Coherent development of dermomyotome and dermis from the entire mediolateral extent of the dorsal somite

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SUMMARY

We have previously shown that overall growth of the myotome in the mediolateral direction occurs in a coherent and uniform pattern. We asked whether development of the dermomyotome and resultant dermis follow a similar pattern or are, alternatively, controlled by restricted pools of stem cells driving directional growth. To this end, we studied cellular events that govern dermomyotome development and the regional origin of dermis. Measurements of cell proliferation, nuclear density and cellular rearrangements revealed that the developing dermomyotome can be subdivided in the transverse plane into three distinct and dynamic regions: medial, central and lateral, rather than simply into epaxial and hypaxial domains. To understand how these temporally and spatially restricted changes affect overall dermomyotome growth, lineage tracing with CM-DiI was performed. A proportional pattern of growth was measured along the entire epithelium, suggesting that mediolateral growth of the dermomyotome is coherent. Hence, they contrast with a stem cell view suggesting focal and inversely oriented sources of growth restricted to the medial and lateral edges. Consistent with this uniform mediolateral growth, lineage tracing experiments showed that the dermomyotome-derived dermis originates from progenitors that reside along the medial as well as the lateral halves of somites, and whose contribution to dermis is regionally restricted. Taken together, our results support the view that all derivatives of the dorsal somite (dermomyotome, myotome and dermis) keep a direct topographical relationship with their epithelial ascendants.

Key words: Avian embryo, Cell delamination, Dermis, Dermomyotome, Desmin, Epithelial-mesenchymal conversion, Myotome, Somite

INTRODUCTION

The paraxial mesoderm of vertebrate embryos segments into metameric units, the somites, which develop stepwise to give rise initially to the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome (DM). Sclerotomal cells then undergo further morphogenetic changes and form the vertebrae and ribs (Brand-Saberi and Christ, 2000; Huang et al., 2000b) (see Kato and Aoyama, 1998). DM precursors give rise to the epaxial muscles of the back, the hypaxial muscles of the body wall and limbs, and the dorsal dermis (Christ et al., 1983; Kalcheim et al., 1999; Huang and Christ, 2000), yet the mechanism by which these derivatives form from the DM is not fully resolved. In the past few years, the existence of an additional cellular domain in the medial epithelial somite has been described. It consists of a specialized subset of early post-mitotic progenitors which in avian embryos, express both MyoD and Myf5. During the process of somite dissociation, these cells bend underneath the forming DM and upon delamination and polarized caudorostral migration, differentiate into the first unit-length myofibers that span the entire mediolateral extent of each segment (Kahane et al., 1998a; Kahane et al., 2002). Notably, these early fibers were suggested to provide a scaffold for further addition of myogenic cells that arise in the overlying DM. These pioneer myoblasts, together with DM-derived myofibers, constitute the myotome from which vertebral muscles of the back and the body wall (intercostal and abdominal) will form (Kalcheim et al., 1999). At variance, limb muscles derive exclusively from the ventrolateral lip (VLL) of the DM (Chevallier et al., 1977) and bear no contribution of pioneer myofibers (N.K. and C.K., unpublished).

A wealth of information is available on early patterning of somite derivatives that involves opposite and mutually inhibitory gradients of factor activity (reviewed by Brent and Tabin, 2002). Yet, the mechanisms by which these basic gradients are translated into morphogenetic processes remain incompletely understood. Ordahl and colleagues (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000) have proposed that growth of both the myotome and DM is driven by two stem cell systems restricted to the dorsomedial and ventrolateral lips (DML and VLL, respectively) of the DM. The DML was suggested to drive medial growth of the myotome and the VLL to be responsible for expansion in a ventral direction (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000; Ordahl et al., 2001). Consequently, both myotome and DM expand incrementally in opposite medial and lateral directions, with the youngest cells approaching the medial and lateral extremes

and a central domain which would correspond to the oldest fibers. This latter intervening space was suggested to remain quiescent in terms of cell growth and contribution to the myotome (Denetclaw and Ordahl, 2000). At variance with this view, we have found that the myotome grows evenly in the mediolateral orientation owing to progressive and simultaneous intercalation of myoblasts from all four lips of the overlying DM among pre-existing pioneer myofibers (Kahane et al., 1998a; Kahane et al., 1998b; Kahane et al., 2001; Cinnamon et al., 1999; Cinnamon et al., 2001). Hence, both young and old fibers evenly spread all along the mediolateral extent and the growth of the myotome follows an overall uniform pattern (Kahane et al., 2002). Altogether, these findings demonstrated that progenitor cells for muscle growth are not restricted to the DML and VLL but also prevail along the entire mediolateral extent of both rostral and caudal DM lips. The origin of the myotome from all four DM lips was now further substantiated (Huang and Christ, 2000; Venters et al., 1999), and the existence of intercalatory mechanisms accounting for coherent myotome growth was independently shown in the mouse embryo using the *LaacZ* method for clonal labeling (Eloy-Trinquet and Nicolas, 2002).

Based on our results inferring a coherent and uniform mode of overall myotome expansion, we asked whether development of the DM epithelium follows a similar pattern, or is, alternatively, controlled by restricted pools of stem cells driving directional growth. Such a question was also relevant for the formation of the DM-derived dermis, which was suggested, based on grafting of half-somites from quail donors into chick hosts, to arise entirely from the medial half of the somite (Olivera-Martinez et al., 2000). To this end, we studied the cellular events that govern DM development from epithelial somites and the regional origin of the dermis. Measurements of cell proliferation, nuclear density and cellular rearrangements revealed that the developing DM can be subdivided in the transverse plane into three distinct and dynamic regions: medial, central and lateral, rather than simply into epaxial and hypaxial domains. To understand how these temporally and spatially restricted changes affect overall DM growth, lineage tracing with CM-DiI was performed. A proportional pattern of growth was measured along the entire DM. These results suggest a model of coherent mediolateral growth of the DM as opposed to a stem cell view implying focal and inversely oriented sources of growth at the medial and lateral edges. In line with the uniform mediolateral growth of the dorsal epithelial somite and the DM, lineage tracing experiments showed that the dorsal dermis derives from progenitors residing along the entire medial to lateral extent of epithelial somites which contribute to dermis in a regionally restricted fashion. Taken together, our results support the view that all derivatives of the dorsal somite exhibit a direct topographical relationship with their ascendants.

MATERIALS AND METHODS

Embryos

Fertile quail (*Coturnix coturnix Japonica*) and chick (*Gallus gallus*) eggs from commercial sources were used in this study. Analysis of DM and dermal development was restricted to interlimb levels of the axis (somites 21-26). Successive stages of somite development were

considered (Fig. 1). The epithelial stage was termed T0 and analyzed in embryos aged 21-28 somite pairs. Approximately 15 hours later, the DM formed at interlimb segments of the axis due to early sclerotome dissociation. This stage was termed T1 and was analyzed in embryos aged 32-34 somites. T2 corresponded to fully dissociated somites, 10-12 hours approximately after T1. The T2 stage was apparent at interlimb levels of the axis in embryos aged 38-40 somite pairs. Finally, T3 was the stage at which the mesenchymal dermis was present [embryonic day (E) 4].

Quail-chick chimeras

Whole epithelial somites or dorsal halves of epithelial somites from quail donors (23-25 somites-old) were grafted into the equivalent site and axial level of age-matched chick hosts as already described (Kalcheim and Teillet, 1989). Chimeras were further incubated till E5, processed for paraffin wax embedding, sectioned at 8 μm and immunolabeled with the QCPN antibody (Developmental Studies Hybridoma Bank) to detect quail nuclei.

Quantification of DM cell proliferation, nuclear density and nuclear layers

Embryos at stages T0, T1 and T2 received a single pulse of 10 mM BrdU and were reincubated for 1 hour, fixed in 4% formaldehyde and processed for paraffin wax embedding. Serial sections (5 μm) were prepared, immunolabeled with a BrdU-specific antibody and counterstained with Hoechst to reveal total nuclei (Burstyn-Cohen and Kalcheim, 2002). Cell proliferation was measured as the proportion of BrdU-positive of total nuclei, the number of nuclear layers per thickness of the pseudostratified epithelium was counted and the actual thickness of the DM was directly measured and presented as arbitrary units. All measurements were performed along the entire mediolateral extent of the dorsal epithelial somite (at T0) or the DM (for T1 and T2) and were conducted in the central domain of each segment midway between the rostral and caudal edges. To this end, the dorsal epithelium was subdivided mediolaterally into equal fields, 625 μm^2 each. The entire mediolateral extent of the dorsal epithelium at T0 was included into three such fields, and the DM at T1 and T2 comprised five and ten contiguous microscopic fields, respectively. To compare between stages, the whole mediolateral length of the epithelium was normalized to 100%, hence 0% represents the medialmost point in the epithelium and 100% the lateral edge. For each parameter, 10-20 alternate transverse sections were measured in two somites per embryo. Results represent the mean±s.d. of three or four independent embryos.

CM-DiI labeling

The medial, central and/or lateral aspects of dorsal epithelial somites or DM were labeled midway between adjacent intersomitic clefts with CM-DiI or with a combination of DiI and DiO as previously described (Kahane et al., 1998a; Cinnamon et al., 2001). Experiments were performed in interlimb-level segments of embryos aged T0 or T1. Embryos were reincubated until T2 or T3, fixed in 4% formaldehyde and processed either for paraffin-wax embedding (CM-DiI-treated embryos) or cryostat sectioning (DiI/DiO) as previously described (Cinnamon et al., 1999). CM-DiI labeling of the sclerotome was performed at T1 after initial dissociation of the somite. To avoid labeling of the DM, the latter was separated microsurgically from the sclerotome, lifted and pulled temporarily aside. Selected sections were counterstained with desmin antibodies (Cinnamon et al., 2001) to delineate the myotome.

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 epithelial somites of 25 somite-stage quail embryos. The lateral domain of two or three successive segments was microinjected using a micropipette positioned parallel to the
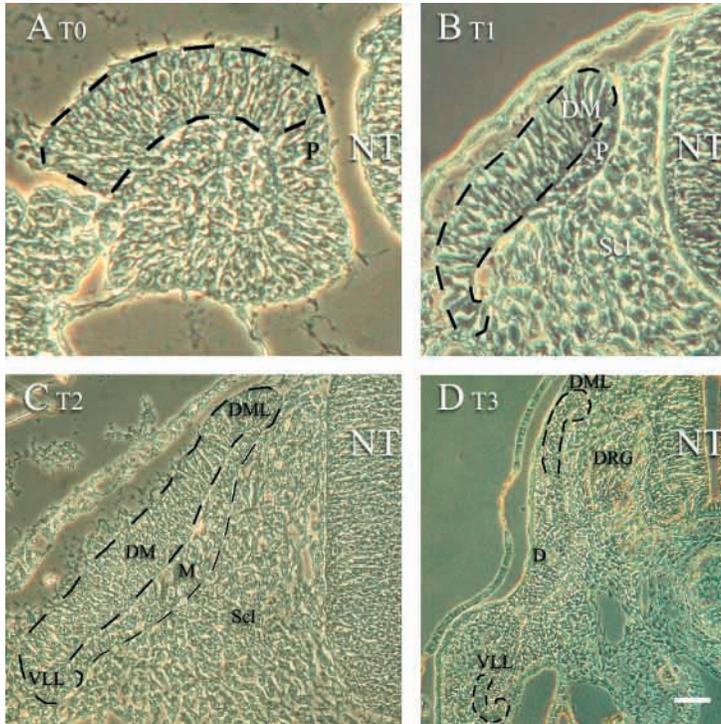
Fig. 1. Successive stages in dermomyotome development at flank levels of the axis. (A-D) Phase contrast images. (A) T0. Epithelial somite stage with the prospective dermomyotome (DM) highlighted by broken lines. (B) T1. Initial formation of the DM following mesenchymalization of the sclerotome (Scl). At this stage the pioneer (P) myoblasts bend underneath the nascent DM. (C) T2. The DM of a fully dissociated somite in which the primary myotome (M) is well differentiated. Note the well defined medial and lateral edges (DML and VLL, respectively). (D) T3. The DM dissociates into dermis except for the DML and VLL, which still remain epithelial (demarcated by broken lines). See text for precise stages. DRG, dorsal root ganglion; NT, neural tube. Scale bar: 8 µm in A; 15 µm in B; 22 µm in C; 80 µm in D.

longitudinal axis of the embryo. To electroporate the dorsolateral half of the epithelium, the negative L-shape tungsten electrode was placed underneath the blastoderm with the tip just ventral to the medial part of somites and the positive electrode was located in a dorsolateral position with respect to the somites. A square wave electroporator (BTX, San Diego, CA) was used to deliver four pulses of current at 20 V, 20 mseconds each. Embryos were reincubated for 7-8 hours to monitor localization of fluorescent protein following initial expression of the transgene and then reincubated till E4. Embryos were fixed in 4% formaldehyde, processed for paraffin wax embedding, sectioned at 10 µm, and immunolabeled with anti GFP (Burstyn-Cohen and Kalcheim, 2002) and desmin antibodies.

Laser-scanning confocal microscopy

Laser-scanning confocal microscopy and digital imaging were performed as already described (Cinnamon et al., 2001). Frozen sections (50 µm) of embryos whose somites were double-labeled with DiI and DiO were scanned at 2 µm increments through the z-axis and sequential images were collected. The confocal images represent cumulative scans of single sections that include, in each case, all fluorescently stained cells.

Measurement of DM growth by DiI labeling

The localization of dye-labeled cells relative to the mediolateral aspect of the epithelium was measured in epithelial somites (T0) of living embryos shortly after injection. Embryos were further incubated until T2, fixed and sectioned, and the position of dye-labeled cells measured in consecutive serial sections. In each case, the localization of the medial and lateral borders of the dye-labeled domain was monitored. The midpoint between the two values was calculated and expressed as a function of total mediolateral length of the corresponding segment. The localization of the midpoint values at T2 relative to T0 was plotted for each injected somite (see Fig. 5).

Dye labeling combined with in situ hybridization for Sim-1

CM-DiI injections were directed to the lateral domain of epithelial somites (T0) and embryos were further incubated till E4 (T3). The dye-injected embryos were first sectioned, analyzed and

photographed. Sections were then subjected to in situ hybridization as previously described (Sela-Donenfeld and Kalcheim, 1999; Kahane et al., 2001) with a chick-specific Sim-1 probe (Pourquie et al., 1996). The relative position of dye-positive cells with respect to the Sim-1 domain was compared in overlays of corresponding images.

RESULTS

Development of the DM and dermis from epithelial somites was studied in interlimb levels of the axis (somites 21-26). As shown in Fig. 1, consecutive stages of somite development were considered (see the Materials and Methods for details). The epithelial stage was defined as T0 (21-27 somite-old embryos, Fig. 1A). T1 corresponded to early sclerotome dissociation when the pioneer myoblasts first bend underneath the forming DM (32-34 somite-old embryos, Fig. 1B) (Kahane et al., 1998a). T2 corresponded to fully dissociated somites when the myotome is already located underneath the whole mediolateral extent of the DM epithelium (38-40 somite pairs, Fig. 1C). Finally, at T3 (E4), a mesenchymal dermis had dissociated from the DM sheath (Fig. 1D).

Spatially and temporally distinct morphogenetic traits characterize development of the DM

Cell proliferation in the dorsal somite and developing DM

The dorsal aspect of the epithelial somite which corresponds to the prospective DM displays a homogeneous pattern of cell proliferation along its entire mediolateral extent with 45-50% of total nuclei having incorporated BrdU following a 1 hour pulse (Fig. 2A,B,E, upper panel). Notably, in the medial aspect of the somite (Fig. 2A,B, asterisk) a subset of BrdU-negative nuclei is apparent that corresponds to the earliest post-mitotic progenitors that will develop into pioneer myofibers, as previously documented (Kahane et al., 1998a). Changes in the
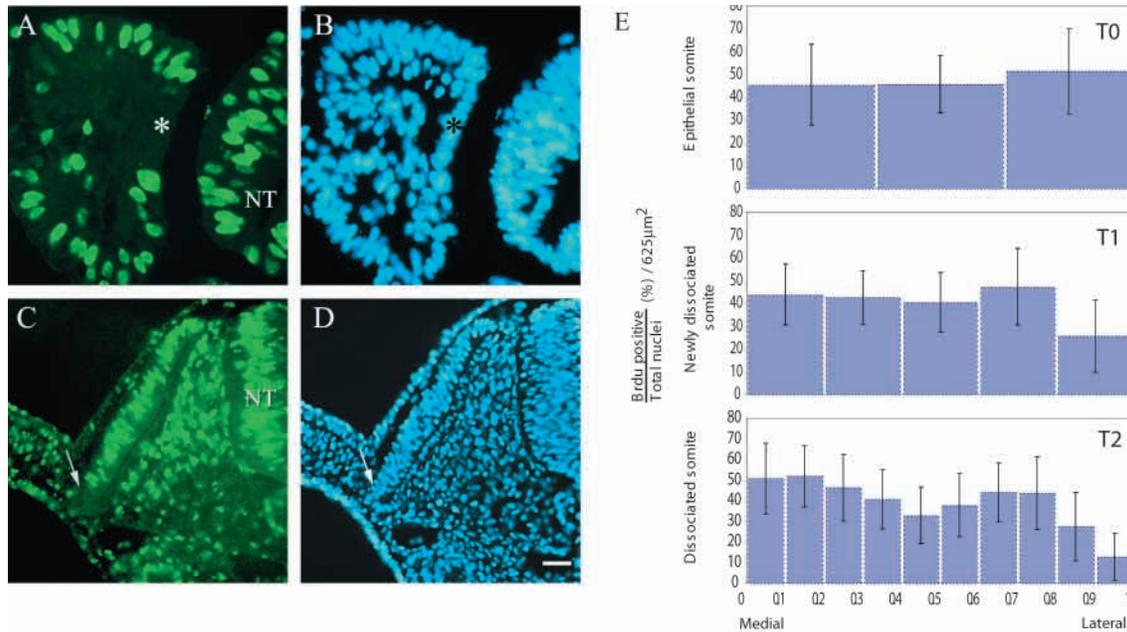
Fig. 2. Cell proliferation in the epithelial somite and developing DM. (A,C) BrdU incorporation into S-phase nuclei following a 1 hour pulse. (A) Note the presence of BrdU-labeled cells throughout the dorsal aspect of the epithelial somite (T0). BrdU-positive figures are restricted to the basal half of the epithelial sheath (compare with B where total nuclei are evidenced by Hoechst staining). A population of medially located epithelial progenitors (*) are BrdU negative, corresponding to prospective pioneer myofibers. (C,D) At T2, BrdU incorporation occurs along the entire mediolateral extent of the DM with an apparent minimum in the ventrolateral region (white arrows indicate Hoechst-positive nuclei in D that are BrdU negative in C). (E) The percentage of BrdU-positive nuclei compared with total nuclei along the mediolateral extent of the dorsal epithelial somite (T0) and developing DM (T1 and T2). Quantifications were performed as described in Materials and Methods. Scale bar: 17 μm in A,B; 22 μm in C,D.

even pattern of proliferation of dorsal cells appeared upon somite dissociation. Quantification of the data revealed that at T1, proliferation along the epithelium remained unchanged including the newly formed DML. An exception to this behavior was the lateral fifth of the DM where an average of 27% of nuclei in the S phase of the cell cycle was measured. This local value further decreased at T2 to 25% in the lateralmost epithelium and attained only 10% in the VLL itself (Fig. 2C,D arrows, and E middle and lower panels). Surprisingly, these low levels of cell proliferation remained at least until E4.5, when the VLL had already entered the somatopleura (not shown). A slightly decreased proportion of BrdU-positive nuclei could also be detected at T2 in the center of the DM when compared with earlier stages (Fig. 2E, lower panel). Taken together, all precursors along the mediolateral DM actively proliferate despite local quantitative differences. Hence, if considering cell proliferation as a major factor driving epithelial growth, the expansion of the DM in the mediolateral direction cannot be accounted for by regionalized proliferative centers restricted to the extreme DML and VLL.

Morphogenetic changes during DM maturation

We next monitored the effect of other cellular parameters on mediolateral growth of the DM. Cell density, the number of nuclear layers per apicobasal thickness of the pseudostratified epithelium and the thickness itself were measured at T0, T1 and T2 along the entire medial to lateral extent of the DM, as described in the Materials and Methods. In the dorsal epithelial somite (T0), comparable values were monitored at all

mediolateral points for the three parameters tested (Fig. 3A,C; data not shown). Upon transition to T1, the lateral fifth of the epithelium underwent a moderate thinning which was reflected both in a decrease in nuclear density and in the number of nuclear layers per epithelial width (Fig. 3C and data not shown). Conversely, the central region of the epithelium enlarged in the apicobasal direction when compared with the corresponding region at T0 (compare red and green bars in Fig. 3C at the central and lateral domains). Between T0 and T1 the mediolateral DM lengthened by 1.8 fold.

The transition between T1 and T2 was characterized by a significant reduction in the thickness of the medial 30-40% of the DM owing to decreased cell density and number of nuclear layers (Fig. 3B,D, compare 3A′ with 3B′, and not shown). This process resembles that observed at T0/T1 for the VLL region but it comprises a significantly larger mediolateral area. Between T1 and T2, the DM epithelium elongated mediolaterally by twofold. Thus, during this period (32ss to 40ss), the medial third of the pseudostratified DM epithelium undergoes a narrowing and extension process by which the magnitude of cellular packing diminishes (Fig. 3A′,B′), yet with no apparent change in cell proliferation (Fig. 2E).

These results show that morphogenetic changes occur everywhere along the growing DM. In the transverse plane, this reorganization defines three distinct and dynamic regions: medial, central and lateral, rather than the two recognized epaxial and hypaxial domains. Moreover, these changes occur with a distinctive timing, comprising two discrete and separable phases, a first phase from T0 to T1 when the lateral
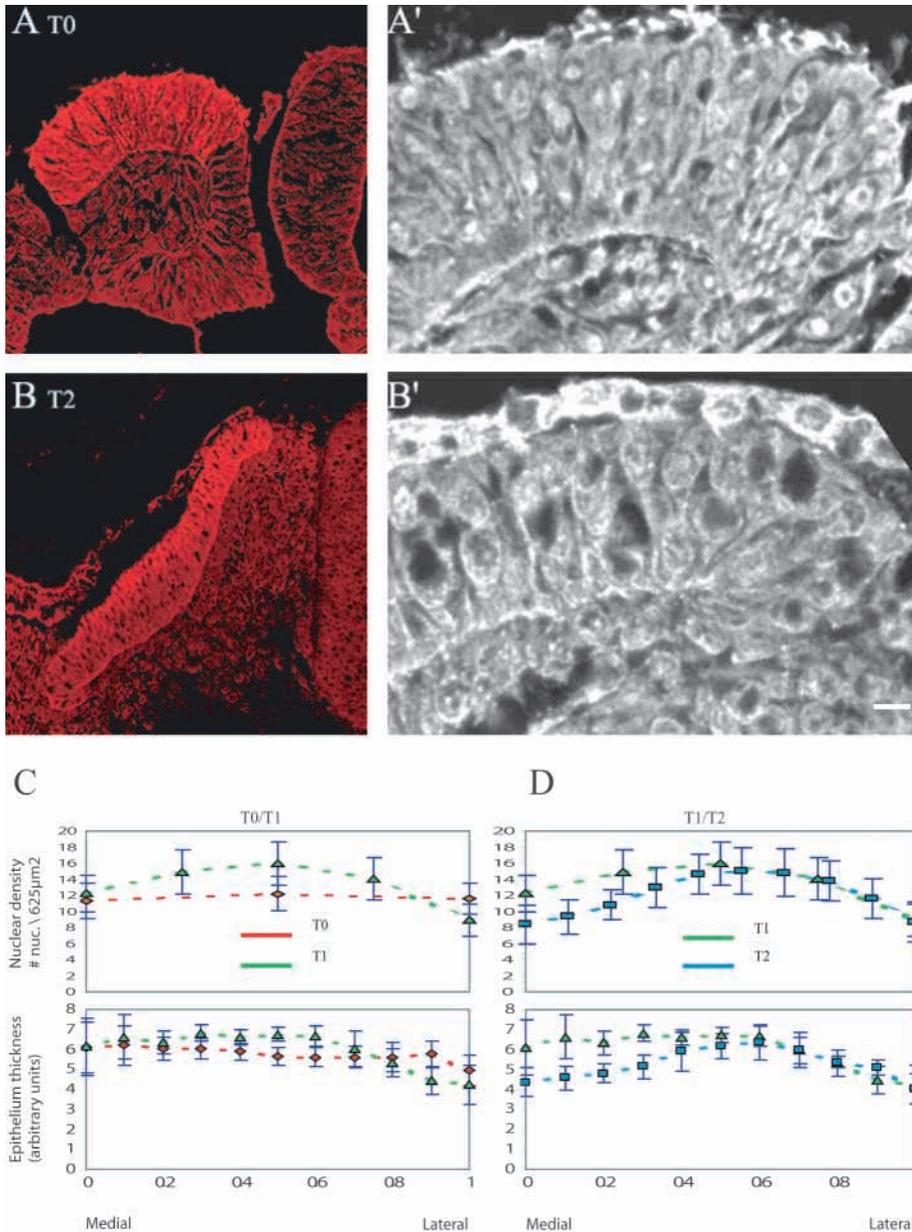
in the DM (n=6, Fig. 4A,B). Likewise, central injections at T0 projected to a central site at T2 (n=7, Fig. 4C,D), and lateral injections projected onto lateral regions of the DM (n=6, Fig. 4E,F), suggesting an overall coherent pattern of DM growth between the two stages considered.

To further examine this question, the projection of the injected midpoints at T2 relative to T0 was plotted as described under Materials and Methods and in Fig. 5A. If growth was proportional, then a similar mediolateral projection of labeled cells is expected at T2 when compared with T0 being graphically represented as a linear correlation between the above values. By contrast, if growth was incremental and solely driven by stem cell systems restricted to the DML and VLL, then cells located at medial positions at T0 would be displaced laterally by T2 and lateral cells would relocate medially, respectively. This would lead to a graphic function that approaches a straight horizontal line. As depicted in Fig.5B, experimental values at T2 occupy similar relative positions along the mediolateral axis as they did at T0 with a deviation not exceeding 15% from an expected linear correlation. Notably, within this range, some of the medial and central injections revealed a slight tendency to shift laterally. This is fully consistent with our observations on the narrowing and extension processes that affect this area relative to the lateral DM. In addition, we observed no significant differences in the relative spreading of injections that included the DML and VLL when compared with more central labeling (data not shown). Thus, a proportional pattern accounts for overall mediolateral growth of the DM inspite of the small local differences monitored in cell proliferation and morphogenetic rearrangements. These results suggest a model of coherent

and central domains change their shape and proliferative behavior, and a second phase from T1 to T2 when the medial third flattens and extends.

The overall mediolateral growth pattern of the DM between T0 and T2 is proportional

To understand how the observed temporally and spatially restricted changes in proliferation and cell rearrangements integrate to affect overall growth of the DM, lineage tracing with CM-DiI was performed. Discrete injections were made at T0 and embryos were reincubated till T2. Fig. 4 shows three representative segments that received the dye at medial, central and lateral positions of the epithelial somite, respectively (Fig. 4A,C,E). Notably, upon DM development, the initial spots of dye-labeled cells spread due to both proliferation and extension movements. Yet, medially directed labeling remained medial
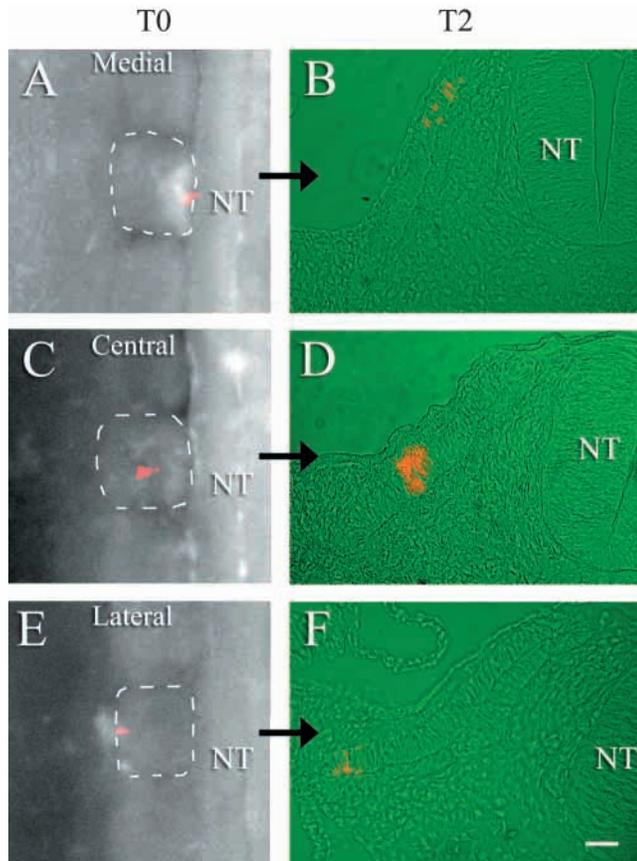
Fig. 4. The position along the DM (T2) of dorsal epithelial somite cells (T0) labeled at distinct mediolateral positions with CM-DiI. (A,C,E) Dorsal view of whole segments labeled at T0 with CM-DiI. Labeling was directed to medial, central or lateral positions of the dorsal somites, respectively. (B,D,F) At T2, the corresponding embryos were sacrificed, serially sectioned and analysed. Representative sections are shown for each type of labeling. Note that the relative mediolateral position of labeled cells at T2 was equivalent to that at T0. NT, neural tube. Scale bar: 25 μm in B,D,F.

mediolateral growth of the DM as opposed to a stem cell view, implying focal and inversely oriented sources of growth restricted to the medial and lateral edges.

The dorsal dermis derives both from medial as well as lateral somite halves

Observation of transverse sections at stages corresponding to dermis formation reveals that dermal cells first dissociate from the center of the DM epithelium and then the process spreads both medially and laterally involving most of the DM, except for the extreme DML and VLL, which remain epithelial for a few additional days. Notably, initial establishment of the subectodermal mesenchyme occurs while the DM epithelium is still largely confined to the epaxial domain, except for the VLL tip which begins extending beyond the ectodermal notch to enter the somatopleura (Fig. 6A-C between arrowheads). In addition, we have shown that the mediolateral extent of the dorsal epithelial somite projects proportionally onto the DM (Figs 4 and 5). As a consequence of the two latter observations, the dorsal dermis would be expected to arise from both medial

as well as lateral halves of the epithelial somite. Yet, based on quail-chick grafts of half-somites of either medial or lateral type, it was suggested that the dorsal dermis derives entirely from the medial but not the lateral half of the somite (Olivera-Martinez et al., 2000). In light of this discrepancy, we reinvestigated the question of the mediolateral origin of the somite-derived dermis.

To this end, lateral halves of somites were labeled with CM-DiI at T0 and embryos were further incubated till T3, when a mesenchymal dermis is already apparent. As shown in Fig. 6D,E, lateral injections performed at flank levels of the axis gave rise to mesenchymal cells localized in a lateral domain of the somite-derived dorsal dermis. This was the case whether injections were directed to locations just lateral to the midline or more laterally approaching the edge of the segment ($n=12$, see also Fig. 7 and data not shown). Likewise, labeling with CM-DiI of the lateral region of epithelial somites at cervical levels also resulted in a lateral mesenchyme containing fluorescent cells (Fig. 6F, $n=5$). To examine this question further using an independent method, a GFP-encoding vector was electroporated into the dorsolateral half of epithelial somites. A few hours after electroporation, GFP-positive cells appeared in the lateral somite confirming the localization of transfections and by E4, the lateral dermis contained fluorescent cells (Fig. 6G,H). This is in addition to the expected labeling of the VLL and consequently, of the lateral myotome. Taken together, results of DiI and GFP lineage tracing demonstrate that the lateral part of the dorsal somite gives rise to the lateral domain of the dorsal dermis.

In order to further examine the relative projection of medial and lateral regions of epithelial somites onto the dorsal dermis, the respective domains were labeled at T0 with both DiI and DiO. Fig. 7A,B shows that cells labeled in each region of the young somite kept a corresponding topographical relationship within the resulting dermis. Furthermore, mesenchymal cells arising in medial as compared with lateral regions of the somite remained topographically segregated from each other, with little or no overlap even if spots were relatively close ($n=4$, Fig. 7A,B, and data not shown). Similar results were obtained when labeling was performed along the DM epithelium at T1 and embryos were further incubated till T3 ($n=3$, Fig. 7C,D). Taken together, our results demonstrate that the dorsal dermis arises from along the entire mediolateral aspect of the dorsal epithelium, and maintains a direct topographical relationship with its epithelial ascendants, at least until T3 (E4).

Dermal cells derived from the lateral half of the somite are included within the Sim-1-expressing domain

The Sim-1 transcription factor was found to be sequentially expressed in the lateral domains of the epithelial somite, then of the dermomyotome and finally of the somite-derived dermis, respectively (Pourquie et al., 1996; Olivera-Martinez et al., 2002). To examine the topographical relationship between dermal cells derived from the lateral region of the epithelial somite vis-à-vis the Sim-1-positive domain of the dermis, the lateral aspect of somites was labeled with CM-DiI at T0 and embryos were further incubated till T3. The localization of DiI-positive cells in the dermis was analyzed first in serial sections, which were subsequently subjected to in situ hybridization as described under Materials and Methods. At T3, Sim-1
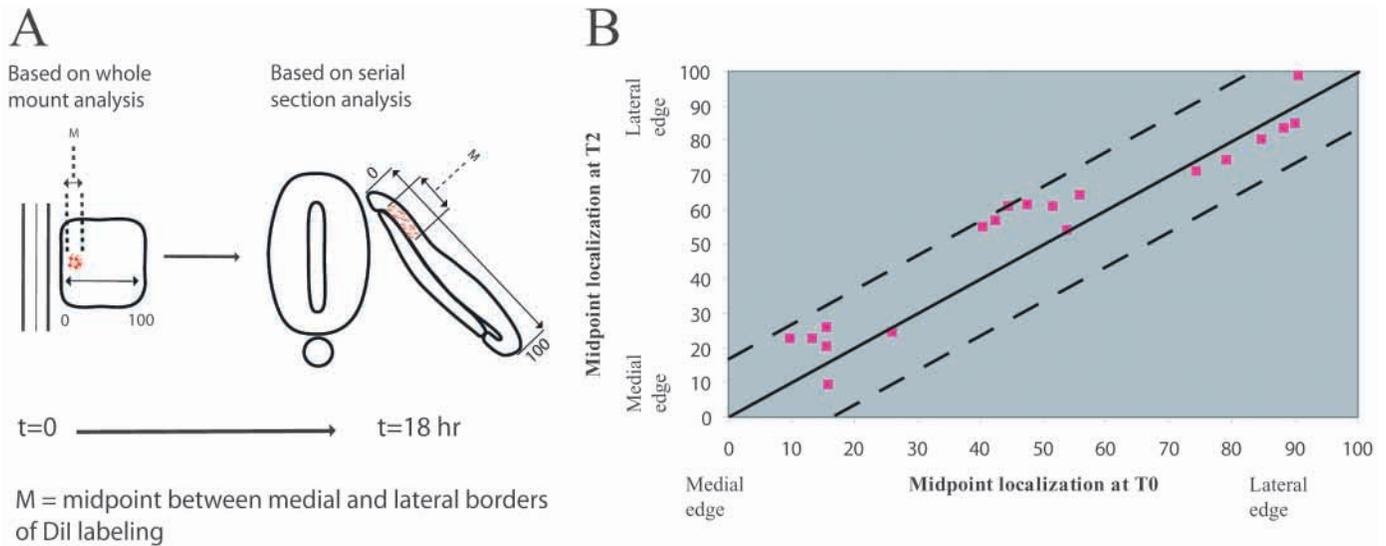
Fig. 5. The overall mediolateral growth pattern of the DM between T0 and T2 is proportional. (A) Scheme illustrating how the positions of dye-labeled cells relative to the mediolateral aspect of the epithelium were measured at T0 and T2 (18 hours). See Materials and Methods for details. (B) The localization of the midpoint values at T2 relative to T0 was plotted for each injected somite. Experimental values at T2 occupy similar relative positions along the mediolateral axis as they did at T0 with a deviation not exceeding 15% (broken lines) from an expected linear correlation (unbroken line).

transcripts were localized to the lateral third of the dermis in addition to the epithelial remnant of the lateral dermomyotome excluding the VLL itself (Fig. 7E,F). At flank levels of the axis, the lateral (or ventral) limit of Sim-1 expression in the dermis corresponded to the ectodermal notch (large arrow), the limit between somite and LPM-derived dermis (Olivera-Martinez et al., 2002; Huang and Christ, 2000; Nowicki et al., 2003). Lateral somitic cells labeled with CM-DiI at T0 developed into dermal cells that were included within the Sim-1-positive territory (Fig. 7G, thin arrows and 7F, *n*=4). Moreover, we also observed CM-DiI-labeled dermal cells localized medial to the Sim-1 domain (Fig. 7F,G arrowhead, and data not shown). The relative abundance of such cells varied according to the mediolateral extent of labeling. These results confirm that the lateral half of epithelial somites gives rise to dermis whose lateralmost cells express the Sim-1 transcription factor. Taken together with our fate mapping experiments revealing a coherent and regionally restricted projection of lateral epithelial somite progenitors onto the corresponding lateral DM and then onto lateral dermal cells, the sequential lateral patterns of Sim-1 expression at corresponding stages indicates that the Sim-1-expressing dermal cells are lineally related with their somitic ascendants.

Origin of the dorsomedial mesenchyme

We next examined the origin of the dorsomedial mesenchyme present between DML and dorsal neural tube. Recent data had suggested that this mesenchyme derives from the DML and is, therefore, of a dermal nature (Olivera-Martinez et al., 2002). Labeling of the medial DM region including the DML at T1 with CM-DiI, gave rise at T3 to fluorescent cells in the myotome, in the dorsal dermis lateral to the DML (arrows in Fig. 8A) and in the DML itself, but not in the dorsomedial mesenchyme (asterisk in Fig. 8A), results which are at variance with the previous suggestion. Therefore, we further explored

this question using quail-chick chimeras. Grafting dorsal halves of quail epithelial somites into the equivalent place of chick counterparts, resulted at E5 in the development of dorsal somite derivatives bearing the quail marker; these included muscle, dorsal dermis and the residual DML. In contrast, the dorsomedial mesenchyme (asterisk in Fig. 8B) was of the host (chick) type like the sclerotome, suggesting it derived from the latter. It was only when whole chick epithelial somites were replaced by their quail counterparts that the dorsomedial mesenchyme was of the quail type (data not shown). These results suggest that the dorsomedial mesenchyme derives from the sclerotome rather than from the DML. To directly examine this issue, CM-DiI was directly applied to the nascent sclerotome as described under Material and Methods. At E5, labeled cells were found both in the sclerotome as well as in the dorsomedial mesenchyme (*n*=4, Fig. 8C) confirming their sclerotomal derivation.

DISCUSSION

Regional traits characterize DM formation and growth

In contrast to the homogeneous pattern of cell behavior apparent in the dorsal epithelial somite, growth of the DM at interlimb levels of the axis is modulated by at least three processes, contribution to the developing myotome, differential cell proliferation, and oriented cell rearrangements involving apicobasal narrowing followed by mediolateral extension. During the first phase between T0 and T1, changes from the homogeneous behavior of the epithelial somite occur in the central and lateral regions of the DM, while during the second phase between T1 and T2 they mainly affect the medial 30-40% of the epithelium. This dynamics defines three contiguous regions with unique properties along the mediolateral aspect of
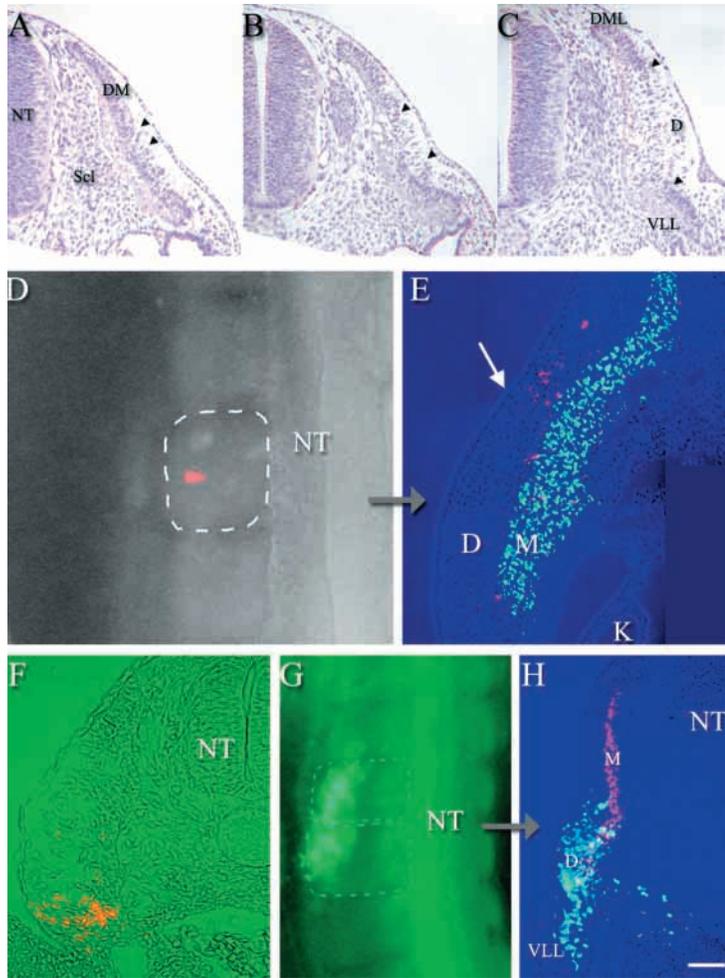
Fig. 6. The dynamics of dissociation of DM into dermis and the contribution of the lateral half of the dorsal somite to dermis formation. (A-C) Hematoxylin-stained sections illustrate successive stages of dermis dissociation from the DM. At flank levels of the axis, dissociation begins by E3 from the central region of the epithelium (A) and progresses medially and laterally (B) until E4, when only the DML and VLL remain epithelial (C). (D) Dorsal view of a living embryo fragment shortly after CM-DiI labeling within the lateral half of a flank-level epithelial somite. (E) The same embryo was further incubated until T3 (E4), when a mesenchymal dermis (D) is already apparent. Representative transverse section shows that lateral injection gave rise to mesenchymal cells localized in a lateral domain of the dorsal dermis (red cells). The arrow indicates the border between somite and lateral plate mesoderm-derived dermis. Because the ectodermal indentation in this section is not significant, the precise localization of the border was determined from adjacent sections. At this stage, the myotome (green as revealed by desmin immunostaining) has already invaded the somatopleura. (F) DiI labeling of the dorsolateral portion of an epithelial somite performed at a cervical level of the axis (somite 10) and fixed at E4.5. Note labeling of the lateralmost dermis located dorsal to the ectodermal notch. Note as well in E and F the presence of CM-DiI+ cells within the desmin-immunoreactive myotome (M, see text for details). (G,H) A GFP-encoding vector was electroporated into the dorsolateral portion of an epithelial somite. Seven hours after transfection, GFP+ cells are apparent in the lateral domain of two successive segments (G). (H) At E4, GFP-positive cells (green) are encountered in the lateral domain of the dorsal dermis, in the VLL and in the lateral part of the desmin-positive myotome (red). Note that the VLL and accompanying myotome have entered the somatopleura, and the lateral dermis containing GFP+ cells remains dorsal, distinctly apart from the somatopleural-derived mesenchyme. Note as well the presence of a few fluorescent cells in the ventralmost sclerotome, likely to derive from transfection of the appropriate progenitors located slightly ventral to the dorsolateral epithelium fated to become DM. D, dermis, DML, dorsomedial lip, K, kidney primordium, NT, neural tube, Scl, sclerotome, VLL, ventrolateral lip. Scale bar: 45 μm in A-C; 30 μm in E; 45 μm in F; 70 μm in H.

the DM (Fig. 9). The distinctive spatiotemporal behavior of these cells further stresses the notion that changes in each domain (medial, central and lateral) are triggered by different signals from the adjacent tissues, which also induce regional molecular traits (reviewed by Sporle, 2001).

Thinning of the ventrolateral domain of the epithelium which is accompanied by a local decrease in cell proliferation is temporally correlated with initial formation of the sclerotome the delamination of which begins precisely in the ventrolateral region of the somite apposed to the prospective VLL of the DM. It remains unknown whether these focal changes are in any way related to the segregation between prospective sclerotome and DM. Notably, in the medial domain of the somite, it is the bending of the epithelial pioneer myoblasts, another cell subset revealing a minimum in DNA synthesis (Kahane et al., 1998a), that defines the separation between ventral sclerotome and the nascent myotome. This minimum in DNA synthesis measured in the VLL persists at least until E4.5 when the growing ventrolateral myotome accompanied by the VLL have already colonized the somatopleura to give rise to intercostal muscles (Christ et al., 1983; Cinnamon et al., 1999). Hence, ventral growth of the DM

and myotome into the somatopleura cannot be solely driven by proliferation of the VLL. Instead, it is likely that the overall growth of the DM and myotome in the mediolateral direction contribute to colonization of the body wall.

In contrast to thinning of the medial and lateral domains, a condensation of epithelial cells is apparent in the central domain of the DM, which causes a local thickening of the structure. As this area is the first to dissociate into dermis, it is tempting to speculate that this process forecasts subsequent cell mesenchymalization. Furthermore, at variance with previous findings suggesting that the central domain of the DM is quiescent relative to the extreme regions (Denetclaw and Ordahl, 2000), direct measurements of cell proliferation and lineage tracing here presented show the dynamic nature of this epithelial subdomain. Along the same line, it has been proposed that the myotome subjacent the central region of the DM contains the oldest fibers and remains static; growth being driven by incremental cell addition lateral and medial to this region (Denetclaw and Ordahl, 2000). Our previous studies have documented instead, that the myotome underlying the central DM region equally expands when compared with adjacent regions by continuous addition of myofibers from the
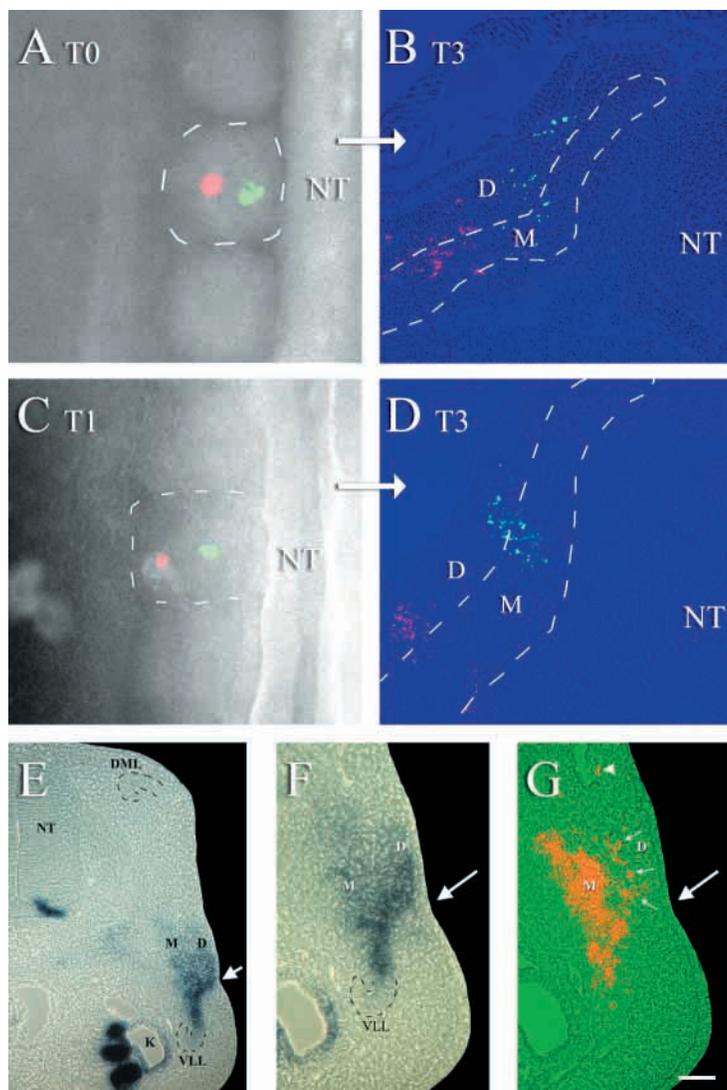
Fig. 7. The dermis arises from the entire mediolateral extent of the dorsal epithelium in a topographically restricted manner. Double DiI/DiO injections to lateral and medial regions of segments, respectively. (A) Dorsal view of a living embryo shortly after labeling at T0. (C) Dorsal view of a living embryo shortly after labeling at T1. Embryos shown in A and C were further incubated until T3 (E4) and cryosectioned. (B,D) Representative transverse sections of A and C, respectively, showing that medial as well as lateral injections contributed to dermal cells localized at medial and lateral positions. Also note at T3 the presence of DiI/DiO-positive cells within the myotome (M, see text for details). (E-G) Dermal cells derived from the lateral somite are included within the Sim-1-expressing domain of the dermis. (E) Expression of Sim-1 mRNA at E4 (T3) in the lateral third of the dermis (D) and myotome (M), the lateral aspect of the dermomyotome excluding the VLL itself (see also F), the kidney primordium and a band of cells in the ventral neural tube lateral to the floor plate. (F,G) Higher magnification of the same section showing that CM-DiI-labeled dermal cells (G, small arrows), which developed from injections in the lateral region of the epithelial somite, are included within the Sim-1-expressing domain of the dermis (F). Note as well the presence of additional labeled cells in the dermis localized dorsomedial to the Sim-1 territory (arrowhead in G). Note that the ectodermal notch (large arrow) corresponds to the limit between somite and LPM-derived dermis as reflected both by the ventral limit of Sim-1 expression within the dermis and by the ventral border of CM-DiI-labeled dermal cells. As expected, lateral injections of CM-DiI in the somite, which comprises the VLL, also give rise to labeled cells in the myotome (M). DML, dorsomedial lip; No, notochord; NT, neural tube; VLL, ventrolateral lip. Scale bar: 50 μm in B; 35 μm in D; 68 μm in E; 38 μm in F,G.

rostral and caudal lips of the DM among pre-existing pioneers, hence containing both old and newly added cells altogether (Kahane et al., 2002).

A coherent pattern of mediolateral development of all dorsal derivatives of the somite

In previous studies, we have found that growth of the myotome in both mediolateral and transverse planes is accounted for by addition of precursors that emanate from all four lips of the overlying DM. The latter progressively intercalate among the pioneer fibers, that originated earlier in the medial epithelial somite (Kahane et al., 1998a; Kahane et al., 1998b; Kahane et al., 2001; Cinnamon et al., 1999; Cinnamon et al., 2001). The integration between these successive waves of myogenic precursors results in an even and proportional pattern of mediolateral growth of the developing myotome (Kahane et al., 2002).

Likewise, direct tracing of the projection of dorsal epithelial somite precursors onto the mature DM revealed an overall pattern of coherent and proportional expansion of this structure along the mediolateral extent. These results are consistent with the outcome predicted from calculating the pattern of DM

growth between T0 and T2 based solely on the measured morphogenetic changes (R.B.-Y. and C.K., unpublished). Hence, the present results further strengthen the topographical association between DM and myotome, suggesting that cells directly translocate from the DM into the corresponding region of the myotome (Fig. 9). Our results are consistent with those of Eloy-Trinquet and Nicolas (Eloy-Trinquet and Nicolas, 2002) who reported, based on clonal analysis in the mouse, on a direct relationship between myotome precursors in the DM and their daughter cells in the myotome. This view therefore refutes the alternative model which suggests that two opposite stem cell systems located in the DML and VLL drive incremental growth. The latter model does not consider the contributions of similar stem cells localized in the extreme rostral and caudal lips of the DM that contribute both to DM and myotome growth and of those progenitors located within the entire DM sheath that actively proliferate and contribute, at least, to continuous expansion of the DM and to subsequent formation of the dermis (Fig. 9).

Consistent with the coherent mediolateral growth of the myotome and DM, the present data using lineage tracing and GFP electroporation of intact segments, demonstrate that the
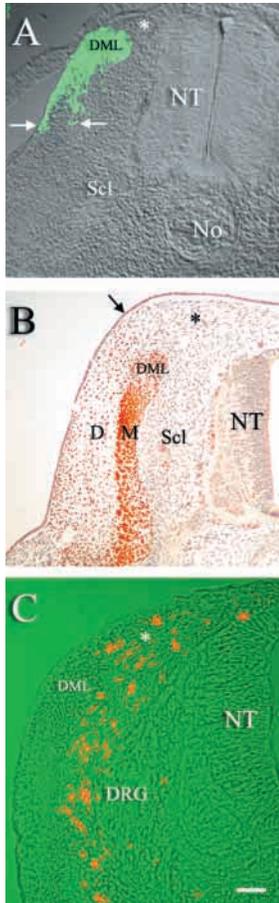
Fig. 8. Sclerotomal origin of the dorsomedial mesenchyme. (A,B) The dorsomedial mesenchyme between DML and dorsal neural tube is not of dermomyotomal origin. (A) Labeling of the medial DM region including the DML at T1 with CM-DiI, gave rise at T3 to fluorescent cells in the myotome, in the dorsal dermis lateral to the DML (arrows) and in the DML itself, but not in the dorsomedial mesenchyme (*). (B) The dorsal half of a quail epithelial somite was grafted into the equivalent place of a chick counterpart at E2. At E5, dorsal somite derivatives reveal the quail marker including muscle (M), dorsal dermis (D) and the residual DML. By contrast, the dorsomedial mesenchyme (*) was of the host (chick) type like the sclerotome (Scl). (C) CM-DiI labeling of the sclerotome at T1 gave rise at T3 to fluorescent cells in the dorsomedial mesenchyme (*). DRG, dorsal root ganglion. Scale bar: 50 μm in A,B; 35 μm in C.

dermis originates from both medial as well as lateral domains of the dorsal epithelial somite which, at an intermediate stage, project into corresponding regions in the DM. In line with our results, a previous study has found that both medial as well as lateral half-somites have the potential to contribute to dermis under experimental conditions (Houzelstein et al., 2000). Our results, however, contrast with previous data that proposed that the dorsal dermis derives entirely from the medial but not the lateral half of the somite (Olivera-Martinez et al., 2000). The latter study was based on quail-chick grafts of half-somites of either medial or lateral type. Studies performed in our laboratory (N.K. and C.K., unpublished) revealed that under such conditions, many grafts resulted in formation of two half-somites that did not fuse and remained separated by adjacent lips (see also Olivera-Martinez et al., 2000). At later stages, we observed that the grafted quail moieties sometimes took over the formation of most of the myotome and dermis (Olivera-Martinez et al., 2000), regardless of their origin as medial or lateral and only when keeping their original proportions was the dermis composed of both quail and chick cells. This variability in the experimental outcomes suggested that the problem of the precise mediolateral origin of the dermis had to be re-examined in intact embryos.

Notably, flank-level DM was also shown to participate to the formation of the scapular blade in avian embryos (Huang et al., 2000a). Although most likely, cells emanating from intermediate and lateral regions of the DM are at the origin of this ossifying structure, the precise mediolateral origin of the

scapular blade progenitors was not determined. It is therefore possible that lateral DM-derived mesenchyme also contributes to the scapular blade. Yet, as labeling of the dorsolateral parts of cervical somites, which do not form cartilaginous structures, also led to the appearance of labeled cells in the subectodermal mesenchyme, we maintain that lateral somite cells are destined to develop into the lateral domain of the dorsal dermis.

Taken together, lineage analysis of the formation of the myotome, DM and dermis consistently suggests that the development of all dorsal derivatives of the somite follows a coherent and continuous mediolateral pattern in which there is, on the one hand, a direct relationship between the epithelial progenitors in the DM and their ascendants in the dorsal somite, and, on the other hand, with their corresponding progeny in the myotome and dermis (Fig. 9). The only exception so far characterized is the development of the pioneer myoblasts which originate in the medial domain of the epithelial somite but upon differentiation into myofibers end up spanning the entire mediolateral extent of the segment (Kahane et al., 1998a). Consequently, this view refutes previously accepted models sustaining that the medial but not the lateral half of the somite gives rise to the whole repertoire of epaxial derivatives up to the lateral somitic boundary separating the somite from LPM derivatives.

Fate of distinct areas of the DM

Progenitors localized in the DML and VLL (Denetclaw et al., 2001; Venters and Ordahl, 2002) as well as in the rostral and caudal lips (our results) give rise to both DM and myoblast descendants. Yet, recent data suggested that the DML also gives rise to the dorsomedial mesenchyme that is apposed to the neural tube at E4-4.5 of avian development (Olivera-Martinez et al., 2002). Based on this putative DML origin and on the expression of the Wnt11 gene, this mesenchyme was interpreted to become dermis. Although it is tempting to speculate that expression of Wnt11 in both the DML and in this mesenchyme reflects a direct lineage relationship between the two, our present DiI labeling results clearly show that the DML does not participate in the formation of the dorsomedial mesenchyme localized between the DML and dorsal neural tube. Consistent with this observation, interspecies chimeras in which the dorsal half of a chick somite was replaced by its quail counterpart, thus giving rise to a DM (including the DML) composed of quail cells and a sclerotome with chick nuclei, revealed that the dorsomedial mesenchyme was composed of chick nuclei. Our results thus confirm previous data based on quail/chick analysis (Huang and Christ, 2000; Christ et al., 2000). It was only when sclerotomal cells were of quail type (Huang et al., 2000b) or when sclerotomal cells were directly labeled with DiI (our results) that the dorsomedial mesenchyme was positively labeled with quail nuclei or DiI, respectively. Hence, the dorsomedial mesenchyme derives from sclerotome and not from DML. Consequently, this mesenchyme develops into a vertebral fate, perhaps as part of the vertebral arch, rather than into dermis. In line with such a possibility, this mesenchyme was shown to participate in the formation of the spinous process of the vertebrae under the influence of BMP4 (Takahashi et al., 1992; Monsoro-Burq et al., 1996; Watanabe et al., 1998). The transcription factor Msx1, a target of BMP4 signaling, is indeed expressed in the dorsomedial mesenchyme
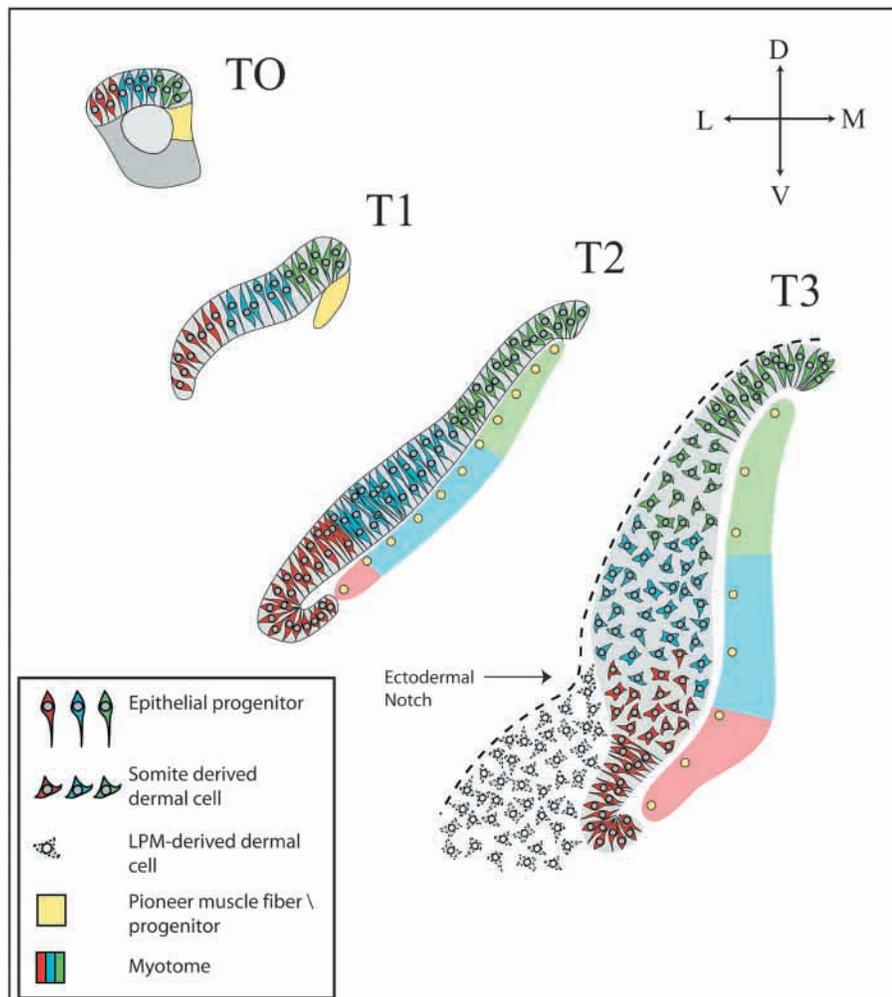
Fig. 9. Coherent development of the DM epithelium followed by the myotome and dermis, from the entire mediolateral aspect of the dorsal somite. The entire mediolateral aspect of the dorsal epithelial somite (T0) proportionally grows to give rise to the DM epithelium (T1 and T2). Despite local differences in morphogenetic traits, active growth occurs in at least three consecutive domains (medial, central and lateral as illustrated by the green, blue and red colors, respectively). Further dissociation of the DM into dermis (T3) also comprises a contribution of epithelial precursors from all three domains. This coherent growth pattern also applies for the development of the myotome, which occurs by progressive intercalation of fibers along the mediolateral aspect of the segment, as they originate in all four DM lips and contribute fibers in a regionalized manner (see text for references). An exception to this uniform and regionalized contributions are the pioneer myofibers (yellow), which (despite of originating at the medial epithelial somite) finally span the entire mediolateral extent of the growing myotome (yellow circles). Initial dermis dissociation from the entire mediolateral DM already occurs, while the latter is largely confined to the epaxial domain. During later development, while the expanding myotome and accompanying VLL colonize the somatopleura to give rise to hypaxial muscles (intercostal and abdominal), the somite-derived dorsal dermis remains restricted to the epaxial domain. LPM, lateral plate mesoderm.

(Houzelstein et al., 2000). Using mouse-chick chimeras in which an *nlacZ* reporter gene was integrated into the mouse Msx1 locus, Houzelstein et al. (Houzelstein et al., 2000) have confirmed that the dorsomedial mesenchyme expressing Msx1 indeed derives from the somite. However, as grafts were of whole somites it was not possible in this study to discriminate between a sclerotomal versus DM origin.

What is then the origin of the dorsomedial dermis forming the dorsal pterylae? This dermis, which develops only at later stages (after E7) is likely to arise from secondary rearrangements of dermal cells that derived initially from the medial DM region and subsequently relocate towards the dorsal midline of the embryo, in a similar way to which sclerotomal cells migrate dorsomedially to form the neural arch.

Our results therefore suggest that the somite-derived dermis arises from the entire DM sheath, including medial and lateral domains. Each region of the DM originates, in turn, at corresponding regions in the epithelial somite (Fig. 9). Consistent with our fate-mapping experiments is the conserved lateral expression of the Sim-1 transcription factor at these three consecutive stages (Fig. 7) (Pourquie et al., 1996; Olivera-Martinez et al., 2002). Surprisingly, we have observed that labeling of the central domain of the dorsal epithelial somite or of the DM sheath also gives rise to cells that colonize the myotome and appear mesenchymal rather than fibers (see for example Fig. 6E,F and Fig. 7B,D). These cells cannot result from accidental labeling of the myotome as no myotome is present at the epithelial somite stage. Moreover, if myotome labeling occurred in injections performed at T1, then these cells would have been detected in segments fixed at T2. However, they become apparent only at E4-4.5 (T3 and older). Therefore, epithelial progenitors from the non-lip regions of the DM may be late contributors of the third wave category of mitotically active progenitors that initially invade the myotome from the rostral and caudal lips (Kahane et al., 2001). The properties and fate of these cells are now under investigation. Hence, the possibility remains open that by the time of delamination, the DM epithelium produces mesenchymal cells which migrate bidirectionally; both superficially to colonize the subectodermal space and give rise to dermis, and also towards the myotome to further contribute to its growth.

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