Oriented cell motility and division underlie early limb bud morphogenesis

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SUMMARY
The vertebrate limb bud arises from lateral plate mesoderm and its overlying ectoderm. Despite progress regarding the genetic requirements for limb development, morphogenetic mechanisms that generate early outgrowth remain relatively undefined. We show by live imaging and lineage tracing in different vertebrate models that the lateral plate contributes mesoderm to the early limb bud through directional cell movement. The direction of cell motion, longitudinal cell axes and bias in cell division planes lie largely parallel to one another along the rostrocaudal (head-tail) axis in lateral plate mesoderm. Transition of these parameters from a rostrocaudal to a mediolateral (outward from the body wall) orientation accompanies early limb bud outgrowth. Furthermore, we provide evidence that Wnt5a acts as a chemoattractant in the emerging limb bud where it contributes to the establishment of cell polarity that is likely to underlie the oriented cell behaviours.

KEY WORDS: Wnt5a, Cell migration, Cell polarity, Limb bud, Morphogenesis, Oriented cell division, Mouse, Zebrafish, Chick

INTRODUCTION
The earliest morphological sign of vertebrate limb formation is an apparent thickening of the lateral plate mesoderm. Forelimb initiation occurs at a stage of development when roughly half of the somites have been laid down, while the rostrocaudal axis of the embryo, including the lateral plate, continues to elongate. Initially, the limb ridge is elongated and grossly symmetrical in the rostrocaudal axis (which corresponds to the anteroposterior axis of the limb). During the first day of outward (proximal to distal) growth, the limb bud acquires a stereotypical shape that is broader posteriorly than anteriorly and is relatively narrow in the dorsoventral axis. Some of the key molecular requirements for limb initiation and early outgrowth have been identified, and include T-box transcription factors (Agarwal et al., 2003; Ahn et al., 2002; Minguillon et al., 2005; Naiche and Papaioannou, 2003) and signalling by fibroblast growth factor 10 (Fgf10) (Min et al., 1998; Sekine et al., 1999). By contrast, the cellular dynamics and tissue mechanisms underlying formation of the initial limb ridge and remodelling of the early limb bud are not well understood.

Several cellular mechanisms that might underlie limb initiation have been proposed. Previous analysis in the chick demonstrated that the limb-forming region of the lateral plate mesoderm maintains a high proliferative rate, while that of the trunk region diminishes during early limb outgrowth (Searls and Janniers, 1971). However, at least for the established limb bud, differential proliferation cannot fully explain outgrowth. Computer simulation of limb bud growth, based solely on the regional distribution of cell proliferation found in the bud (Fernandez-Teran et al., 2006), fails to predict the generation of an appropriately shaped elongating limb bud, but rather predicts the generation of a relatively spherical morphology (Boehm et al., 2010). Therefore, anisotropic or oriented cell behaviours might contribute to limb bud outgrowth and the generation of its stereotypical shape.

Evidence for the presence of cell movement in the limb bud is found in both mouse and zebrafish models. In mature mouse limb buds at embryonic day (E) 11.0, four-dimensional time-lapse imaging revealed outward, rotatory movement of the surface ectoderm (Booth et al., 2008), although these studies were not at cellular resolution and neither mesoderm nor early stages were assessed. Lateral plate mesoderm cells lacking tbx5 in zebrafish (Ahn et al., 2002) fail to enter the pectoral fin bud. Furthermore, Fgf1 mutant cells in mouse embryo chimaeras fail to populate the limb, in contrast to wild-type (WT) cells, which do (Ciruna et al., 1997; Saxton et al., 2000). It has also been shown that Fgf4, which is secreted by the apical ectodermal ridge (AER), can act as a chemoattractant (Li and Muneoka, 1999). An intriguing alternative mechanism is that Fgf4 might function to increase the liquid-like cohesiveness of mesoderm in the limb field (Damon et al., 2008; Heintzelman et al., 1978). This might cause the limb field to phase separate from the adjacent lateral plate mesoderm. In isolation, this property would cause the limb field mesoderm to be engulfed by the lateral plate. However, the lateral plate exhibits a unique active-rebound response that promotes limb bulging (Damon et al., 2008). Micromass culture data suggest that differential adhesiveness is an important mechanism that underlies the segregation of cells in the mature limb bud into proxiomdistal domains (Barn and Niswander, 2007).

Another signalling molecule that might contribute to cell movement during limb outgrowth is Wnt5a. The Wnt5a gene is expressed in the elongating tail bud and in the early ventral limb bud ectoderm, then shortly thereafter in the distal limb bud ectoderm and mesoderm, among other areas of outgrowth (Gavin et al., 1990; Yamaguchi et al., 1999). Mouse embryos lacking Wnt5a exhibit shortened rostrocaudal body axes and limbs (Yamaguchi et al., 1999). Wnt5a is able to cause directional cell movement in vitro by reorienting the cytoskeleton in response to a
chemokine gradient (Witze et al., 2008). It is conceivable that a similar mechanism might contribute to limb bud outgrowth in addition to the known positive effect of Wnt5a on mesoderm proliferation (Yamaguchi et al., 1999).

By contrast, it has been suggested that cell movement is a feature of limb formation only in lower vertebrates, and not in mouse or chick (Rallis et al., 2003). However, a direct survey of individual cell behaviours during early limb outgrowth in the mouse or chick has not previously been undertaken. The possibility that orientated cell division occurs during limb bud outgrowth has been addressed, although not systematically tested (Hornbruch and Wolpert, 1970). Here we utilise genetic, live-imaging and lineage-tracing techniques to directly survey the movements, shapes and division planes of mesodermal cells in mouse, chick and zebrafish embryos to define the morphogenetic mechanisms that generate the early limb bud and address whether equivalent cell behaviours drive this event across vertebrates. Our studies reveal the directional movement of mesoderm into the early limb bud, as well as spatially distinct biases in cell shape and cell division plane between the lateral plate and limb bud across species. A transition of these largely parallel parameters accompanies, and is likely to contribute to, early outgrowth of the bud. Cell polarity, which is partially conferred by Wnt5a, is likely to underlie these oriented cell behaviours.

MATERIALS AND METHODS

Embryo culture

CAG::H2B-EGFP (Hadjantonakis and Papaioannou, 2004) and CAG::myr-Venus (Rhee et al., 2006) transgenic mouse lines were used, and crossed with Wnt5a+/– mutants (Yamaguchi et al., 1999). E9.25–9.5 embryos [corresponding to late Theiler stage 14 (18-20 somites) to stage 15 (21-25 somites); Bard et al., 1998] were dissected and decapitated in DMEM containing 10% fetal calf serum. For live imaging, embryos were submerged just below the surface of optimised media (see Results) containing 25% DMEM and 75% rat serum. Cheese cloth or fragments of 1% agarose were used to position the lateral plate mesoderm and early limb bud directly against a coverslip at the bottom of a metallic confocal well, such that the entire depth of the tissue under study could be visualised. Time-lapse imaging experiments were performed for periods of up to 3 hours in a humidified chamber at 37°C in 5% CO2. The presence of pyknotic nuclei disqualified live-imaging experiments from analysis.

Two transgenic zebrafish lines, h2af/z:gfp (Pauls et al., 2001) and β-actin:hras-egfp (Cooper et al., 2005), were used. Embryos were cultured using Mesab (tricaine, Sigma) anaesthetic in egg water at 28°C for up to 3 hours in air. Some zebrafish embryos were cultured in egg water in the presence of 4 μM latrunculin A or its carrier 0.1% DMSO.

Image acquisition

Laser-scanning confocal data were acquired using a Zeiss LSM 510 META microscope system and a LiveCell culture chamber (Neue Biosciences). GFP and Venus fluorophores were excited using a 488 nm argon laser. For microscope system and a LiveCell culture chamber (Neue Biosciences). Laser-scanning confocal data were acquired using a Zeiss LSM 510 META.

Lineage tracing

Chick embryos at Hamburger and Hamilton stage (HH) 15-17 were exposed through a window in the shell. Hand-pulled glass needles were used to deliver small spots of Dil to the lateral plate mesoderm. Embryos were photographed using a Leica MZ16 F stereomicroscope to document the position of the dye, and then cultured in ovo at 39°C until HH 20 prior to being rephotographed.

Zebrafish embryos were injected at the one-cell stage with DMNB-FITC for ubiquitous distribution and allowed to develop in the dark until 16 hours post-fertilisation (hpf). Cells in the lateral plate were then labelled through photo-activation of the FITC with a 365 nm laser focused through a 20× objective on a compound microscope. Embryos were allowed to develop until 28 hpf and were then immunostained against FITC to amplify the signal.

Chemotaxis assays

Acrylic beads were soaked for 1 hour in Wnt5a (1 mg/ml), FgR8 (1 mg/ml), sonic hedgehog (Shh) (1 mg/ml), retinoic acid (RA) (10 μg/ml) (Helms et al., 1994), or carrier (PBS; DMSO for RA). HH 16 chick embryos were accessed in ovo through a window. The ectoderm overlying the lateral plate mesoderm was incised just enough to allow implantation of an acrylic bead into the mesoderm. Small spots of Dil were placed within 200 μm rostral and caudal to the bead for optimal sensitivity based on the findings of a previous chemotaxis assay (Li and Muneoka, 1999). Distance was measured directly from captured images using the scale bar. Embryos were photographed using a Leica MZ16 F stereomicroscope to document the position of the dye relative to the bead and then cultured in ovo at 39°C until HH 20, prior to being rephotographed. Maintenance of separation between the bead and the Dil was taken to imply a lack of chemotraction, as was seen with all carrier-soaked beads. Approximation of previously separated Dil to the bead in the form of a contiguous streak was taken to imply chemotraction of mesoderm to the bead.

Image processing

Particle image velocimetry (PIV; DaVis, LaVision, Göttingen, Germany) was used to determine bulk tissue motion based on the trajectory of fluorescent nuclei over successive time-lapse frames in two dimensions.

Cell polarity

Paraffin-embedded sections were stained against GM130 (Golga2), a Golgi matrix protein, and counterstained with the nuclear marker DAPI. The angle, from 0-359°, at which the Golgi lay with respect to the centre of the nucleus was measured for individual cells using the rostrocaudal embryo axis as the 0/180° line of reference. Confocal microscopy was used to obtain multiple z-stacks through the entire specimen under investigation. The nucleus-Golgi angles were measured for all cells while avoiding duplication by selecting z-stacks separated by one cell diameter. Measurements were grouped into twelve segments of 30° and were represented on a polar plot.

Orientation of cell division

Time-lapse movies of CAG::H2B-EGFP and h2af/z:gfp transgenic mouse and zebrafish limb buds imaged in the coronal plane were inspected frame-by-frame using Volocity software (Improvision). Confocal z-stacks, together encompassing the entire depth of the tissue under study, were assessed individually. Care was taken not to record duplicate mitoses on adjacent z-stacks by ensuring that they were separated by one cell depth. Two regions were analysed separately: lateral plate mesoderm medial to where the limb bud protrudes beyond the trunk, and the limb bud lateral to that line. Tracing paper was used to draw a line precisely representing the metaphase-to-telophase transition of every single mitosis identified. Angles of these lines were measured from 0-180° using a compass with reference to the rostrocaudal axis of the embryo as derived from low-magnification images, with 0 representing the rostral end. The planes of cell division were grouped into six segments of 30° and were represented on a polar plot with segments shaded symmetrically about the centre. Segment length was used to denote the proportion of divisions within that range. This methodology is capable of detecting a bias in cell division along the rostrocaudal and proximodistal axes, but not the dorsoventral axis.
RESULTS

The trajectory of tissue movement into the mouse early limb bud

To characterise cell movements during early limb bud outgrowth, we first established a system facilitating the ex utero development of intact lateral plate and early limb bud mesoderm in which those tissues could be live imaged. Transgenic CAG::H2B-EGFP mouse embryos (Hadjantonakis and Papaioannou, 2004) were used because localisation of the H2B-GFP fusion reporter to chromatin facilitates the visualisation of nuclei during all phases of the cell cycle, such that cells can be segmented and individually tracked in four dimensions (three-dimensional time-lapse). We optimised 3-hour static culture conditions of decapitated E9.0-9.5 (18-25 somites) mouse embryos by testing various media and rat serum concentrations. Using the quality of extracted ribosomal RNA bands as a readout, we determined that in a 5% CO2 environment maintained at 37°C, 75% rat serum supplemented with DMEM provided optimal ex utero development of static samples in a chamber resting on the microscope stage (see Fig. S1A in the supplementary material). We confirmed that short-term culture under these conditions did not result in increased cell death as assessed by LysoTracker Red staining (see Fig. S1B in the supplementary material), nor in dysmorphology of the limb bud (see Fig. S1C in the supplementary material). Using this formulation coupled with rapid dissection and short-term (up to 3 hours) time-lapse imaging experiments, pyknotic nuclei (a sign of cell necrosis, see Materials and methods) were rarely observed in CAG::H2B-EGFP transgenic tissues during live-image data acquisition.

As forelimb and hindlimb development might be subject to distinct kinetics, we chose to focus our studies on the forelimb. During Theiler stage 14 (~E9.0-9.25, 18-20 somites) the prospective mouse forelimb field appears as a barely perceptible thickening of lateral plate mesoderm between somites 8 and 12. Live imaging at this site revealed tissue movement in a rostral-to-caudal direction relative to somite boundaries (n=8 embryos studied) (Fig. 1A; see Movie 1 in the supplementary material). This finding explains previous fate-mapping (Chaub, 1959) and Tbx5 gene expression (Agarwal et al., 2003; Ahn et al., 2002; Zhao et al., 2009) studies that suggest a caudal progression of the prospective forelimb-forming region of the lateral plate mesoderm relative to the somites, prior to overt limb morphogenesis.

At Theiler stage 15 (~E9.25-9.5, 21-25 somites), lateral plate mesoderm was seen entering the early forelimb bud at an oblique angle from rostral positions (Fig. 1B,C), and laterally from more central positions relative to the limb bud (Fig. 1D; see Movie 2 in the supplementary material). The relative velocity of movement gradually increased from proximal to distal within the bud (Fig. 1D,E). This finding suggests that velocity is additive within the bud, and that the substrate for movements is the neighbouring cells themselves. Movement into the limb bud from caudal positions relative to the bud was not observed. Rather, tissue in the posterior aspect of the limb bud demonstrated rotational movement, such that cells at the posterior edge moved slightly back towards the embryo (n=10) (Fig. 1E; see Movie 2 in the supplementary material). This pattern of movement is partially analogous to the rotational motion of later limb ectoderm (Boot et al., 2008) and to polonaise movements that occur during primitive streak formation (Cui et al., 2005; Voiculescu et al., 2007), suggesting that rotation of tissue commonly accompanies longitudinal growth during development. In summary, these data indicate that lateral plate mesoderm cells migrate into the early mouse limb bud, and do so primarily from rostral and lateral positions (Fig. 1F).

Wnt5a has been implicated in the regulation of cell polarity and directional cell movement in a number of contexts, including the morphogenesis of early mesoderm (Sweetman et al., 2008; Witze et al., 2008). Wnt5a mutant mice exhibit severe shortening of the body axis and limbs (Yamaguchi et al., 1999). To determine
whether this mutant phenotype might be due, in part, to a cell migration defect, we used the CAG::H2B-GFP reporter to live image Wnt5a−/− embryos. At Theiler stage 14, lateral plate mesoderm cells of Wnt5a−/− embryos lacked the rostral-to-caudal movement seen in WT littermates (n=5) (Fig. 1G; see Movie 3 in the supplementary material). At Theiler stage 15, a corresponding shortening of the lateral plate mesoderm (and entire long-body axis) was clearly evident (Fig. 1H). This shortening is underscored by the unusual proximity of the hindlimb bud field (as identified by expression of Tbx4, a specific marker of the hindlimb) to the forelimb (Fig. 1H'). Cells in the early Wnt5a−/− forelimb bud moved with a vector comparable to that of WT littermates, albeit at a reduced velocity (Fig. 11; see Movie 4 in the supplementary material). The mean velocity of randomly selected cells individually traced frame-by-frame from z-stacks midway through the dorsoventral axis for 1.5 hours in WT embryos was 20.2 µm/hour (s.d.=2.6), compared with 10.8 µm/hour (s.d.=2.3) in the mutants, ($P<0.0001$, n=20 cells traced in three embryos each). Therefore, these data suggest that Wnt5a is necessary to maintain the normal velocity of mesoderm cell movements.

**Tissue validation in chick**

To cross-validate our directional movement findings from the mouse embryo, we studied tissue displacements in the chick embryo by lineage tracing. The chick wing bud arises from lateral plate mesoderm adjacent to somites 18-22 at HH 16 (26-28 somites total) (Chaubie, 1959). HH 15-17 chick embryos were labelled with Dil and cultured in ovo at 39°C until HH 20 (40-43 somites). Lateral plate mesoderm labelled in the rostral portion of the wing bud-forming region, adjacent to somites 17 and 18, left a trail of dye entering the early wing bud in a caudal and lateral direction (n=10) (Fig. 2A,B). Mesoderm that was labelled more centrally and inferiorly, adjacent to somites 19-21, resulted in a more directly lateral trail of Dil entering the bud (n=6) (Fig. 2C,D). Tissue caudal to somite 21 was not found to enter the wing bud, but rather became displaced just caudal to the bud (n=7; data not shown). The linear displacement of dye that we observed, as opposed to the displacement of intact spots, could result from a number of mechanisms, including directed cell migration and oriented cell division. Nonetheless, the pattern of mesoderm movement into the early wing bud, however, was similar to that of the mouse limb bud.

**Wnt5a as a chemoattractant**

To identify candidate molecules that might function to draw mesoderm into the early limb field, we performed chemotaxis assays in chick embryos. We tested Wnt5a, Fgf8, sonic hedgehog (Shh) and retinoic acid (RA). Wnt5a is expressed in the early ectoderm and distal mesenchyme of the nascent limb bud (Gavin et al., 1990), whereas Fgf8 is expressed by the AER (Crossley and Martin, 1995). Shh is expressed in the posterior limb bud mesoderm (Riddle et al., 1993), and RA is expressed in lateral plate mesoderm prior to, and during, limb initiation, and is necessary for forelimb induction (Zhao et al., 2009). Acrylic beads were soaked in protein or carrier for 1 hour prior to implantation into HH 16 chick embryo lateral plate mesoderm. Spots of Dil were placed rostral and caudal within 200 µm of the bead for optimal sensitivity of the assay (Li and Muneoka, 1999), and the position of these spots relative to the bead was documented. Following overnight incubation in ovo, the position and configuration of the Dil relative to the bead was again documented. Beads soaked in Wnt5a, but not PBS, frequently became surrounded by Dil that was contiguous with the previously distinct spot of dye ($P=0.04$, Fisher’s exact test) (Table 1, Fig. 2E-H). Neither carrier alone, nor any of the other proteins tested, exhibited evidence of chemotaxis. These data suggest that Wnt5a acts as a chemoattractant of mesoderm. Our observations are consistent with similar findings in the developing mouse palate (He et al., 2008).

**Tissue movement in zebrafish**

We extended our tissue movement analyses to zebrafish (*Danio rerio*) to establish whether the early mechanisms of budding are conserved despite the divergent structural features of the mature limb and fin. The zebrafish pectoral fin bud becomes morphologically distinct from lateral plate mesoderm adjacent to somites 2 and 3 at 24 hpf (26 somites). To localise the region of the lateral plate mesoderm that gives rise to the pectoral fin bud, we performed lineage tracing. Ubiquitously distributed, but non-emitting fluorescein (DMNB-FITC), was uncaged in lateral plate mesoderm adjacent to specific somites using a focused laser at 18 hpf (18 somites), prior to fin initiation (Fig. 3A). Embryos developed until 28 hpf (30 somites) and were then immunostained

<p>| Table 1. Wnt5a acts as a chemoattractant of mesoderm |
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<th>Bead</th>
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<td>Retinoic acid in DMSO</td>
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Shown are the number of beads exhibiting evidence of chemotaxis in chemotaxis assays performed in chick embryos.
Tissue motion is halted (but not with 0.1% DMSO carrier) as demonstrated by the lack of arrows under PIV analysis. 

To assess cell movements in the more mature pectoral fin bud, but prior to cartilaginous condensation, we live imaged h2af/z:egfp transgenic zebrafish embryos. With the embryo in the sagittal plane, views of the broad two-dimensional proximodistal-anteposterior surface of the pectoral fin bud, comparable to those of the mouse forelimb bud, were achieved (Fig. 3F,F'). Limited cell movements were seen between 28 and 40 hpf (see Movie 5 in the supplementary material). From 40 to 45 hpf, fin mesoderm cells could be seen to move in a rotatory fashion, from proximal-anterior to distal-posterior (n=16) (Fig. 3G; see Movie 6 in the supplementary material). This movement was analogous to those in the mouse limb bud at Théler stage 15 (Fig. 3H), taking into account the different orientation of the limb and fin buds. Tissue motion was abolished in the presence of 4 μM latrunculin A, but not 0.1% DMSO carrier (n=4) (Fig. 3I; see Movie 7 in the supplementary material), indicating that actin-based mechanisms are required for cell movement. 

Regional differences in cell shape and polarity 

Having shown that the movement of cells is regionally distinct in the forelimb-forming region of each organism, we sought to establish whether mesoderm cells in these regions exhibit a distinct shape and orientation. We performed live imaging of CAG::myr-Venus transgenic mice (Rhee et al., 2006) and βactin:hras-egfp (Cooper et al., 2005) transgenic zebrafish. These transgenic reporters illuminate the cell perimeter, avoiding potential artefactual changes in cell morphology that might be associated with tissue fixation. Multiple z-stacks encompassing the entire depth of the relevant tissue were taken in the coronal plane. By imaging multiple planes and identifying the distinct morphological characteristics of the single-cell layer of ectoderm (Fig. 4), we ensured that only mesoderm was analysed. Regional differences in mesoderm cell shape were identified in both mouse and zebrafish. Mouse lateral plate mesoderm cells at Théler stage 14 (n=11) and those just medial to the early limb bud at Théler stage 15 (n=9) displayed elongated shapes that ran parallel to the rostrocaudal axis of the embryo body (Fig. 4A,C). However, in Wnt5a−/− mutants, elongated cell shapes were not apparent at either stage (n=7 and 4, respectively) (Fig. 4B,D). WT (n=9) and Wnt5a−/− mutant (n=9) limb bud cells lateral to the trunk at Théler stage 15 exhibited isotropic shapes without a longitudinal bias (Fig. 4A-F). Little to no intervening space between cells was evident by live imaging (Fig. 4A-F). 

To test whether mesoderm cells exhibit polarity that corresponds to their direction of movement, we determined the location of the Golgi apparatus relative to the nucleus. The Golgi apparatus is commonly located near the leading edge of a motile cell (Nabi, 1999). At Théler stage 14 (20 somites), we identified a caudal and lateral bias in the position of the Golgi in WT mesoderm cells (n=3) (Fig. 4G,I). However, in Wnt5a−/− mutant mesoderm, this bias was not apparent (n=3, P<0.05, χ² test) (Fig. 4H,J). These findings correlate with the direction of mesoderm movement in WT embryos as well as with the diminished movements seen in Wnt5a−/− mutants, and suggest that cell polarity is required for directed cell movements. 

In zebrafish, lateral plate cells at the base of the pectoral fin bud also exhibited a longitudinal bias parallel to the rostrocaudal axis of the embryo (n=6) (Fig. 4K). Most of the cells within the fin bud were isotropic between 35 and 45 hpf, as in the mouse bud, but cells at the proximal-anterior portion of the 40-45 hpf fin bud displayed a distinctive mediolateral longitudinal orientation, parallel to the proximodistal axis of the fin bud and perpendicular to the rostrocaudal embryo axis (n=10) (Fig. 4L,M). Silencing of the βactin:hras-egfp transgene resulted in mosaic reporter expression and facilitated the visualisation of individual cell morphologies. We observed actin-rich protrusions pointing postero distally from the distal end of the elongated cells (Fig. 4K,M). These protrusions were likely to be filopodia, localised to the leading edge of cells and extending in the direction of movement.
Our data revealed parallels between cell orientation and the direction of tissue movement. A transition of cell behaviours is likely to involve active remodelling of the cytoskeleton because filopodia and Golgi localise to the leading side of mesoderm cells and latrunculin treatment impairs cell movement. The lack of apparent intercellular matrix seen by live imaging supports the proposal that the substrate upon which motion occurs is the cells themselves. Cell division might therefore be capable of influencing tissue motion.

**Regional differences in the orientation of cell division**

We sought to integrate data on cell division planes with our other findings. Time-lapse images of CAG::H2B-EGFP and h2af/z:gfp transgenic mouse and zebrafish buds, respectively, captured in the coronal plane were inspected frame by frame. Analysis of individual z-stacks facilitated the identification of mitotic planes (see Movie 8 in the supplementary material). The angle of the metaphase-to-telophase transition of every mitosis identified throughout a given specimen was measured with respect to the rostrocaudal axis (Fig. 5A).

In both mouse and zebrafish embryos, we found evidence for spatially distinct preferences in cell division planes. In lateral plate mesoderm at Theiler stage 14, the preferred plane of division was parallel to the rostrocaudal axis of the embryo (n=2 embryos, 61 mitotic angles measured; Fig. 5B). This bias was similar in lateral plate tissue medial to the early limb bud at Theiler stage 15 (n=2, 66 mitoses; Fig. 5C). In the early bud itself, a bias in the plane of cell division was identified perpendicular to the long axis of the embryo (n=2, 224 mitoses; Fig. 5D). The distribution of division planes was significantly different between WT lateral plate mesoderm and limb bud (P=0.007, χ² test). Cell division planes in Wnt5a mutant lateral plate mesoderm lacked the distinct orientation bias of WT embryos (n=2, 50 mitoses, P=0.05; Fig. 5E). However, in the Wnt5a mutant limb bud, there remained evidence of a bias in division plane largely perpendicular to the long axis of the embryo, similar to that of WT embryos (n=2, 59 mitoses, P=0.9; Fig. 5F).

Zebrafish embryos demonstrated similarities in cell division planes to mouse embryos. At 35 hpf, lateral plate mesoderm cells divided with a bias similar to that of mice (n=2, 24 mitoses; Fig. 5G), whereas fin bud cell divisions were more strikingly biased perpendicular to the embryo long axis (n=2, 30 mitoses, P=0.02; Fig. 5H). By 44 hpf, a preferred plane of cell division was no longer apparent in the lateral plate as compared with 35 hpf embryos (n=2, 20 mitoses, P=0.3; Fig. 5I). A cell division bias did persist in the 44 hpf fin bud, where it was comparable to that of 35 hpf embryos (n=2, 70 mitoses, P=0.6; Fig. 5J). Collectively, these data indicate that distinct preferences in cell division plane occur between lateral plate mesoderm and the early limb bud.

**DISCUSSION**

We have investigated the individual cell behaviours driving the tissue-scale morphogenetic mechanisms by which the vertebrate limb bud arises. Our findings challenge the notion that cell movement is a feature of only lower vertebrate early limb outgrowth (Rallis et al., 2003). Our data explain the previously observed caudal displacement of lateral plate mesoderm (Chaubé, 1959) and of the Tbx5 expression domain that marks the forelimb field prior to initiation (Agarwal et al., 2003; Zhao et al., 2009). Importantly, cell motion and division planes are reoriented during early limb outgrowth. Taken together with previous fate mapping
of the later limb bud (Saunders, 1948; Vargesson et al., 1997) and genetic studies (Hasson et al., 2007; Naiche and Papaioannou, 2007; Ros et al., 1996), our results suggest that limb initiation and subsequent outgrowth represent distinct lateral plate-dependent and -independent phases of development.

Our comparative analyses reveal that cell movement occurs similarly in the mouse and chick. Mesoderm movement also occurs during early fin outgrowth, albeit in a different pattern that later becomes analogous to that of the mouse. Our data confirm that the apparent condensation of the tbx5 expression domain during zebrafish pectoral fin initiation (Ahn et al., 2002) is secondary to cell movement, rather than to the loss and gain of expression among different cell populations. Differences in tissue movement during early forelimb outgrowth between the mouse and zebrafish might be attributable to differences in the position of the limb bud relative to the active site of rostrocaudal axis elongation at the tail end. In zebrafish, the posterior growing end of the embryo is, in relative terms, further away from the pectoral fin field, and the lateral plate mesoderm adjacent to the pectoral fin bud does not exhibit caudal-ward motion. The pre-existing caudal-ward vector adjacent to the mouse forelimb field is likely to influence cell movements into the early bud.

An active transition of cell polarity might provide the necessary coordinate behaviour that underlies initial bud outgrowth. We found that the oriented migration and division of mesoderm cells are linked. Disruption of cell polarity in the Wnt5a mouse mutants simultaneously resulted in the loss of longitudinal cell organisation, of oriented cell division and of directional movement. Although multiple forces, such as oriented cell division and directional cell movement, are likely to contribute to limb budding, these Wnt5a mutant data suggest that all such behaviours result from positional polarity possessed by mesoderm cells.

Our data are consistent with a model in which Wnt5a is involved both in establishing mesodermal cell orientation and in signalling the transition of cell polarity that is necessary to generate a limb bud. Expression of Wnt5a in the elongating tail bud is likely to contribute to the orientation of cells in the lateral plate mesoderm. By virtue of a new expression domain in the nascent limb field (Gavin et al., 1990), Wnt5a is likely to provide a repolarisation cue for mesodermal cells to move toward the new Wnt5a source. There are likely to be redundant factors contributing to this transition, as the limb bud was not as strongly affected as the lateral plate mesoderm in the absence of Wnt5a. A subset of the numerous Wnt and Frizzled genes that are expressed in the limb bud (Summerhurst et al., 2008) might perform this function, among other candidates.

Although the mechanism linking cell polarity with directional movement through cytoskeletal changes in response to Wnt5a has been explored in depth (Witze et al., 2008), the relationship of oriented division to directional movement remains unclear. We speculate that cell division generates a force that is capable of influencing the direction of movement of neighbouring cells in early mesoderm, where they are closely apposed. Although Fgf8 was not found to be a chemoattractant in our assay, it is likely to facilitate the entry of cells into the nascent limb bud by rendering the limb field mesoderm more fluid in nature (Damon et al., 2008) and by promoting cell survival (Sun et al., 2002). Directional movement and increased rates of proliferation also explain why the limb field, which exhibits higher tissue cohesiveness, is not engulfed by the lateral plate mesoderm. Our data therefore suggest that multiple mechanisms contribute to early limb bud outgrowth. Improvements in our ability to visualise and measure physical
forces in living embryos will facilitate further dissection of the nature and complexity of the morphogenetic mechanisms that generate the limb bud.

Acknowledgements
We thank Dr Terry P. Yamaguchi for generating the limb bud.

nature and complexity of the morphogenetic mechanisms that


Minguillon, C, Del Buono, J. and Logan, M. P. (2005). Tbx5 and Tbx4 are not sufficient to determine limb-specific morphologies but have common roles in limb outgrowth.


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