myofibers and could also generate endothelial cells, muscle satellite cells etc. An additional similarity between our study and that of Kardon et al. (Kardon et al., 2002) is that both revealed the existence of clones containing single cell types along with clones containing mixed progeny. In both studies, the presence of mixed clones was, in general, positively associated with clonal size, in further support of a stochastic model of lineage segregation.

A shift in the plane of cell division precedes fate segregation of individual ID progenitors

In the young pseudostratified DM epithelium, mitoses occur at the apical pole (Fig. 7) and DNA synthesis in the basal half of the epithelium (Ben-Yair et al., 2003). At this stage, the orientation of the vast majority of cell divisions is parallel to the plane of the epithelium. We observed that in the mature DM, a progressive shift in the plane of cell division becomes apparent; and with initial dissociation of the DM into the aggregated ID, the orientation of 71.7% of the cells changes to a perpendicular direction. This value resembles the proportion of mixed clones obtained at E5 (68.3%). These results raise the intriguing question of the significance of this shift to lineage segregation of DM cells into its derivatives. In the nervous system, it has been hypothesized that parallel divisions serve to expand the progenitor pool, whereas the perpendicular type of mitoses produce two different daughter cells, one that remains in the ventricular zone as a progenitor and one that migrates away to differentiate (e.g. Kornac and Rakic, 1995; Chenn and McConnell, 1995; Doe, 1996). Along the same line, we propose that the early DM contains a pool of self-renewing progenitors (Fig. 9) that, by dividing in a parallel orientation, contribute to mediolateral expansion of the DM, a process found to occur uniformly along this structure (Ben-Yair et al., 2003). Also similar to the neuroepithelium, we find that the orientation of cell divisions changes in the DM prior to dissociating into PMPs and dermis. Unlike the neuroepithelium, however, the central portion of the DM dissociates in its entirety, giving rise to the aggregated ID, which in itself lasts for 1.5 days approximately. A subset of ID cells are also likely to self-renew because progeny in this domain are found at both 24 and 48 hours following transfection. Nevertheless, at this stage, a major outcome of the ID is the production of mixed clones containing both PMPs and dermis. By analogy to the neuroepithelium, it is tempting to speculate that this fate segregation results from the change in orientation of cell divisions. Direct demonstration that this is the case is still lacking due to the technical difficulty of tracing in ovo the final fates of individual cells that specifically divide perpendicular to the epithelium.

Supportive evidence for such a mechanism could potentially derive from asymmetric allocation of cell-fate-determinants to one of two daughter cells dividing in the perpendicular orientation, as demonstrated in invertebrates (e.g. Rhyu et al., 1994; Knoblich, 2001; Lu et al., 2000). NUMB is one such protein shown to inhibit NOTCH signaling and also to divide asymmetrically in the nervous system of vertebrates (Chenn and McConnell, 1995; Zhong et al., 1996; Zhong et al., 1997; Cayouette et al., 2001; Cayouette and Raff, 2003; Wakamatsu et al., 1999). However, we have not observed any asymmetric allocation of NUMB to the central DM sheet or to ID cells (R.B.-Y. and C.K., unpublished).

The integrity of intercellular junctions could also play a role in maintaining the orientation of mitoses. N-cadherin is expressed in the epithelial DM with adherens junctions located apically (Duband et al., 1987; Duband et al., 1988). The observed shift in mitotic orientation could be secondary to, and perhaps also result from, a loss of N-cadherin-associated signaling that occurs upon dissociation of the epithelium. Consistent with such a possibility, the stereotypic orientation of mitoses in the zebrafish neuroepithelium was found to be partially perturbed by loss of function of specific junctional components (Geldmacher-Voss et al., 2003). Factors emanating from the adjacent ectoderm or myotome could also influence the observed shift in planes of cell divisions in the ID. Consistently, the pigmented epithelium was reported to control the plane of cell division in the adjacent retina (Cayouette et al., 2001). In spite of environmental modulation, it appears that the orientation of cell divisions is intrinsic to the epithelium, as cultured dissociated cells keep their original orientations ex vivo (Cayouette et al., 2001; Estvill-Torrus et al., 2002). It will be interesting to elucidate whether similar general mechanisms operate during DM ontogeny and to determine their precise molecular identity.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/4/689/DC1

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