LGN-dependent orientation of cell divisions in the dermomyotome controls lineage segregation into muscle and dermis

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SUMMARY

The plane of cell divisions is pivotal for differential fate acquisition. Dermomyotome development provides an excellent system with which to investigate the link between these processes. In the central sheet of the early dermomyotome, single epithelial cells divide with a planar orientation. Here, we report that in the avian embryo, in addition to self-renewing, a subset of progenitors translocates into the myotome where they generate differentiated myocytes. By contrast, in the late epithelium, individual progenitors divide perpendicularly to produce both mitotic myoblasts and dermis. To examine whether spindle orientations influence fate segregation, early planar divisions were randomized and/or shifted to a perpendicular orientation by interfering with LGN function or by overexpressing inscuteable. Clones derived from single transfected cells exhibited an enhanced proportion of mixed dermomyotome/myotome progeny at the expense of 'like' daughter cells in either domain. Loss of LGN or Gαi1 function in the late epithelium randomized otherwise perpendicular mitoses and favored muscle development at the expense of dermis. Hence, LGN-dependent early planar divisions are required for the proper allocation of progenitors into either dermomyotome or myotome, whereas late perpendicular divisions are necessary for the normal balance between muscle and dermis production.

KEY WORDS: Apicobasal polarity, aPKC, Avian embryo, Dermis, Desmin, Epithelium, Lineage segregation, Myotome, Somite, ZO-1, Quail, Chick

INTRODUCTION

The dermomyotome (DM) is a transient epithelium formed during somite development that gives rise to myofibers, mitotic muscle progenitors, dorsal dermis, and also the smooth muscle and endothelium of adjacent blood vessels. Lineage analysis has shown that segregation into the above lineages occurs in a spatiotemporally regulated fashion (Ben-Yair and Kalcheim, 2008; Buckingham and Montarras, 2008; Buckingham and Vincent, 2009; Kalcheim and Ben-Yair, 2005; Kalcheim et al., 2006; Scaal and Christ, 2004). Therefore, the DM is an attractive model with which to study cell fate decisions operating in an epithelium.

The DM is composed of a central sheet limited by four lips. Only the lips have been shown to generate fully elongated myocytes or myofibers (Cinnamon et al., 2006; Cinnamon et al., 1999; Gros et al., 2004; Huang and Christ, 2000; Kahane et al., 1998b; Kahane et al., 2002) that intercalate among a scaffold of earlier pioneer myocytes and depend on its integrity for proper patterning (Kahane et al., 2007; Kahane et al., 1998a). The central sheet dissociates, producing dermis and Pax3/7-positive muscle progenitors that remain mitotically active within the myotome (Ben-Yair and Kalcheim, 2005; Kahane et al., 2001); later, these progenitors develop into fibers or satellite cells (Gros et al., 2005; Kassar-Duchosoy et al., 2005; Relaix et al., 2005). Importantly, both mitotic myoblasts and dermis originate from single DM cells (Ben-Yair et al., 2003; Ben-Yair and Kalcheim, 2005). The

separation of these two lineages is correlated with a striking shift in the plane of epithelial cell division from an initial planar orientation into a perpendicular orientation prior to cell dissociation, but it remains unclear whether this shift plays a role in lineage segregation. Since this shift is coupled to the asymmetric segregation of N-cadherin to the apical but not to the basal daughter cell, it could lead to differential fate acquisition (Ben-Yair and Kalcheim, 2005; Cinnamon et al., 2006).

Spindle orientations during mitosis play an important role in progenitor self-renewal, as opposed to the generation of differentiated progeny (Doe, 2008; Siller and Doe, 2009; Yu et al., 2006). Studies in various species have documented the function of G-protein regulators in controlling spindle positioning (Bellaiche and Gotta, 2005; Morin et al., 2007; Schaefer et al., 2000; Yu et al., 2000). For example, Leu-Gly-Asn repeat-enriched protein (LGN) [also known as G-protein signaling modulator 2 (Gpsm2)] is the vertebrate homolog of *Drosophila* Partner of inscuteable (Pins; Raps – FlyBase), which is essential for spindle positioning by linking the cell cortex with the mitotic spindle (Du and Macara, 2004; Du et al., 2001; Gotta et al., 2003; Sanada and Tsai, 2005; Siller et al., 2006). The C-terminal GoLoco domain of LGN binds a unique form of cortical Gαi (also known as Gnai1), whereas its N-terminus binds to NuMA/Mud, which in turn associates with spindle components (Blumer et al., 2006; Du and Macara, 2004; Du et al., 2001; Izumi et al., 2006; Siller et al., 2006). Binding of Gαi and NuMA/Mud to LGN/Pins promotes cortical localization of LGN/Pins and reorients the mitotic spindle (Nipper et al., 2007). LGN/Pins homologs maintain planar cell divisions in the epithelium, and cells lacking LGN function exhibit spindle randomization, leading to abnormal fates and mislocalized daughter cells (Konno et al., 2008; Morin et al., 2007; Sanada and Tsai, 2005). Inscuteable (Insc) has been shown to link Pins with the apically localized Par-3 (Bazooka)/Par-6/aPKC

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complex (Schaefer et al., 2000; Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000), hence directing cell divisions into a perpendicular orientation (Konno et al., 2008; Kraut and Campos-Ortega, 1996; Zigman et al., 2005).

Here, we explored the significance of the plane of cell divisions in cell localization and fate segregation in the DM. We find that whereas the mature DM produces mitotic myoblasts and dermis following perpendicular divisions, single progenitors in the early central sheet, which mostly divide with a planar orientation, either self-renew or generate unit-length myocytes, a previously unknown fate of the young epithelium. Inhibition of LGN activity or gain of Insc function performed at both early and late DM stages altered the normal orientation of mitoses. At the early stage they caused a striking increase in divisions, producing both myotomal and DM progenitors at the expense of 'like' divisions. At a later stage, in which most divisions were perpendicularly oriented, interfering with LGN or Gαi1 activity enhanced muscle at the expense of dermis development. These results suggest that LGN-dependent planar cell divisions in the early DM sheet are required for the maintenance of symmetric divisions that allocate progenitors to either DM (self-renewing progenitors) or myotome (terminal mitoses). Furthermore, the normal 90° shift in the plane of cell division prior to epithelial dissociation is essential for generating a balance between muscle and dermal fates. Our data underscore, for the first time, the significance of the orientation of cell divisions in fate acquisition in the DM epithelium.

MATERIALS AND METHODS

Embryos
Fertile quail (*Coturnix coturnix Japonica*) and chick (*Gallus gallus*) eggs from commercial sources (Moshav Orot and Moshav Mata) 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).

Electroporation and single-cell transfections
The flank somites/DM of E2.0 (HH15) to E3.0 (HH18) embryos were injected with concentrated DNA (~4 μg/μl) encoding the following constructs: pCAGGS-IRES-GFP (from A. Klar, Hebrew University, Jerusalem, Israel); YFP-C1-Lyn (from T. Meyer, Stanford University, CA, USA); pCAGGS-IRES-Ct-cLGN, pCAGGS-hLGN, pCAGGS-IRES-mInsc and pCAGGS-Venus-Gαi1 (see cloning details below). For electroporation, the negative L-shaped tungsten electrode was placed underneath the blastoderm with the tip positioned ventral to the central part of the DM and the positive electrode located in a dorsal position with respect to the DM. A square wave electroporator (BTX, San Diego, CA, USA) was used to deliver a single pulse at 20 volts for 10 milliseconds. Embryos were re-incubated for 16-45 hours.

Single-cell transfections were carried out as previously described (Ben-Yair and Kalcheim, 2005). Cells in the central domain of the dorsal epithelial somite were injected with either pCAGGS-IRES-GFP, pCAGGS-IRES-Ct-cLGN or pCAGGS-IRES-mInsc (~0.5 μg/μl). The success rate of injections was 10.3±0.15% for all constructs. Embryos were further incubated for 24 or 45 hours, fixed and stained for Pax7, desmin and GFP (see below).

In the two paradigms used, only embryos with labeled cells in the central portion of the DM were considered for further analysis, unless otherwise noted.

Electroporation of antisense morpholinos
A fluorescein-tagged morpholino (MO) for chicken LGN knockdown was designed to target the first intron splice site according to the manufacturer's instructions (Gene Tools). The antisense sequence was as follows: 5′-ACTAAAATTCATCATCTTACCTGTA-3′. The working concentration of LGN-MO and of a standard control-MO was 2 mM. Morpholinos were injected directly into the dorsal epithelium prior to application of the electric pulse.

Cloning of ct-LGN, hLGN, Gαi1 and mInsc
The C-terminus of chicken LGN (cLGN) was PCR amplified from cDNA (forward, 5′-GCGAATTCAGTAGGACGTCGTCACAGTATGGAG-3′; reverse, 5′-GCCTCGAGTCAGCTAGAACTTGGTCCTTTAATAGCAG-3′) and then subcloned into the pCAGGS-IRES-GFP backbone to generate pCAGGS-Ct-cLGN-IRES-GFP. Full-length human LGN (hLGN) was from L. Y. Jan (Wiser et al., 2006). pCAGGS-mInsc-IRES-GFP was generated from pCAGGS-HA-mInsc by subcloning mouse Insc (mInsc) into the same backbone (Konno et al., 2008). pCAGGS-Venus-Gαi1 was generated by subcloning Venus-Gαi1 from pK-Venus-Gαi1 (Zheng et al., 2010) into pCAGGS.

Embryo processing and sectioning
Embryos were fixed with 4% formaldehyde or with Fornoy (for in situ hybridization on sections) and processed for paraffin wax embedding as previously described (Cinnamon et al., 1999). For LGN immunostaining, embryos were fixed for 30 minutes in 1% TCA at 4°C. Serial 10-μm transverse sections were mounted on Superfrost/Plus slides (Menzel Glaser, Braunschweig). Immunostaining for Pax7 and MyoD was performed on whole embryos, as previously described (Ben-Yair and Kalcheim, 2005).

In situ hybridization
Embryos were subjected to in situ hybridization on sections (Kahane et al., 2001a) with avian-specific probes for *Alx4* (Cinnamon et al., 2006), *Dermo1* (Scaal et al., 2001) or *Lgn*. The *Lgn* probe was prepared by subcloning a 500 bp *Eco*RI-*Sac*I fragment of ct-cLGN into the pBlueScript SKII vector.

Immunohistochemistry
Immunostaining was carried out with monoclonal antibodies to desmin (ICN# 10519, 1:100), GFP (Molecular Probes, 1:200), Pax7 and myosin (MF20) (Developmental Studies Hybridoma Bank, 1:10), and with polyclonal antibodies to ZO-1 (Zymed 40-2200, 1:100), aPKC (Santa Cruz sc-216, 1:60), laminin (Sigma L9393, 1:100), LGN (Sigma HPA007327, 1:100), MyoD [from B. Paterson (NIH, Bethesda, MD, USA), 1:1000], and γ-tubulin (Sigma T3559, 1:500). Secondary antibodies were coupled to Cy2, Cy3 or Cy5 (Jackson ImmunoResearch, 1:200). Mounting medium was from DakoCytomation.

Data analysis
Determination of the plane of cell divisions was performed 16 hours after electroporation as described (Ben-Yair and Kalcheim, 2005). Briefly, embryos were immunolabeled with γ-tubulin antibodies to label the centrosomes and further processed for paraffin wax embedding. Serial sections were subjected to Hoechst nuclear staining. Individual mitoses were identified and respective γ-tubulin and Hoechst stainings separately photographed with a ×100 objective. Since the two centrosomes belonging to individual mitoses were sometimes found at slightly different focal depths, each mitosis was documented by capturing two to three 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 Photoshop 7 (Adobe) (see Fig. S1 in the supplementary material).

Clones containing GFP^{+} cells were analyzed in serial sections as previously detailed (Ben-Yair and Kalcheim, 2005). Labeled cells were identified as myotomal, DM epithelial, intermediate domain (ID) or dermal by their position and by expression of specific markers such as desmin, *Alx4*, Pax7 and *Dermo1* (see Results). Clonal composition in the different treatments was defined as the proportion of clones containing specific cell types among total clones. In addition, the distribution of labeled cells per domain was calculated as a function of the total labeled cells monitored in all clones that received a given treatment.

Images were taken with a BX51 microscope (Olympus) with Uplan FL-N 10×/0.30, 20×/0.5 and 40×/0.75 dry objectives (Olympus) at room temperature using DP controller v1.2.1.108 acquisition software (Olympus) coupled to a DP70 (Olympus) cooled CCD digital camera. Confocal scanning was performed using an Olympus Fluoview FV1000 with 40×/0.9 dry and 100×/1.35 oil objectives, software version 1.7c.

Samples were optically screened at 0.6-1 µm increments through the *z*-axis. For figure preparation, images were exported into Photoshop CS2 (Adobe). If necessary, the brightness and contrast were adjusted for the entire image and images were cropped without color correction adjustments or γ adjustments. Final figures were prepared using Photoshop CS2.

Statistical analysis

The association between two categorical values (clone composition or cell distribution per experimental group) was analyzed using Fisher's exact test. Comparison of angles of cell division between the groups was performed by applying the ANOVA test (using the Brown-Forsythe correction for unequal variances) with the Dunnett-T3 multiple comparison post-hoc test. In addition, the above variable was analyzed using the non-parametric Kruskal-Wallis test. All tests applied were two-tailed with $P \leq 0.05$.

RESULTS

The central sheet of the early DM is a source of differentiated myocytes

Sequential production of myocytes followed by muscle progenitors from the central DM

Single progenitors in the central portion of epithelial somites or of the nascent DM were injected with pCAGGS-GFP. Six hours later, the central location of the labeling was confirmed (Fig. 1A,C), consistent with previous findings (Ben-Yair et al., 2003). Thirty-six hours following the early E2 injections, GFP$^+$/desmin$^+$/Pax7$^-$ fibers appeared in the underlying myotome (Fig. 1B-B″,F,H) of many segments. Further characterization of this novel contribution revealed that 27% of the clones contained myocytes (n=48 labeled clones resulting from 648 injected somites in 54 embryos).
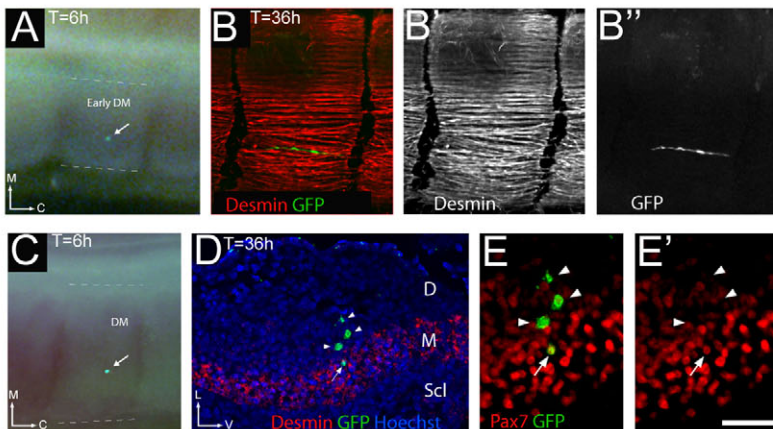

Fig. 1. The central DM first generates differentiated myocytes and then muscle progenitors. (**A-B″**) Time-lapse images of myocyte formation from the central epithelium. Clonal injection of pCAGGS-GFP into the central epithelial somite at E2 reveals, 6 hours later, the presence of a labeled cell in the center of the nascent DM (A, arrow). (**B-B″**) Confocal image showing that at 36 hours, a labeled (GFP$^+$) muscle fiber develops within the desmin$^+$ myotome. (**C-E′**) Time-lapse images of a clonal injection into the central DM at E3 (C, arrow, 6 hours) that produced a Pax7$^+$/desmin$^-$ myoblast (arrow in D,E) and also labeled cells in the dermal domain (arrowheads in D-E′) with weaker Pax7 immunostaining. (B,D). Nuclei are stained with Hoechst. (C,D) Note that the progeny of the centrally injected cell remained in an equivalent position 36 hours later. (**F,G**) Quantification of the proportion of Pax7$^+$ cells in the myotomes of segments injected at E2 (F, n=35 cells) or E3 (G, n=120 cells). (**H,I**) Schemes depicting the sequence of lineages issued from the central DM. Single cells in the central epithelium (left panels in H,I) were injected with pCAGGS-GFP at E2 or E3. Between E2 and E3, this domain generates either myofibers or additional DM progenitors (H). A day later, it produces dermis and mitotic myoblasts (I). C, caudal; M, medial; L, lateral; V, ventral; DM, dermomyotome; ES, epithelial somite; NT, neural tube; No, notochord; D, dermis; Scl, sclerotome; M, myotome. Scale bar: in E′, 25 µm for E,E′, 42 µm for D.
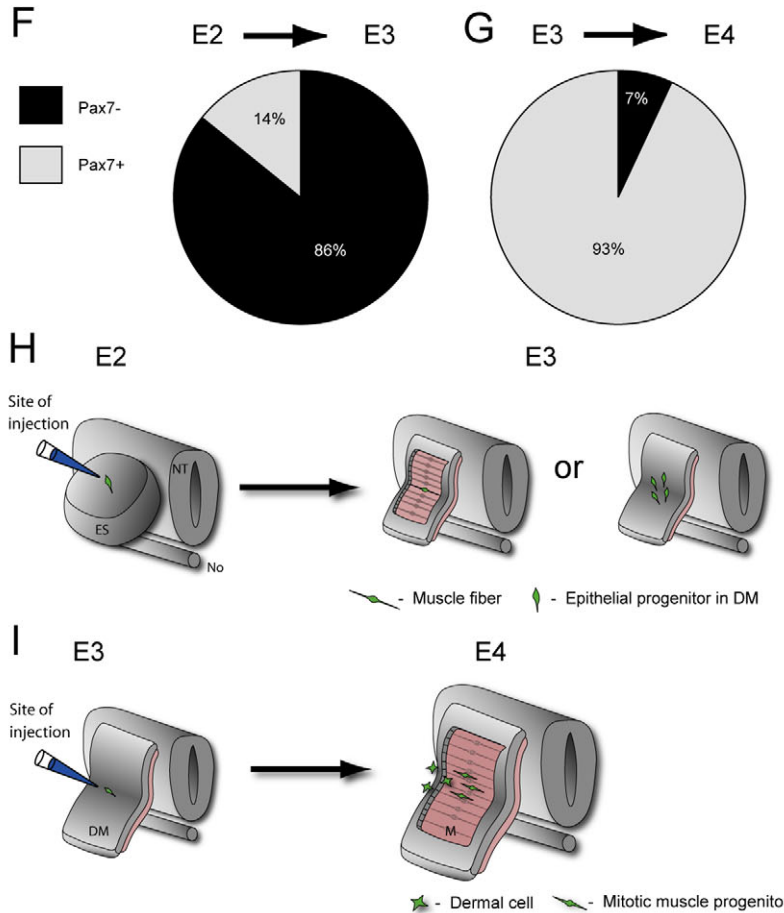
Notably, most of the muscle fiber-containing clones (12/13) had no additional cell types, suggesting that they stemmed from restricted DM progenitors. In addition, these clones were composed of either two fibers each (14.5%) or of a single fiber (12.5%), indicating that they differentiated following a terminal mitosis or without previous cell division, respectively. In the remaining clones, labeled cells extensively proliferated and were found either in the DM epithelium or in DM-derived progeny (see Fig. 3).

By contrast, when the established DM was clonally injected at E2.5-3, before dissociation, most labeled cells that translocated into the myotome remained mesenchymal, mitotically active, and expressed Pax3 and Pax7, as previously described, in addition to generating dermis (Fig. 1C-E′,G,I; data not shown) (Ben-Yair and Kalcheim, 2005). Therefore, the central DM sequentially generates two muscle sublineages: the early, fully epithelial DM sheet is a source of differentiating myocytes, here reported for the first time, and this contribution precedes the massive colonization of the myotome by muscle progenitors that begins by E3.5, coinciding with the dissociation of the central epithelium. Altogether, in the early epithelium single progenitors either generate myofibers or self-renew (Fig. 1H). This self-renewing population is, in turn, the source of central DM progenitors from which dermis and mitotic myoblasts derive. Consistently, when multiple cells in the central portion of the epithelial somite were labeled (see Fig. S2A in the supplementary material) and embryos analyzed 36 hours later, both muscle fibers and mesenchymal progeny were generated in muscle and dermis (see Fig. S2 in the supplementary material).

Early central DM-derived progenitors transiently maintain an epithelial-like morphology within the myotome while downregulating Pax7 and upregulating MyoD

The above results suggest that, while still epithelial, the central DM generates differentiated myocytes. To further characterize this contribution, pCAGGS-GFP or membrane-tethered GFP (Lyn-GFP) was centrally electroporated at E2. Labeled fibers were not detected until at least one day after transfection (Fig. 1B,H, see Fig. S2 in the supplementary material), prior to which (at 16-20 hours) labeled cells that translocated into the myotome retained an epithelial-like morphology, spanned the entire myotomal thickness and exhibited typical endfeet (Fig. 2). Lyn-GFP labeling of these endfeet colocalized with markers of epithelial polarity, such as laminin in the basal aspect of the myotome (Fig. 2C) and ZO-1 (also known as Tjp1), aPKCλ and N-cadherin at the apical surface abutting the DM (Fig. 2D-F, arrowheads). These observations confirm that, on the way to differentiating, central DM-derived myoblasts transiently retain apicobasal polarity within the myotome. In addition, the transient epithelial cells downregulated Pax7 (Fig. 1F, Fig. 2G) and expressed MyoD, but most were still negative for myosin (Fig. 2H,I).

Hence, at E3, the myotome is composed of an array of fully elongated myocytes interrupted by scattered cells that retain epithelial features; these cells are likely to be specified myoblasts that progressively differentiate following a delay of ~20-24 hours.

Planar divisions in the early DM are necessary for the production of like progeny in either DM or myotome

Cell divisions in the early DM are predominantly symmetric

During early DM development, most cell divisions are planar, with both daughter cells localized parallel to the mediolateral extent of the epithelium (Fig. 3D). Based on these data, we suggested previously that such divisions are symmetric in outcome and account for DM expansion (Ben-Yair and Kalcheim, 2005). The findings described above show that the early DM also contributes to muscle colonization, raising the possibility that oriented cell divisions are also involved in myogenesis. To better understand this process, we clonally injected single cells in the central epithelial somite with pCAGGS-GFP. Labeled progeny were observed 24 hours later in DM, in both DM and myotome, or in myotome only (Fig. 3A-C). Only clones containing two or more cells, i.e. where cell divisions took place, were analyzed further. Fifty-six percent of injections gave rise to cells restricted to the DM, 18% generated cells in myotome, and 13% in both DM and myotome. In addition, 11% of injections produced both DM cells and nascent sub-ectodermal mesenchyme, and 2% only sub-ectodermal mesenchyme (Fig. 3G). These results demonstrate that between E2 and E3, when most divisions (67%) in the central DM are planar, a corresponding majority of clones (76%) exhibit a symmetric outcome.

ct-LGN and Insc increase the proportion of asymmetric cell divisions in the early DM

To examine the significance of early planar divisions, we transfected cells with the GoLoco domain of chick LGN (ct-LGN), which is known to interfere with the function of the endogenous protein and to randomize mitoses in neural systems (Konno et al., 2008; Kraut and Campos-Ortega, 1996; Morin et al., 2007; Zigman et al., 2005), although its possible role in the mesoderm remains unknown. Chick *Lgn* mRNA and protein are expressed in early and late DM, suggesting the possibility of a similar function in this epithelium, but are not detectable in the post-mitotic myotome (Fig. 4). In contrast to chick *Lgn*, the second vertebrate homolog of *Pins*, *Ags3* (also known as *Gpsm1*), is not transcribed in the DM (not shown) (see Morin et al., 2007). To further verify the importance of spindle orientations, we also tested the effect of mouse Insc, which has been reported to generate perpendicularly oriented divisions (see Introduction). In contrast to *Lgn*, *Insc* mRNA is not detected in the DM (data not shown) and therefore its overexpression was used as an ectopic tool to manipulate mitotic orientations.

First, we determined their effects on the cleavage plane of DM cells. The planes of cell division were determined at telophase, 16 hours following electroporation. As described above, control cells predominantly exhibited planar cell divisions (n=31 mitoses, Fig. 3D). By contrast, electroporation of ct-LGN efficiently randomized the plane of cell divisions, part of which also became perpendicularly oriented (n=47, Fig. 3E; P<0.001). Likewise, overexpression of Insc enhanced vertically oriented at the expense of planar divisions (n=35, Fig. 3F; P=0.006).

Next, we clonally injected epithelial somites with either ct-LGN or Insc. Embryos were reincubated for an additional 24 hours and only clones containing two or more cells were considered. ct-LGN significantly increased the proportion of clones with asymmetric progeny that contained daughter cells in both DM and myotome (P<0.001). This increase was at the expense of both types of symmetric divisions yielding either myotomal cells or DM cells only (Fig. 3G,H).

Likewise, overexpression of Insc also enhanced the proportion of clones with asymmetric progeny (P=0.02), and this was at the expense of clones composed of only myotomal cells. The proportion of additional clone types was not significantly altered (Fig. 3G,I). As expected, the proportion of clones composed of single myotomal cells did not change significantly upon treatment
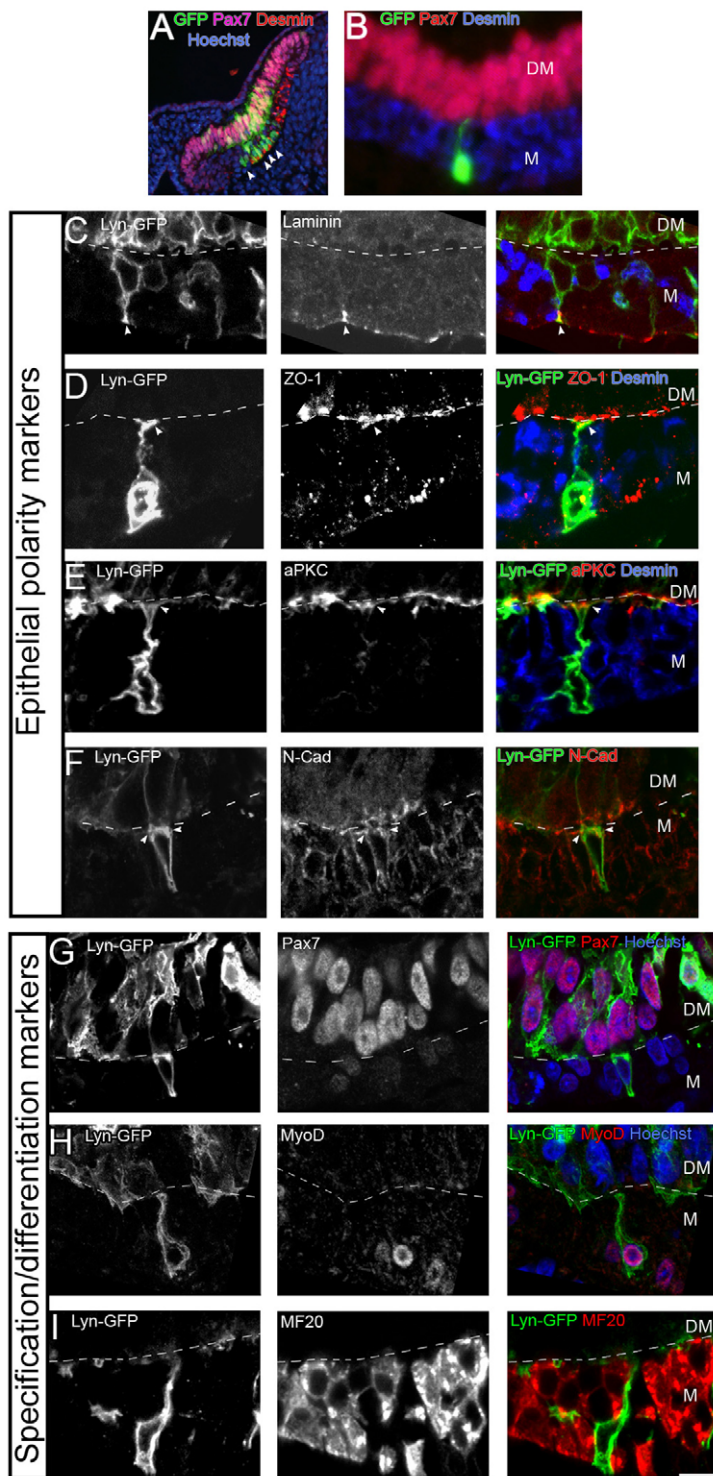
Fig. 2. Early central DM-derived progenitors transiently maintain an epithelial-like morphology within the myotome. (**A**) Electroporation of pCAGGS-GFP into the central domain of the dorsal somite at E2 yields, a day later, labeled cells both in the central DM epithelium and also in myotome, where they span its entire apicobasal extent (arrowheads). (**B**) Clonal injection (similar timing as in A) showing a single labeled cell extending across the apicobasal domain of the myotome (M). (**C-F**) Electroporation with Lyn-GFP reveals that the basal endfeet of epithelial-like myotomal progenitors contact the laminin[+] basal membrane (C), and the apical endfeet co-express ZO-1 (D), aPKC (E) and N-cadherin (F) (arrowheads). Note that N-cadherin also decorates additional sites on the cell surface. (**G-I**) The GFP[+] epithelial-like cells in the myotome lack Pax7 immunoreactivity (G) and are negative for MF20 (I), whereas cells in the overlying DM are GFP[+]/Pax7^{+} and in the myotome differentiated myocytes are MF20^{+}. By contrast, epithelial cells already express MyoD (H). Dashed lines separate DM from myotome. Scale bar: in I, 50 μm for A, 11 μm for B,G, 8 μm for C,D,H, 5.5 μm for E, 7 μm for F,I.

with either ct-LGN or Insc (data not shown; *P*>0.5 for both), confirming that only clones derived from dividing progenitors were sensitive to ct-LGN or Insc. Despite the dramatic changes in the outcome of single mitotic events, the overall distribution of injected cells to the different domains was comparable at E3 in all treatments (Fig. 3J-L), except for a modest increase in myotomal cells in ct-LGN-treated segments (*P*=0.02). This is reasonable, first because epithelial and myotomal cells can derive from both symmetric as well as asymmetric divisions, and second because having entered the myotome progenitors cease to divide, whereas their counterparts in the DM continue proliferating. Consequently, when monitoring absolute numbers of cells per domain, the relative impact of the post-mitotic cells is low.

We previously reported that the normal parallel-to-perpendicular shift in the plane of cell divisions in DM cells coincides with the appearance of the first dermal progenitors (Ben-Yair and Kalcheim, 2005). If the shift in mitotic orientations were the sole factor determining the onset of dermis development, we would expect
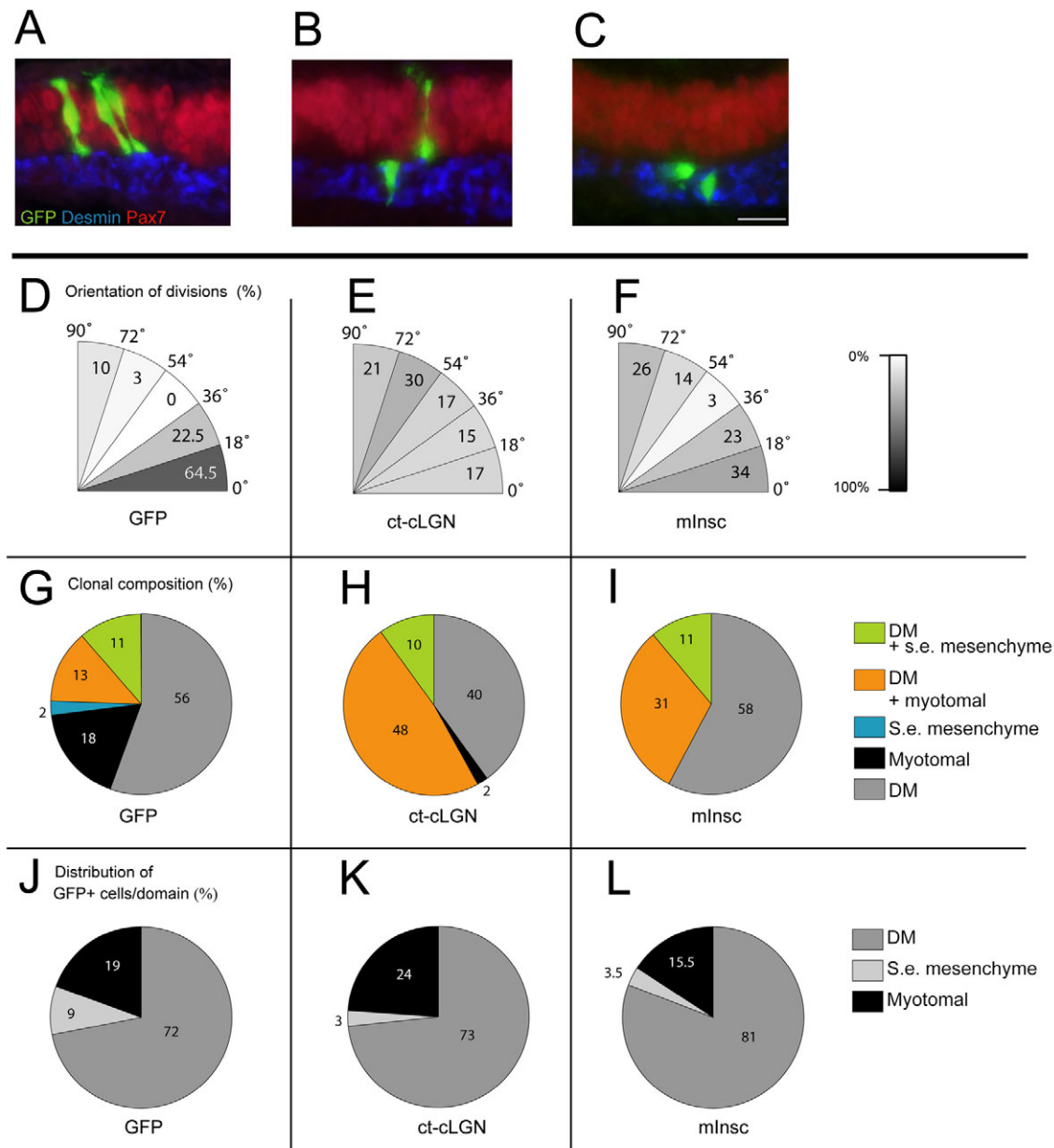
Fig. 3. Early treatment with ct-LGN or Insc randomizes otherwise planar cell divisions and increases asymmetric outcomes. (**A-C**) Outline of the derivatives obtained 1 day following clonal injections into the central DM at E2. Labeled progeny are apparent in the Pax7+ DM (A), in both DM and desmin+ myotome (B), or in myotome only (C). (**D-F**) Quantification of the axis of division relative to the apical DM surface at telophase. Measurements were performed 16 hours post-transfection. (D) pCAGGS-GFP (30 electroporated somites/10 embryos, 31 mitoses monitored). (E) ct-LGN (60 segments/20 embryos, 47 mitoses monitored). (F) Mouse Insc (mInsc) (30 somites/11 embryos, 35 mitoses examined). The value in each 18° section is the percentage of cases falling within the depicted orientation. Most control cells divide with a planar orientation (0-18°), whereas randomization, including an increase in oblique/perpendicular divisions (54-90°), is seen with ct-LGN and Insc treatment. (**G-I**) The percentage of clones containing labeled progeny in: DM + subectodermal (s.e.) mesenchyme; DM + myotome; subectodermal mesenchyme only; myotome; or DM. (G) 528 segments were clonally injected with pCAGGS-GFP in 88 embryos leading to 54 labeled clones comprising 1-10 cells each. (H) 660 segments were injected with ct-LGN in 110 embryos leading to 69 labeled clones. (I) 570 segments were injected with Insc in 95 embryos leading to 59 labeled clones. Note the increase in mixed DM + myotome clones with both ct-LGN and Insc at the expense of myotome-only clones. ct-LGN did not affect the average clone size relative to control GFP (3.79 versus 3.97 cells/clone, respectively). By contrast, clone size of progenitors that received Insc was slightly reduced (from 3.97 cells/clone in control GFP to 3.54 cells/clone), reciprocal to what has been observed upon loss of Insc function (Zigman et al., 2005). Insc might have mildly impaired cell division, as the proportion of clones containing only a single DM cell/clone increased to 32%, versus 9% in controls or in ct-LGN-injected segments (P=0.005). (**J-L**) The overall distribution of labeled cells to DM epithelium, subectodermal mesenchyme or myotome. Scale bar: 15 μm.

both ct-LGN and Insc to enhance, prematurely, the proportion of clones with subectodermal mesenchyme (presumptive dermal progenitors). However, the frequency of such clones did not change significantly when compared with controls (Fig. 3G-I). Therefore, a premature increase in the frequency of perpendicularly oriented divisions is not sufficient for generating dermis.
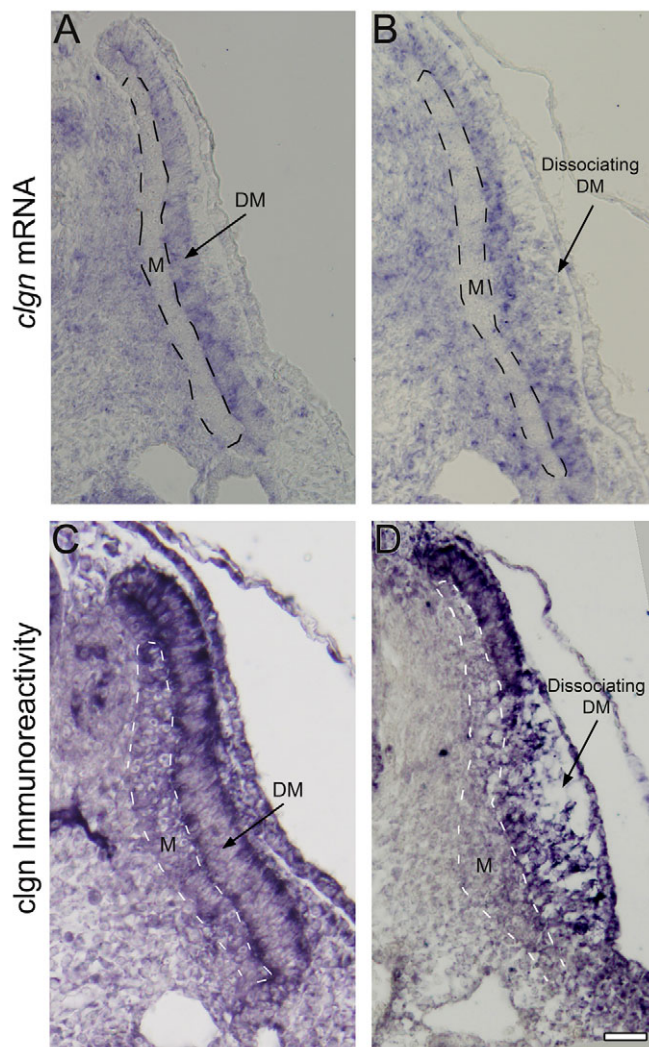
Fig. 4. Expression of chick *Lgn* mRNA and protein in the early and late DM. (**A**,**B**) In situ hybridization with a chick *Lgn* probe reveals patchy expression in the early epithelium (A) and late dissociating DM (B). (**C**,**D**) LGN immunoreactivity (alkaline phosphatase activity) is detected in the basal and apical aspects of the epithelial DM and in dissociating DM progenitors. Note the lack of LGN signal in the early post-mitotic myotome (M). The myotome is delimited by dashed lines. Scale bar: 42 μm.

These data demonstrate that the planar orientation of cell divisions in the early DM (E2 to E3) is required for the normal topographic segregation of DM progenitors into either the DM epithelium to contribute to its expansion, or into the myotome where they generate early myocytes.

ct-LGN-dependent randomization of mitoses is associated with unequal N-cadherin inheritance

In early planar mitoses, N-cadherin is inherited by both daughter cells. By contrast, in oblique and perpendicular divisions taking place in the mature DM N-cadherin differentially segregates to the daughter cell facing the myotome (Cinnamon et al., 2006). We examined whether the premature shift from planar to oblique or perpendicular orientations induced by ct-LGN (Fig. 3) is accompanied by unequal partitioning of N-cadherin. Epithelial somites were electroporated and the allocation of N-cadherin

recorded in telophase. In pCAGGS-GFP controls, most cell divisions were still planar at the time of fixation and N-cadherin was inherited by both daughter cells (Fig. 5A, arrows). Ct-LGN caused a 2.4-fold decrease in mitoses with symmetric N-cadherin distribution and a 2.2-fold increase in mitoses exhibiting N-cadherin immunostaining only in the daughter cell facing the myotome (i.e. asymmetric) (Fig. 5B, arrows, 5D; $P<0.009$). In the latter case, apical daughters were sometimes already apparent in the myotome that expresses N-cadherin (Fig. 5C, arrows). Thus, ct-LGN creates premature asymmetry as assessed both by the production of mixed clones and by unequal N-cadherin inheritance.

Perpendicularly oriented divisions in the mature DM maintain the balance between muscle and dermis production

Starting at E3, prior to DM dissociation into dermis and muscle progenitors, cell divisions in the central DM are mostly perpendicular to the mediolateral extent of the epithelium (Ben-Yair and Kalcheim, 2005) (Fig. 6A). When electroporated at E3, ct-LGN efficiently randomized the plane of cell divisions (Fig. 6B; $P<0.01$). We further validated the role of LGN using two additional and independent approaches. First, we knocked down LGN using a specific antisense morpholino oligonucleotide (MO). Its efficiency in reducing LGN immunoreactivity was assessed in the neuroepithelium, where expression is highest. Electroporation into hemi-tubes caused a marked reduction in LGN protein expression levels 24 hours after treatment as compared with the non-treated side or to a control-MO (see Fig. S3A-A″ in the supplementary material; data not shown). As expected, LGN-MO randomized cell divisions when compared with control-MO ($P=0.03$) and yielded similar results to those of ct-LGN (Fig. 6G,I). This effect was rescued upon co-transfection of the LGN-MO together with full-length human LGN ($P=0.001$) (Fig. 6I,J), whereas human LGN had no effect on its own when compared with control GFP or control-MO ($P=0.8$ for both). Second, we overexpressed Gαi1 N-terminally tagged with enhanced YFP (Venus). Venus-Gαi1 fails to localize to the plasma membrane and has been reported to randomize the plane of cell divisions by sequestering LGN in the cytosol (Du and Macara, 2004; Zheng et al., 2010). Electroporation of this construct into E3 DM indeed randomized mitotic spindles in a similar manner to both ct-LGN and LGN-MO (Fig. 6E; $P=0.04$).

Next, we assessed the effects of randomizing otherwise perpendicular cell divisions on the production of the normal derivatives of the central DM, i.e. dermis and mitotic myoblasts. If mitotic orientations play a role in fate segregation, it is expected that randomization at this stage would shift to different extents the balance between derivatives by generating cells that divide along a variety of planes. To examine this assumption, we electroporated pCAGGS-GFP, ct-LGN, LGN-MO, Venus-Gαi1 or mouse Insc into the central DM at E2.5-3, and embryos were reincubated until E4. Control GFP+ and control-MO+ cells homed to both dermis and myotome (Fig. 6D,K). By contrast, treatment with ct-LGN, LGN-MO or Venus-Gαi1 severely reduced the production of dermis (Fig. 6E,F,M) without inducing apoptosis (see Fig. S3B,C in the supplementary material; data not shown). Notably, segments that were co-transfected with both LGN-MO and full-length human LGN exhibited a phenotype similar to controls (Fig. 6D,K,N) and to human LGN alone (Fig. 6L), further confirming the specificity of the LGN-MO. Moreover, this phenotypic rescue was consistent with the
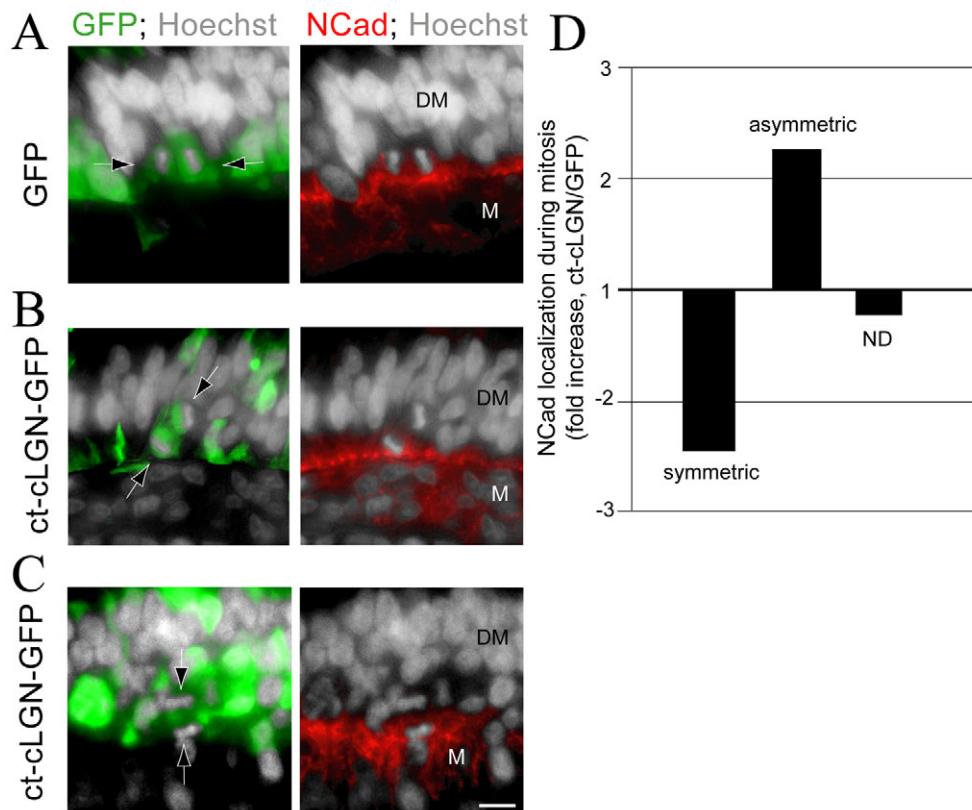
Fig. 5. ct-LGN-dependent randomization of mitoses is associated with asymmetric N-cadherin allocation to daughter cells adjacent to the myotome. (**A-C**) Electroporation of pCAGGS-GFP (green) into the dorsal epithelial somite at E2 reveals, 16 hours later, a planar mitosis with equal partition of N-cadherin (red) to both daughter cells (A, arrows). Oblique (B) and perpendicular (C) telophases resulting from transfection of ct-LGN (green, arrows), showing N-cadherin associated with the daughter cells closest to the myotome. In C, note that the lower cell (arrow) has translocated into the N-cadherin$^+$ myotome (M). Arrows and arrowheads depict the localization of daughter cells. Nuclei are stained with Hoechst. (**D**) Quantification of the localization of N-cadherin in cells at telophase. ct-LGN (35 mitoses in 13 embryos) caused a 2.2-fold increase in the asymmetric distribution of N-cadherin (present only in the apical progeny) and a 2.4-fold reduction in the symmetric pattern (N-cadherin present in both daughter cells) compared with control GFP (41 mitoses monitored in 13 embryos; $P<0.009$). ND, distribution of N-cadherin not determined for technical reasons. Scale bar: 8 μm.

orientation of cell divisions being reverted to normal. As expected, treatment with Insc at this stage of DM development did not alter the already perpendicularly oriented divisions and had no effect on fate segregation (data not shown).

To examine whether randomization of cell divisions merely affected the topographical localization of DM-derived cells or also affected their fate, the expression of dermal and muscle markers was analyzed. Three domains were considered: myotome, dermis and an intermediate domain (ID) between dermis and myotome that is composed of DM-derived mesenchymal progenitors (Ben-Yair and Kalcheim, 2005). Control GFP-transfected progenitors that homed to the dermis and ID co-expressed *Alx4* (Fig. 7A-A″) and those reaching the dermis adjacent to the ectoderm also expressed *Dermo1* (also known as *Twist2*) (Fig. 7C-C″). In addition, most labeled cells that colonized the myotome at this stage retained Pax7 (Fig. 7E,E′, arrowheads). As expected, few labeled cells exhibited desmin immunoreactivity (Fig. 7G,G′, arrowheads), representing the last differentiated myocytes stemming from the early central epithelium (Fig. 1G). GFP$^+$ cells in the ID were Pax7$^+$/*Alx4*$^+$/*Dermo1*$^-$ (Fig. 6). As described above, treatment with ct-cLGN strongly favored myotome colonization at the expense of dermis (Fig. 7B,D,F,H). In the myotome, ct-cLGN-labeled cells co-expressed myotomal markers, in particular Pax7

(Fig. 7F,F′,H,H′), but did not express either *Dermo1* or *Alx4* ectopically. Labeled cells in the ID were positive for Pax7 and *Alx4*, as in the control situation (Fig. 7F′; data not shown). Hence, loss of LGN function results in a change in cellular fates that accompanies their preferential translocation into myotomal versus dermal primordia.

To further challenge the above results we adopted a clonal approach. Single cells were injected with either pCAGGS-GFP or ct-LGN. At E4 in controls, the majority of clones were mixed, containing cells in both dermis and myotome, consistent with previous findings (Ben-Yair and Kalcheim, 2005). By contrast, ct-LGN significantly decreased the proportion of mixed clones ($P=0.05$) as well as that of clones containing dermis (dermal and dermal + ID, $P=0.05$) while favoring the development of progeny in the myotome ($P=0.05$; Fig. 8A,B). Moreover, assessment of the distribution of labeled cells in the different domains further revealed a decreased contribution to dermis ($P<0.001$) and a corresponding increase in muscle colonization ($P<0.001$; Fig. 8C,D). In addition to being consistent with the results obtained upon mass electroporation, clonal analysis further demonstrates that perpendicular divisions that characterize the mature central DM are necessary to maintain the proper balance between the production of dermis versus muscle progenitors by generating
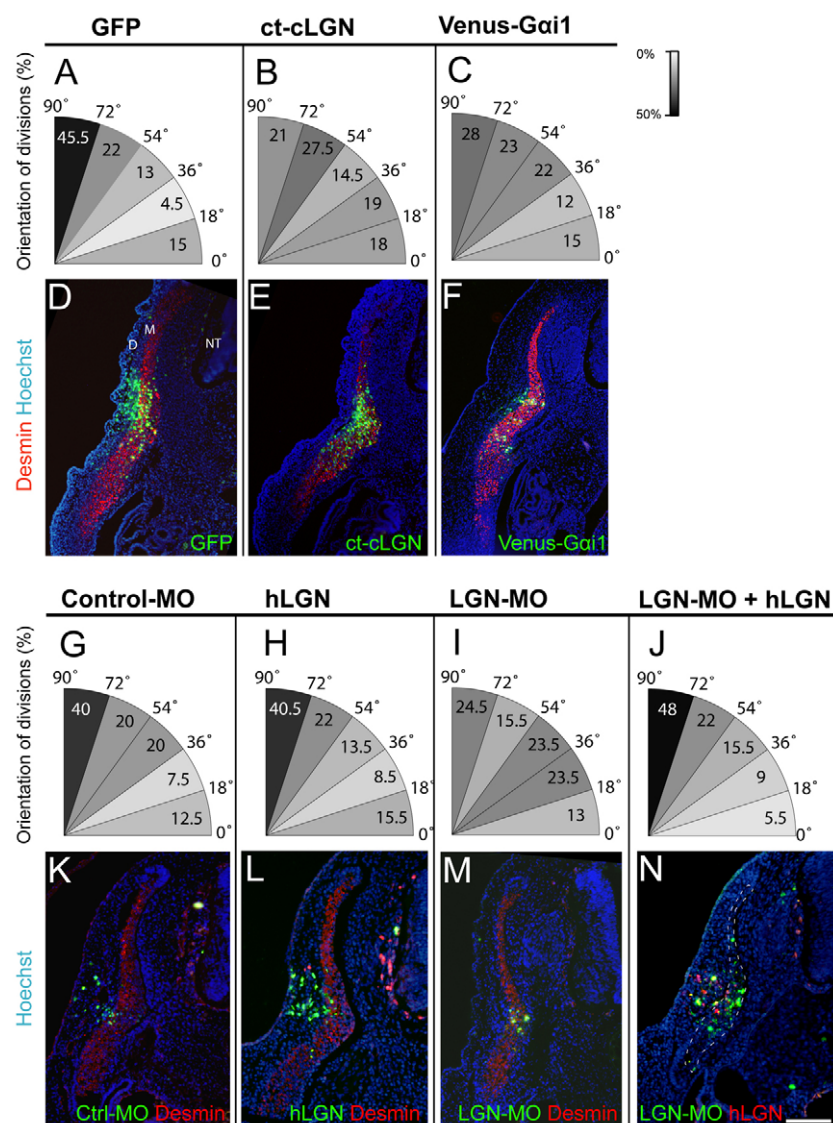
Fig. 6. Interference with late-stage perpendicular mitoses enhances muscle at the expense of dermis development. (**A-C,G-J**) Quantification of the axis of division in the late DM relative to the apical DM surface at telophase. (A) pCAGGS-GFP (66 electroporated somites/11 embryos, 68 mitoses monitored). (B) ct-LGN (96 segments/16 embryos, 62 mitoses monitored). (C) Venus-Gαi1 (102 segments/17 embryos, 82 mitoses monitored). (G) Control morpholino (control-MO) (28 segments/7 embryos, 40 mitoses monitored). (H) Human LGN (hLGN) (36 segments/9 embryos, 59 mitoses monitored). (I) LGN-MO (40 segments/12 embryos, 77 mitoses monitored). (J) hLGN + LGN-MO (80 segments/22 embryos, 145 mitoses monitored). The value in each 18° section is the percentage of cases falling within the depicted orientation. (A,G) Most control cells divide with oblique/perpendicular orientations (54-90°). ct-LGN, LGN-MO and Venus-Gαi1 randomize mitotic orientations. hLGN rescues the randomization caused by LGN-MO but has no autonomous effect on mitotic orientations. (**D-F,K-N**) Electroporation of the E3 DM with pCAGGS-GFP (D; n=7 embryos), control-MO (K; n=8) or hLGN (L; n=12) yields, at E4, labeled progeny in both myotome (M) and dermis (D). By contrast, ct-LGN (E; n=7), LGN-MO (M; n=11) and Venus-Gαi1 (F; n=9) biased the localization of labeled cells toward the muscle at the expense of dermis. hLGN + LGN-MO (N; n=22) yielded a normal cell distribution to both anlage. Scale bar: 105 μm.

asymmetric progeny from single epithelial cells. Hence, we show for the first time the importance of oriented cell divisions in the fate segregation of skeletal progenitors.

DISCUSSION

The earliest differentiated myocytes arise from the medial epithelial somite (Kahane et al., 1998a) and then from the four lips of the DM (Kahane et al., 1998b). We report here that the central epithelium of the early DM is also a source of differentiated fibers, substantiating the notion that the entire DM contributes to the post-mitotic myotome. Moreover, this region is of particular interest because it subsequently contributes to the growth phase of the myotome by generating mitotic muscle progenitors in addition to dermis (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchosoy et al., 2005; Relaix et al., 2005). Thus, the central DM sequentially produces unit-length myocytes and then muscle progenitors.

Common to both cell types is the fact that they are generated from Pax3/7-positive progenitors. The early cells translocate into the myotome while transiently retaining an epithelial-like conformation but losing Pax7 expression, and remain as such for

several hours before differentiating. This contrasts with the late contribution of proliferative progenitors, which enter the myotome as non-epithelial cells and maintain Pax3 and Pax7 expression. These differences might indicate that the early fiber precursors can differentiate only from an epithelial source, whereas the late progenitors translocate into the myotome as the DM dissociates. It is possible that these structural features enable early progenitors to access basement membrane-anchored factors that promote muscle differentiation (Bajanca et al., 2006). Another characteristic of these progenitors was revealed by clonal analysis. Most clones containing fibers, whether derived from dividing or non-dividing progenitors, do not contain additional cell types, suggesting that the fiber progenitors are fate restricted. Consistently, they downregulate Pax7 and begin expressing MyoD while still at the intermediate epithelial stage. Our data do not address, however, whether this is due to intrinsic specification within the DM or, alternatively, to myotomal factors operating on ingressing multipotent progenitors.

A relevant question stemming from the observation that the young DM sheet generates differentiated myocytes concerns the mechanism by which these cells enter the myotome from an intact
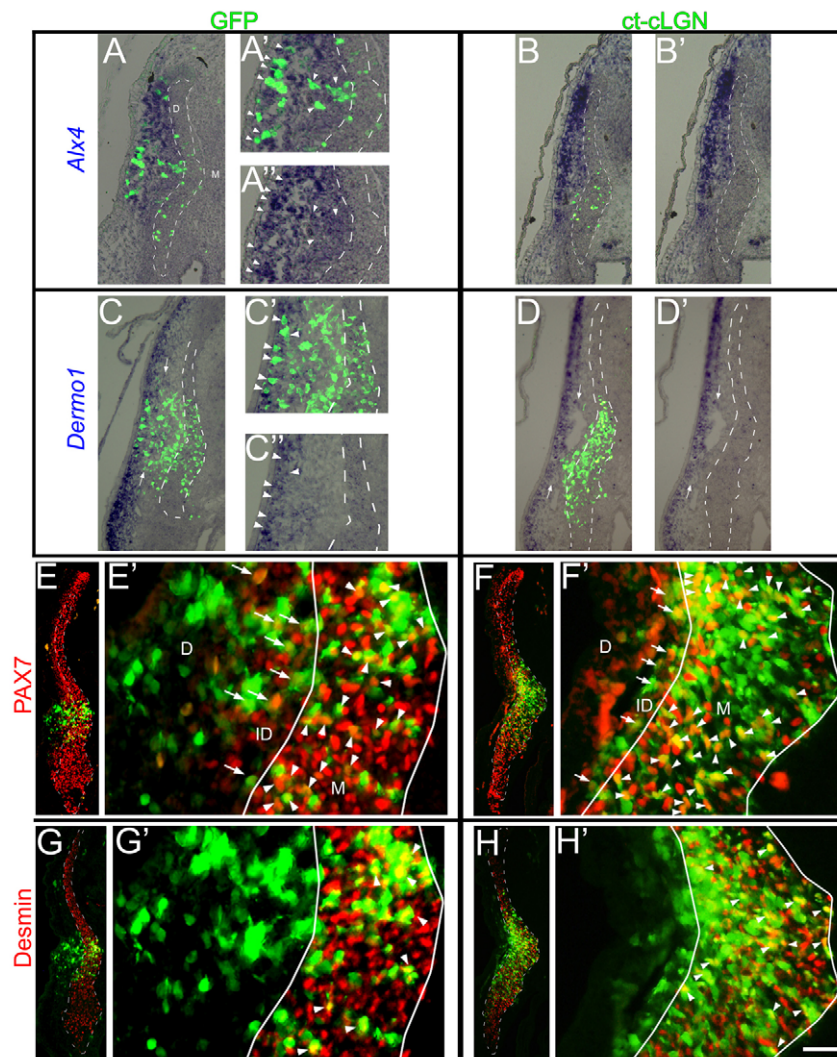
Fig. 7. The effect of ct-LGN on colonization of muscle versus dermis. (**A-A″,C-C″,E,E′,G,G′**) Control GFP+ cells colonize the dermis, where they co-express *Alx4* (arrowheads in A′,A″), and the superficial *Dermo1*+ domain of the dermis (arrowheads in C′,C″). Control labeled cells also home to the myotome, where most co-express Pax7 (arrowheads in E′) and some are desmin+ (arrowheads in G′). GFP+ cells are also Pax7+ in the intermediate domain (ID) between superficial dermis (D) and muscle (M) (arrows in E′). (**B,B′,D,D′,F,F′,H,H′**) ct-LGN-treated cells do not reach the Dermo1+ domain and remain in the ID where they co-express Pax7 (arrows in F′). Reciprocally, most ct-LGN+ cells home to the myotome (B,D,F,H), where most co-express Pax7 (arrowheads in F′) and some differentiate into desmin+ myocytes (arrowheads in H′). Arrows in C,D delimit the *Dermo1*-expressing domain. Myotomes are delineated by dashed or continuous lines. Scale bar: in H′, 103 μm for A,B,B′,C,D,D′, 62 μm for A′,A″,C′,C″, 97 μm for E-H, 14.5 μm for E′-H′.

epithelium. Although the precise mechanism remains unknown, epithelial cells from the DM lips have been shown to translocate into the underlying myotome to generate fibers while the lips remain epithelial (Denetclaw et al., 2001; Gros et al., 2004). In the central sheet, observation of static images suggests at least two possible modes of cell translocation: DM progenitors might lose their pseudostratified structure to lie in an intermediary position between DM and myotome (see Fig. S4A-C in the supplementary material) and/or directly ingress into the myotome (see Fig. S4D-F in the supplementary material) prior to generating, there, the transient epithelial cells. Regardless of the exact mechanism, we show that the apicobasal polarity of the transient epithelial cells becomes inverted relative to that of DM cells. Time-lapse analysis coupled to real-time visualization of polarity markers would be necessary to resolve the dynamics of this process.

The finding that the DM sheet sequentially produces myocytes and mitotic myoblasts raises fundamental questions concerning the mechanisms underlying the switch between these two cell types. A possible link is illustrated by data showing that the myotome itself signals to the overlying DM to undergo dissociation via FGF/Snail signaling (Delfini et al., 2009). This might be the first stage in a series of events required for the transition between fibers and muscle progenitors. An additional mechanism is the observed shift in the plane of cell division between the two consecutive phases.

Since early mitoses in the DM are planar and late divisions turn oblique or perpendicular, we previously proposed that the former account for expansion of the DM, whereas the latter generate fate diversity (Ben-Yair and Kalcheim, 2005). Here, we directly examined whether spindle orientation is involved in the two sequential phases of central DM ontogeny, first, in the switch between production of muscle fibers versus DM progenitors, and later, in the segregation of myotomal progenitors from dermis. We confirmed that in the early DM, interfering with LGN function randomizes the plane of otherwise planar divisions, thus increasing the proportion of oblique/perpendicular mitoses, as shown for the nervous system (Morin et al., 2007), and that Insc similarly enhances vertical mitoses (Konno et al., 2008; Zigman et al., 2005). The two treatments caused a significant increase in asymmetric progeny producing both myotomal and epithelial cells. This was at the expense of all symmetric divisions, both proliferative (producing two epithelial daughter cells) and terminal (producing two myotomal cells). In the nervous system, the switch of neuroepithelial and radial glial cells from symmetric proliferative to asymmetric neurogenic divisions is accompanied by a deviation of the cleavage plane from the apicobasal orientation. This deviation is often only relatively small but nonetheless results in the apical plasma membrane of neuroepithelial cells being bypassed (rather than bisected) by the cleavage furrow and, hence,
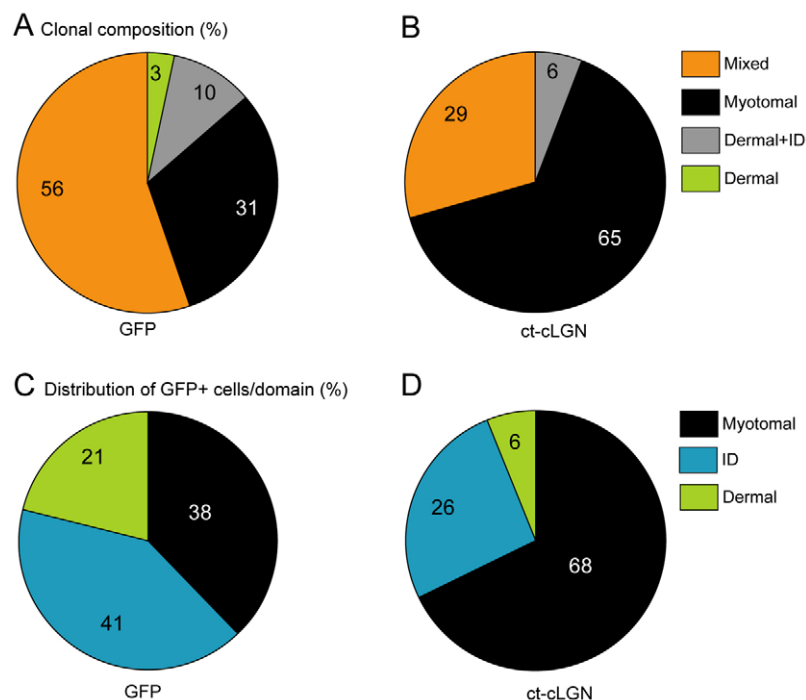
Fig. 8. Late clonal injections of ct-LGN enhance the proportion of clones that contain only muscle at the expense of mixed muscle-dermal clones. Clonal injections of pCAGGS-GFP (48 clones produced from injection of 528 somites in 44 embryos) or ct-LGN (35 clones produced from injection of 636 somites in 53 embryos) into the dorsal somite followed by fixation 45 hours later. Clones resulting from successful injections were serially analyzed. (**A**,**B**) The percentage of clones containing labeled progeny in both myotome and dermis (mixed), in myotome only, in dermis + intermediate domain (ID) and in dermis only. Note in ct-LGN the increase in clones containing only muscle at the expense of mixed clones and of clones containing dermis. (**C**,**D**) The overall distribution of labeled cells to myotome, dermis or ID. ct-LGN enhances muscle colonization at the expense of dermis and ID.

being inherited by only one of the daughter cells (Huttner and Kosodo, 2005; Kosodo et al., 2004). In the most extreme case, the cleavage plane is oriented perpendicular to the apicobasal axis (Gotz and Huttner, 2005). Consistently, we find that the premature symmetric-to-asymmetric switch caused by ct-LGN is associated with an enhanced number of mitoses with unequal partitioning of the apical cell surface determinant N-cadherin, which has previously been shown to affect lineage segregation in the DM (Cinnamon et al., 2006).

A second consequence of randomizing spindle orientation involves the balance between the production of muscle and dermis from the late DM, where most cell divisions are vertically oriented. In this context, ct-LGN, LGN-MO and Venus-Gαi1 strikingly favored the muscle fate at the expense of dermis. How does randomization affect these lineages? We suggest that segregation into muscle and dermis requires the coupling of two simultaneous processes: on the one hand, vertically oriented divisions in the late-stage DM, and on the other hand, the gradual dissociation of the DM sheet (Ben-Yair and Kalcheim, 2005) (this study). The shift to a vertical orientation could favor both an asymmetric inheritance of apical determinants and the physical separation of apical and basal daughter cells into distinct domains, particularly when tied to the dissociation of the epithelial architecture. Experimentally increasing vertically oriented divisions without accompanying cell dissociation did not lead to premature dermis development. Thus, vertical divisions alone are insufficient for dermis formation, yet they are necessary because late randomization impaired dermis production. Instead, this treatment increased the generation of like progeny that colonized the muscle, probably by enhancing the symmetric inheritance of otherwise asymmetrically localized determinants. N-cadherin is a likely candidate as it is asymmetrically distributed in vertical and oblique divisions in the DM, its distribution is sensitive to ct-LGN misexpression (this study), and its overexpression favors myotomal at the expense of dermal production (Cinnamon et al., 2006). Future studies should clarify the identity and mechanism of action of these additional

determinants and how they generate cellular diversity on a background of structural changes in the DM from epithelium to mesenchyme.

This study is the first to address the significance of the orientation of cell divisions in the localization and fate of DM progenitors. Similarities can be drawn between skeletal and neural systems, where most studies have been performed (Siller and Doe, 2009). In both cases, epithelial progenitors produce daughter cells with a higher degree of specification; these relocate into spatially distinct domains in a manner that depends on controlled orientations of cell divisions (Konno et al., 2008; Morin et al., 2007; Zigman et al., 2005) (this study). However, in contrast to the central nervous system, where mitotic progenitors become mislocalized to the differentiative domain as a result of loss of LGN function, DM progenitors undergo a true change of fate. It remains to be determined whether this results from an altered segregation of cell fate determinants or, alternatively, from a primary effect on cell localization, as a result of which bipotent DM progenitors reach their homing sites where they are instructed to adopt correct fates. Spindle orientation is therefore likely to be part of a conserved mechanism for the correct localization and/or balanced production of differentiated derivatives.

Competing interests statement
The authors declare no competing financial interests.

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