Development 125, 983-994 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV1231

Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis

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Accepted 6 January 1998: published on WWW 17 February 1998

SUMMARY

We have taken advantage of the optical transparency of zebrafish embryos to investigate the patterns of cell division, movement and shape during early stages of development of the central nervous system. The surface-most epiblast cells of gastrula and neurula stage embryos were imaged and analysed using a computer-based, time-lapse acquisition system attached to a differential interference contrast (DIC) microscope. We find that the onset of gastrulation is accompanied by major changes in cell behaviour. Cells collect into a cohesive sheet, apparently losing independent motility and integrating their behaviour to move coherently over the yolk in a direction that is the result of two influences: towards the vegetal pole in the movements of epiboly and towards the dorsal midline in convergent movements that strengthen throughout gastrulation. Coincidentally, the plane of cell division becomes aligned to the surface plane of the embryo and oriented in the anterior-posterior (AP) direction. These behaviours begin at the blastoderm margin and propagate in a gradient towards the animal pole. Later in gastrulation, cells undergo increasingly mediolateral-directed elongation and autonomous convergence movements towards the dorsal midline leading to an enormous extension of the neural axis. Around the equator and along the dorsal midline of the gastrula, persistent AP orientation of divisions suggests that a common mechanism may be involved but that neither oriented cell movements nor shape can account for this alignment.

When the neural plate begins to differentiate, there is a gradual transition in the direction of cell division from AP to the mediolateral circumference (ML). ML divisions occur in both the ventral epidermis and dorsal neural plate. In the neural plate, ML becomes the predominant orientation of division during neural keel and nerve rod stages and, from late neural keel stage, divisions are concentrated at the dorsal midline and generate bilateral progeny (C. Papan and J. A. Campos-Ortega (1994) *Roux's Arch. Dev. Biol.* 203, 178-186). Coincidentally, cells on the ventral surface also orient their divisions in the ML direction, cleaving perpendicular to the direction in which they are elongated. The ML alignment of epidermal divisions is well correlated with cell shape but ML divisions within the neuroepithelium appear to be better correlated with changes in tissue morphology associated with neurulation.

Key words: Morphogenesis, Cell movement, Cell division, Cell shape, Epiblast, Ectoderm, Neural plate, Gastrulation, Neurulation, Zebrafish

INTRODUCTION

While great strides are being made in the understanding of many aspects of development, we are still largely ignorant of important details about individual cell behaviours during morphogenesis and growth. We have undertaken a study of such behaviours for the cells in the surface layer of the zebrafish epiblast as they undergo gastrulation and neurulation. Gastrulation entails a wholesale rearrangement of the blastula to create the primary germ layers and overtly establish the embryonic axes (Trinkaus, 1984a). It is also a time when cells of the embryo begin to show regionally patterned gene expression that anticipates partitioning of the body and central nervous system (Sasai and de Robertis, 1997). Many molecules are now known to be involved in this patterning process; however, far less is known of how this relates to morphogenesis. In this paper, we look specifically at the patterns of cell divisions and movements within the surface layer of the epiblast during gastrulation and neurulation and relate these patterns to other coincident morphogenetic events.

Gastrulation in zebrafish spans from hours 5 1/4 to 10 postfertilisation (Kimmel et al., 1995) and neurulation, as detected by a thickening of the neural plate, begins shortly after, at about the 2-somite stage (Schmitz and Campos-Ortega, 1994). A number of classes of cell movement that underlie gastrulation in zebrafish have previously been described: epiboly (Warga and Kimmel, 1990), radial intercalation (Wilson et al., 1995), involution and/or ingression (Shih and Fraser, 1995; Warga and Kimmel, 1990) and convergence movements. During the course of teleost gastrulation, a convergence of cells towards the dorsal midline may proceed by two distinct mechanisms. Firstly, in the early *Fundulus*

gastrula, cells of the dorsal germ ring migrate towards and then enter the embryonic shield (Trinkaus et al., 1992). Such movements have not yet been reported for zebrafish but the early convergence within the surface epiblast, which we report below, is most probably the result of an analogous process. Secondly, during the latter half of gastrulation, convergence of cells within the plane of the epiblast leads to the extension of the dorsal axis (Warga and Kimmel, 1990). It is clear from fate maps of the early gastrula (Kimmel et al., 1990; Woo and Fraser, 1995) that, although extensive, movements are stereotyped over most of the embryo, although cell behaviour within the shield region may be more complex (Shih and Fraser, 1995; but see also Melby et al., 1996). Coincident with these movements, in zebrafish, there are several rounds of cell division (Kimmel et al., 1995). There is growing evidence both during morphogenesis (Morriss-Kay and Tuckett, 1987; Schoenwolf and Alvarez, 1989; Sausedo et al., 1997) and later (Adams, 1996) that cell divisions may not be random. Indeed in zebrafish, Kimmel and his colleagues (Kimmel et al., 1994) showed that cells in lineages contributing to the hindbrain and spinal cord, often divided parallel to the anterior-posterior (AP) axis of the embryo during divisions 14 and 15 but then to realign to a mediolateral (ML) orientation for division 16, which takes place during neurulation.

Despite the central role of cell division in development, the mechanisms by which it is spatially controlled are poorly understood. Histology suggests, in general, that cells divide in a characteristic plane relative to their location. This tells us something of the underlying organisation of the cells in tissues and may sometimes also reflect developmental decisions that are taking place at the time of division (Strome, 1993). We have used time-lapse DIC microscopy to study the surface layer of deep cells of the zebrafish epiblast and neural plate, cells immediately beneath the enveloping layer (EVL). All images were collected directly into a computer-based system. This powerful method for accumulating two- and three-dimensional time-lapse sequences permits rapid, quantitative analysis of cell behaviour, which is essential for a comprehensive understanding of the mechanisms of morphogenesis. In this paper, we specifically analyse the patterns of cell movement and shape and their relation to the orientation of division in three different dorsoventral regions at the level of the equator and areas along the anterior to posterior extent of the dorsal midline during the important periods of gastrulation and neurulation. We have addressed the hypothesis that simple cell properties may be responsible for determining the direction in which cells divide. Our findings are that these parameters are not sufficient to account for the highly stereotyped alignment of most cell divisions that we see in these embryos and that other organising principles must control the direction of cell division.

MATERIALS AND METHODS

Embryos

General maintenance and embryo collection of the zebrafish (*Danio rerio*) were carried out according to *The Zebrafish Book* (Westerfield, 1995). Embryos were maintained and manipulated in embryo medium (Westerfield, 1995) at 28.5°C. Each embryo was morphologically staged according to Kimmel et al. (1995) before and after filming.

Imaging

The embryos were dechorionated with watchmakers' forceps, gently inserted into a drop of 0.20% agarose in embryo medium (Westerfield, 1995) and mounted in a custom-built chamber having a coverslip on the bottom. Using a hairloop, they were carefully oriented. After adding few drops of embryo medium, a second coverslip was sealed above with silicone grease to prevent evaporation. Two precautions were taken to avoid compression of the embryos and retain their spherical shape during filming: a distance of more than 2.0 mm was maintained between the top and bottom coverslips; the chamber was slightly underfilled with medium.

An inverted microscope (Olympus Ltd.) equipped with DIC optics was used for time-lapse imaging. A computer-controlled system (Kinetic Imaging Ltd.) performed image acquisition from a slow-scan, cooled CCD camera (Astrocam Ltd). Digital images were typically collected at 20 to 90 second intervals and stored directly onto the computer hard disk.

Time-lapse computer analysis

Sequences of images were quantitatively analysed on a Macintosh computer using the public domain program NIH-Image (developed at the US National Institute of Health). Time-lapse movies were analysed for the orientation of cell division, speed and path of cell movement and parameters of cell shape. Orientation was expressed in degrees relative to the dorsal midline within the range of 0° to 180° – taking the animal-most angle between the line and the dorsal midline. The dorsal midline at early stages was defined as the focus of deep cell convergence, then later as the line midway between the lateral borders of the notochord. Data from both sides of the embryo were pooled since each displayed nearly identical distributions of angles for cell division and movement (data not shown).

The orientation of cell division was determined by drawing a line between the poles of separating daughter cells during cytokinesis – perpendicular to the plane of cleavage. Freehand outlines of individual cells were used to measure planar cell areas and an ellipse was fitted to this; the length of the major (M) and minor (m) axes of the ellipse were used as a measure of cell elongation (M/m). The orientation of the major axis corresponds to the axis of cell elongation. Paths of cell movement were traced by following cell nuclei between frames of the movies. Customised routines were written to perform all of these tasks.

Statistical analysis

Statistical analyses of data describing the direction of cell movement, elongation and cell division used tests designed for circular data (Fisher, 1993). Data were first compared to a random (uniform) model of distribution by calculating the Watson U^2 statistic, any data that could not be distinguished from random by this test at a critical value for $P<0.05$ were not further analysed. Axial data (orientation of division and elongation) were 'doubled' for the purposes of analysis; calculated values for mean orientation were subsequently halved after calculation. Data were designated as directional if a unimodal von Mises model could be fit: a maximum-likelihood estimate of the von Mises κ parameter and the estimated sample mean were used to calculate an U^2 statistic which was rejected if its value exceeded that of the critical value at $P<0.05$. Directional data were clustered around a given direction (AP or ML axis of the embryo, orientation of cytokinesis) if the 95% confidence interval for the estimate of the mean encompassed that reference direction. To compare two groups of directional measurements (movement and division), a non-parametric statistic comparing the mean and dispersions of the two sets of data was calculated and compared to the critical value for the 95% point of the χ^2_1 distribution (Fisher, 1993).

Data presentation

Measurements of the direction of movement, orientation of division and direction of elongation were grouped into 10° bins and plotted

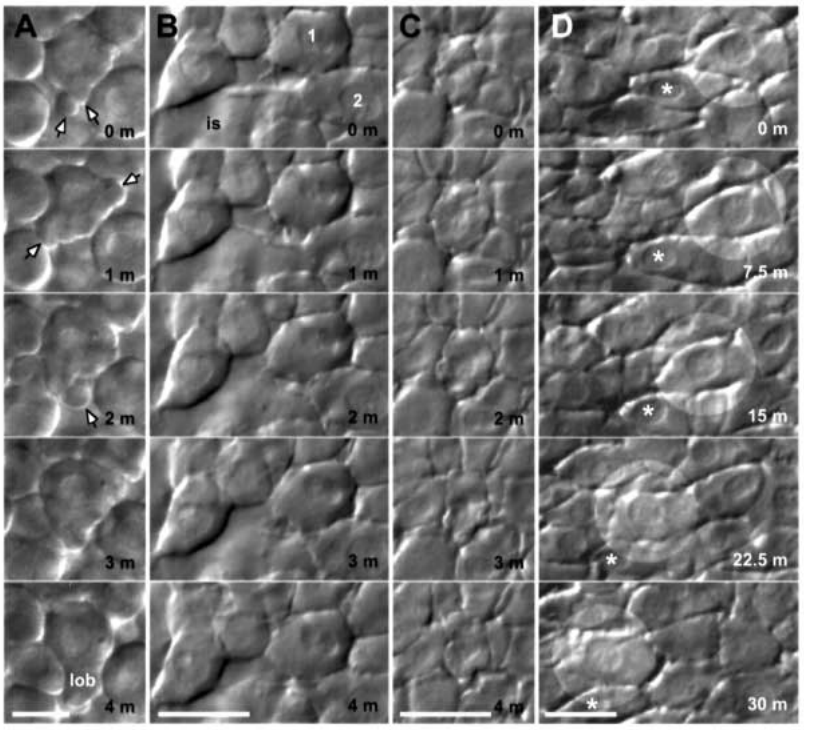

Fig. 1. Cell behaviours observed during gastrulation in the epiblast. (A) Deep cells in the late blastula and early gastrula continuously change their shape, producing lobopodia (lob) or blebs (arrows) and, less frequently, lamellipodia or filopodia. (B) Later in gastrulation, filopodia (cell 1) and lamellipodia (cell 2) become restricted to occasional regions of intercellular space (is). (C) Occasional, persistently blebbing, cells move extensively amongst other cells, unaffected by contact inhibition. (D) Late in gastrulation, cells of the dorsal epiblast develop protrusive activity as they undergo mediolateral cell intercalation towards the dorsal midline (left in D). (A) Early gastrula (50% epiboly), animal pole; (B-D) late gastrula (75-90% epiboly), equator. (B,C) Dorsolateral; (D) dorsal. Time in minutes (m). Scale bars, 20 μm.

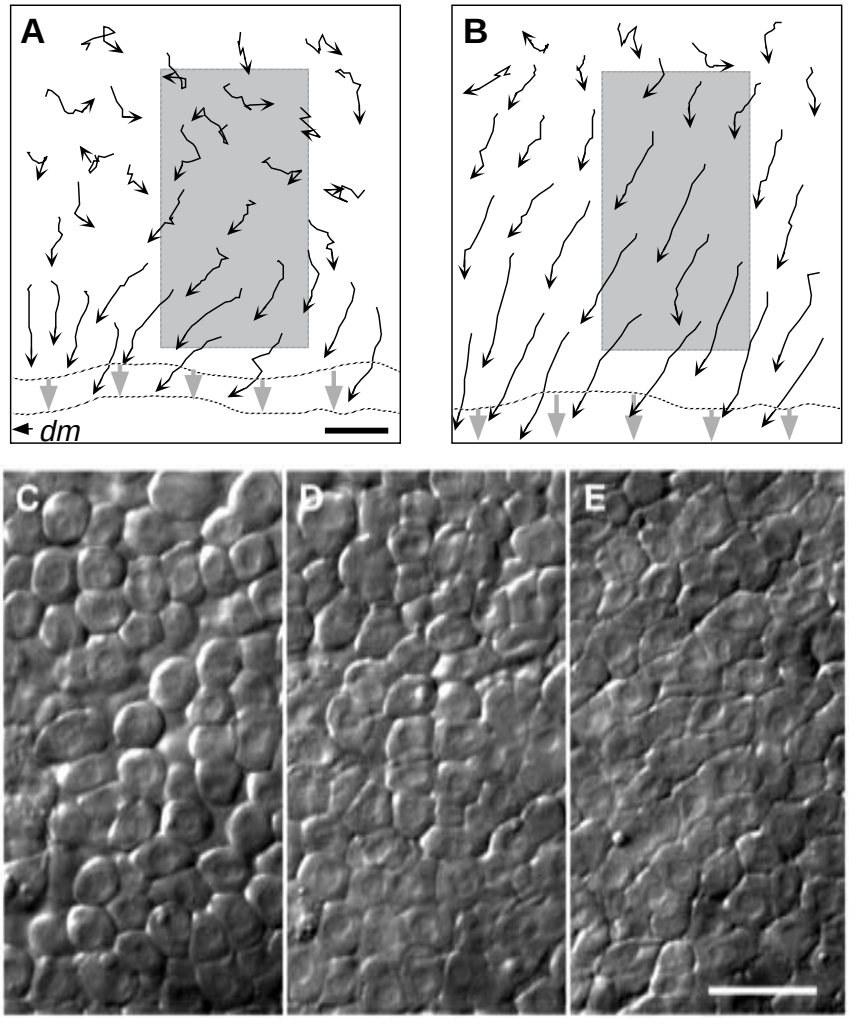

Fig. 2. Changes in cell contact and movement within the superficial-most layer of deep cells, at the dorsal margin of the early gastrula. Changes in the pattern of cell movement as gastrulation begins can be seen from the tracks of cells over two consecutive 1 hour periods beginning at 50% epiboly (A,B). Individual images from the movie are shown corresponding to the 0, 1 and 2 hour time points (C-E). Images from the shaded regions in A and B are shown in C (5 hours), D (6 hours) and E (7 hours). Large arrows indicate the displacement of the margin. Scale bars, 40 μm. In the early gastrula (50% epiboly), deep cells are spherical, loosely organised and separated by regions of intercellular space (C). Cells make small random autonomous movements (upper region, A). During the first hour of gastrulation, cells closest to the margin begin forming close contacts with each other (D), change shape and move co-ordinately towards the blastoderm margin (grey line) and the dorsal midline (dm) (A). These changes propagate towards the animal pole as gastrulation proceeds (B,E).

either as simple angular histograms, in which the length of each bar is proportional to its relative frequency, or as rose diagrams, in which the area of each sector is proportional to its relative frequency (radius proportional to the square root of frequency). The arrow inside simple angular histograms corresponds to the mean angle of that sample (Figs 3, 4).

RESULTS

Changes in cell activity and contact during the morphogenetic movements of epiboly, convergence and extension of the epiblast

Deep cells of the late zebrafish blastula are spherical and loosely packed, surrounded by significant regions of intercellular space. They make short, random and incoherent movements as individuals (Kane and Kimmel, 1993), constantly changing shape (Fig. 1A). Their behaviour changes dramatically as gastrulation begins: cells closest to the margin pack together, forming close contacts and appear more closely associated with the overlying enveloping layer (EVL). Intercellular space decreases as cell density rises and cells enter the surface layer by radial intercalation from deeper layers (Warga and Kimmel, 1990; Wilson et al., 1995). Previously spherical cells begin to display a greater diversity of polygonal shapes (Fig. 2) as they congregate with the appearance that they have accumulated into an adherent sheet; this view is reinforced by later cell behaviours on the ventral surface (see below) and the reported accumulation of cells into clusters in *Fundulus* (Trinkaus et al., 1992). By the shield stage, the epiblast is moving as a co-ordinated sheet towards the blastoderm margin, converging towards the dorsal midline (Fig. 3). These changes in behaviour begin at the blastoderm margin and propagate animalward in a wave, arriving at the animal pole at about 80% epiboly. Analysis shows that the superficial-most epiblast is now moving as an organised sheet in which there is very little mixing within the plane. Most cells now display contact inhibition of movement but when small

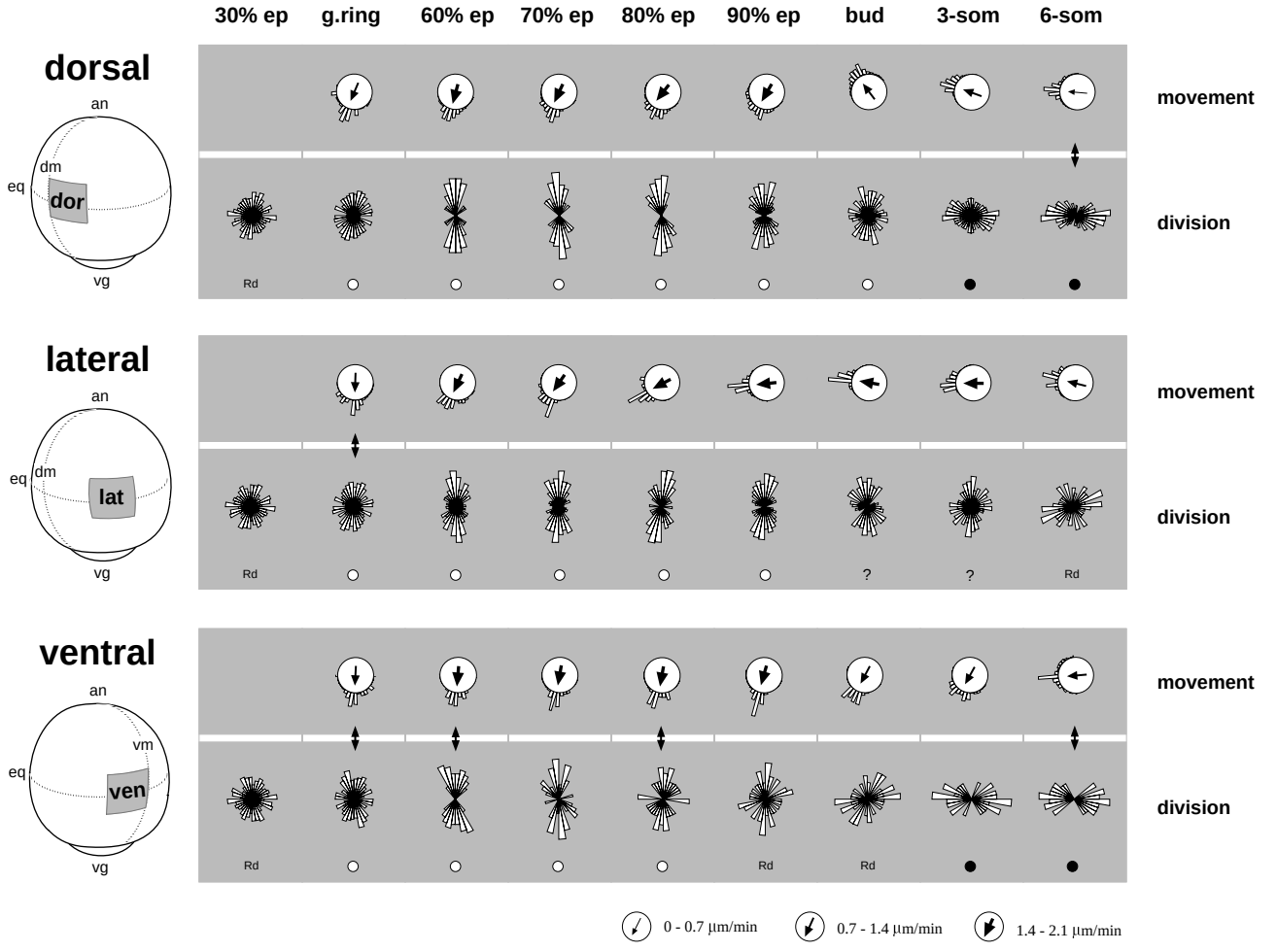

Fig. 3. Cell movement and division in the epiblast are highly aligned during gastrulation and the beginning of neurulation. The direction of movement and orientation of division of cells of the epiblast is shown at the level of the equator for three regions: dorsal (dor), lateral (lat) and ventral (ven). Movement (upper) and divisions (lower) are plotted in circular histograms at approximately one hour intervals. The mean orientation of movement is indicated by an arrow, its thickness indicative of the average velocity. Key for symbols: Rd, divisions randomly distributed; open circles, data fits a unimodal distribution aligned to the AP axis; closed circles, data fits a unimodal distribution aligned to the ML axis; ?, sample non-random but not aligned either axis; double-headed arrows, significant correlation between the mean of direction of cell movement and orientation of division. dm, dorsal midline; vm, ventral midline; an, animal pole; vg, vetegal pole; eq, equator.

pockets of intercellular space are available cells do develop filopodia and/or lamellipodia (Fig. 1B). Only a small population of cells (less than 5%), moving exclusive by blebbing locomotion (Trinkaus, 1973), does not show contact inhibition and move within and between the surface epiblast and lower layers (Fig. 1C).

In the second half of gastrulation (70% epiboly to bud stage), cells on the dorsal surface of the epiblast lose contact inhibition as they elongate and develop protrusive activity in the ML direction. These cell-autonomous movements are the basis of mediolateral intercalation whereby cells move between their neighbours towards the dorsal midline and, in so doing, extend the dorsal axis (Fig. 1D). This kind of cell behaviour has been observed in amphibia (Shih and Keller, 1992) and is likely to be the mechanism of the axial extension common to many vertebrate species: fish (Kimmel et al., 1994; Warga and Kimmel, 1990; this study), amphibia (Jacobson and Gordon, 1976; Keller et al., 1992a,b) and birds (Schoenwolf and Alvarez, 1992).

Spatiotemporal changes in the patterns of cell movement within the epiblast

The net direction of displacement of epiblast cells during gastrulation can be regarded as the combination of two motions: vegetalward epiboly and lateromedial convergence towards the dorsal midline. We have looked in detail at the displacements of cells on the dorsal, lateral and ventral faces of the embryo at the level of the equator. During the first half of gastrulation, epiboly predominates over convergence (Fig. 3), but over time, convergence gradually grows in significance. Early convergence movements probably correspond to the dorsad migrations of cells described in the marginal germ ring of *Fundulus* (Trinkaus et al., 1992). The force from this migration may be passively transmitted from the margin by the cells within the epiblast sheet. This movement differs radically from convergent-extension which takes place along the length of the dorsal axis at later stages. The change in net direction of cell movement is most clearly seen on the lateral equatorial face, between 75% and 100% epiboly, where movement reorients by approximately 60°, to become almost lateromedial (Fig. 3) meanwhile movement at the ventral midline remains predominantly AP. One consequence of the coincidence of epiboly and extension on the dorsal surface is that there is a region along the midline with no net AP extension; cells posterior to it move vegetalward and those anterior, move animalward. In Fig. 4, this transition point is located at about 150 – 200 μm above the equator of the embryo. This stationary point is likely to be present in other species in which convergent extension and epiboly are coincident and has already been reported for *Xenopus* (Keller et al., 1992b). Progressively, this region of transition moves vegetalward, rapidly descending to the vegetal pole at the end of epiboly. Subsequently, convergence persists along the whole length of the embryonic axis, expanding anteriorward until neural plate stage (Fig. 3). Active lateromedial movements continue in neurulation as the neural plate thickens and the neural keel forms.

Changes in cell shape and adhesion within the epiblast

In the early gastrula, the planar areas of all cells within the

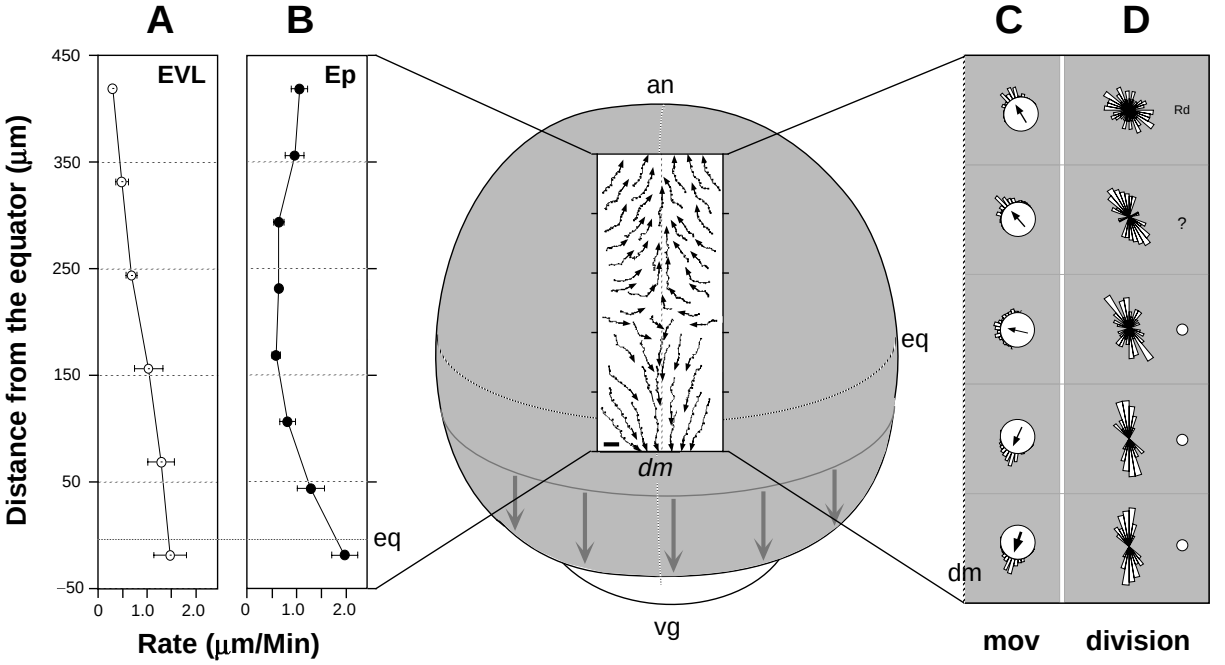

Fig. 4. Direction of movement and orientation of cell division varies along the dorsal midline of the late gastrula. Cells of the dorsal epiblast were traced between 75% and 90% epiboly (8-9 hours; inferred movement of margin is shown with large arrows in schematic). Data accumulated from different animal-vegetal regions were combined and divided into five animal-vegetal positions. The velocity of cell movement (mean ± s.d.) within the EVL (A) and epiblast (B) was calculated by following the movements of individual nuclei. Direction of movement (C) and orientation of division (D) are plotted. No significant correlation was observed between the mean orientations of cell movement and division. Abbreviations and symbols used as in Fig. 3. Scale bar, 25 μm.

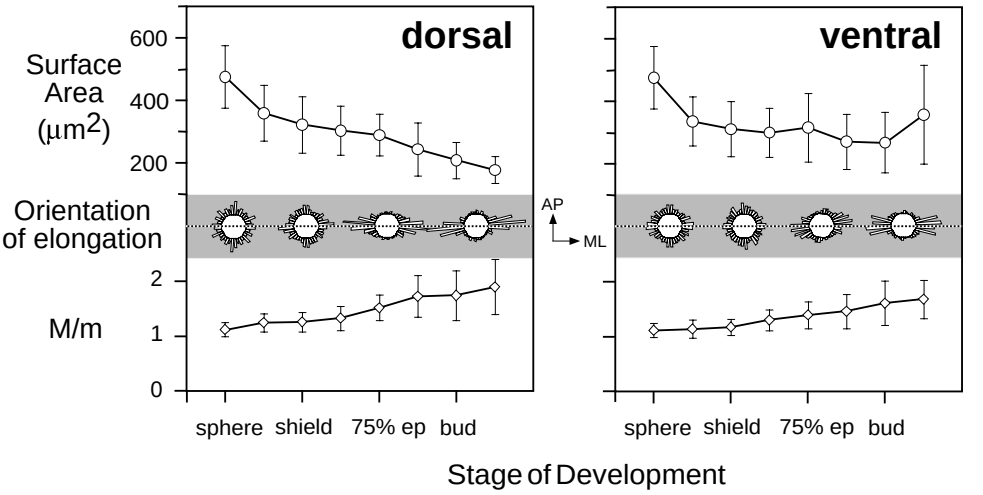

Fig. 5. Cells of the dorsal and ventral epiblast become elongated during gastrulation. During gastrulation there is a major movement of cells to the dorsal surface of the embryo. On the dorsal surface there is an increase in cell density and elongation in the ML direction, and a decrease in average cell area within the surface plane. Conversely, on the ventral surface, cells also become elongated mediolaterally but here, as a consequence of the dorsalward stretching of the cell sheet, they increase their average area relative to those of the dorsal surface. Data shows cell areas from freehand outlines, and M/m, the ratio of the longest to shortest axes, after fitting the closest ellipse.

surface plane of the epiblast are essentially equal. Dorsalward convergence during gastrulation increases cell density and reduces area on the dorsal face while ventral cells show a relative greater area and a flattening such that, by the 3-somite stage, ventral cells have about twice the area of those dorsal (Fig. 5). Cell shape also changes dramatically during this period, as the initially spherical deep cells on both the dorsal and ventral faces of the blastula become elongated in the ML direction, but by different mechanisms. On the dorsal surface, cells actively elongate while intercalating; however, on the ventral side, depletion of cells appears to stretch the remaining thin epithelium in the ML direction.

Co-ordinated cell movement may depend upon increased adhesion between cells during gastrulation, as judged by the elimination of intercellular space and an increase in the extent of close cell apposition and polygonal cell shape (Fig. 2). These changes are seen both on the dorsal surface where cell density is rising but the strongest evidence comes from the ventral surface where density is decreasing. For example, in the ventral evacuation zone (VEZ) of the embryo (Kimmel et al., 1995), cell contacts are maintained even as cells become stretched to a flattened monolayer; contacts are finally lost only at 90% epiboly when cell density no longer permits continuous cover. Remaining cells then move on the yolk as individuals by means of lamellipodia. A similar depletion and stretching is seen at the equatorial ventral surface during the bud stage, but here the epithelium remains intact.

Behaviour of dividing cells

Time-lapse imaging reveals extensive cell division in the surface layer of the epiblast throughout the period of this study, encompassing approximately three cell cycles from the beginning of gastrulation to the 10-somite stage. Dividing cells are clearly visible using DIC microscopy and a typical sequence is shown in Fig. 6. Following division, daughter cells may retain for many minutes an intercellular bridge that can

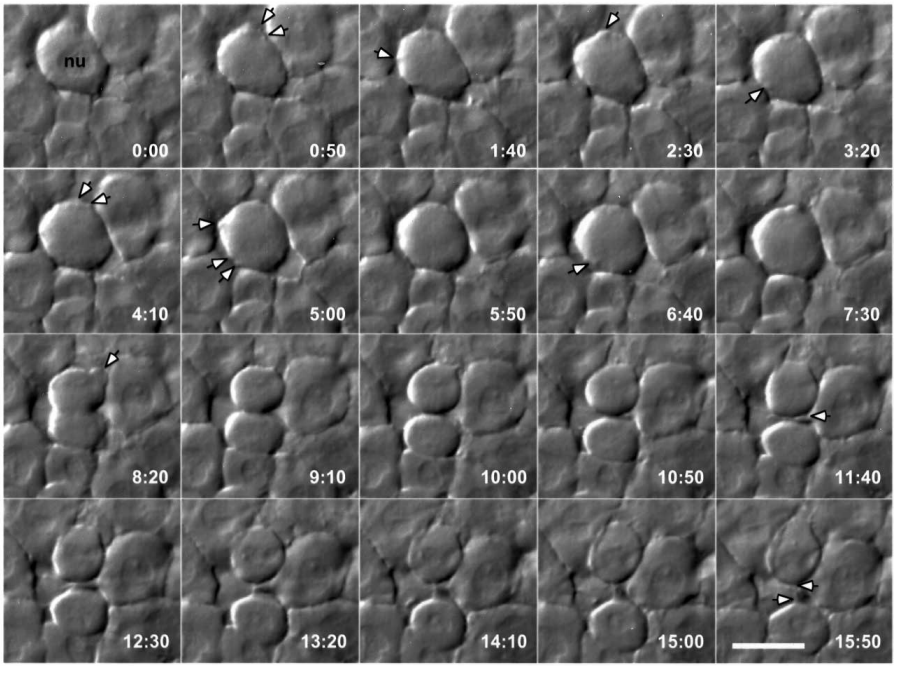

Fig. 6. Behaviours of dividing cells in the epiblast. Cell division begins with the disappearance of the nucleus (nu), as the nuclear envelope breaks down. Extensions are retracted as the cell rounds-up and separates from its neighbours, although elongated cells often retain an asymmetry at this stage. Blebs are often formed over the entire surface of the cell (arrows between 0:05 and 6:40 minutes) during mitosis, and at the poles (8:20 minutes) during cytokinesis. As the cleavage furrow forms, cells quickly divide into two spherical daughter cells of about equal size (8:20-10:00 minutes), whose nuclei later reappear. Neighbouring cells often send extensions towards the dividing furrow (arrow at 11:40 minutes). An intercellular bridge between daughter cells often remains (arrow at 15:50 minutes). Images are from the dorsolateral epiblast (75% epiboly). Scale bar, 20 μm.

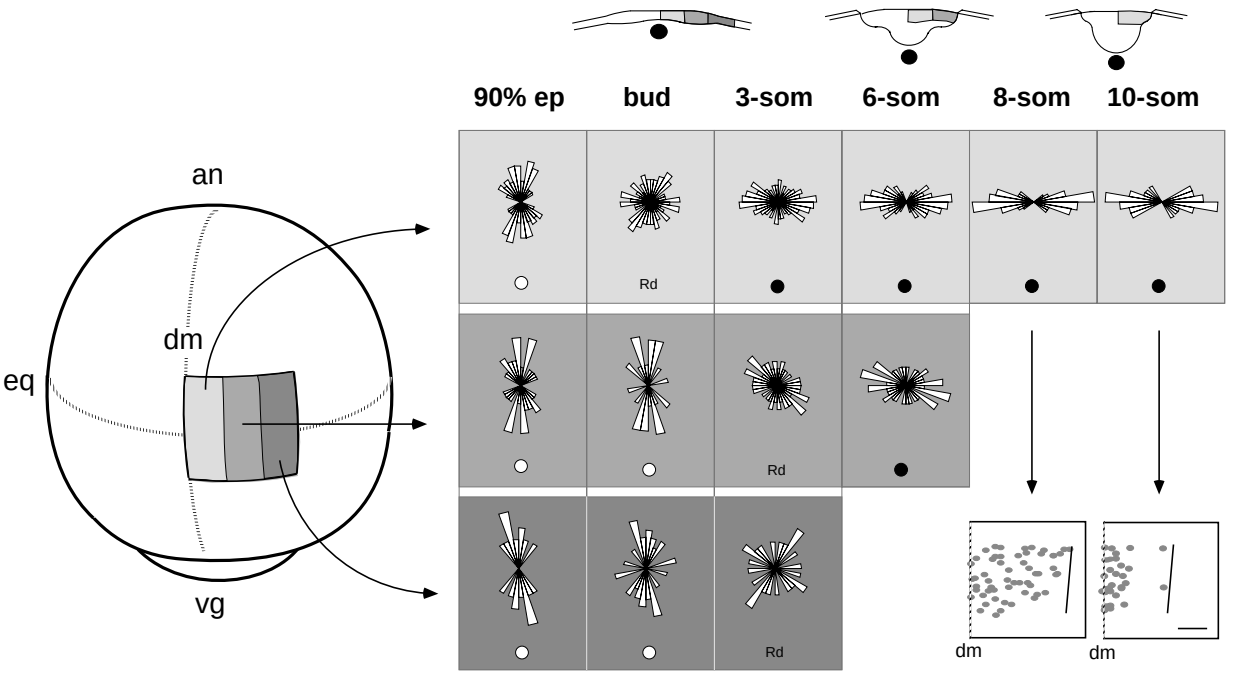

Fig. 7. The orientation of cell division changes during neurulation as a function of both time and distance from the dorsal midline. The orientation of cell division changes from AP to ML on the dorsal surface of the embryo during neurulation. This is first seen in neural plate cells dividing closest to the dorsal midline (top, bud to 3 somites) and spreads throughout the neural plate as the neural keel begins to form. As neurulation proceeds, cell division gradually becomes restricted to the dorsal midline (dm) (see insets on the right bottom of the figure). Divisions at the midline produce daughters that distribute to opposite sides of the neural keel (Kimmel et al., 1994; Papan and Campos-Ortega, 1994). Data are grouped for three locations relative to the dorsal midline, 0-70 μm (top), 70-140 μm (middle) and 140-210 μm (bottom). Abbreviations and symbols as in Fig. 3. Scale bar, 20 μm.

elongate to 30 μm during subsequent neighbouring cell intercalation (Kageyama, 1987; Schoenwolf and Alvarez, 1992). The duration of mitosis and cell division (from the onset of nuclear envelope breakdown through cytokinesis) varies slightly from cell to cell and with stage of development: ranging from 7.0±0.5 minutes (mean ± s.d.) during early gastrulation (50-75% epiboly) to about 9.0±0.5 minutes later in gastrulation and in early neurulation (70% epiboly to 6 somites).

Division of epiblast cells becomes aligned to the anterior-posterior axis after the onset of gastrulation

Cell division of deep cells (immediately beneath the EVL) during the last hour of blastula stage (dome to 50% epiboly) is at all angles relative to the surface of the embryo: planar – parallel to the surface plane; radial – parallel to a radius into the embryo and oblique – at an angle intermediate to the other two. At the animal pole, all orientations are equally represented i.e. random, while closer to the blastoderm margin, where cells are more densely packed (Schmitz and Campos-Ortega, 1994), most divisions are either planar or oblique. When gastrulation begins, divisions in the superficial layer of deep cells gradually become planar. Further, these planar divisions become aligned such that their daughters separate in the AP direction of the embryo. About 85% of divisions are planar in the equatorial region of the epiblast by 70% epiboly, the rest of the divisions are oblique (10%) and radial (5%). Divisions first become planar around the blastoderm margin and this propagates towards the animal pole during gastrulation, coincident with the other changes in behaviour described above. Most divisions at the animal pole have become planar by 75% epiboly. Alignment of planar divisions to the AP axis also begins close to the blastoderm margin (Fig. 3) and spreads towards, but never quite reaches, the animal pole. The resulting gradient of alignment, which remains throughout gastrulation, is seen in Fig. 4D, where it parallels a decrease in speed of movement both of the EVL (Fig. 4A) and the epiblast (Fig. 4B). At the end of gastrulation, more than 90% of divisions on the ventral and lateral surfaces are planar. On the dorsal surface, however, oblique and occasional radial divisions become more prevalent (together 30%) as cells mix during planar intercalation.

Cell division in dorsal and ventral epiblast becomes aligned ML during neurulation

When neurulation begins there is a gradual 90° rotation in the orientation of cell division from AP to ML for cells on both the dorsal and ventral faces of the embryo (Figs 3, 7). At the dorsal equator, this transition begins as the neural plate starts to form, beginning with those cells closest to the midline (Fig. 7, bud stage) and, by the stage of neural plate contraction and neural keel formation (3-6 somites), ML has become the predominant orientation of division over the entire neural plate. Divisions of cells lateral to the neural plate are not ML-aligned. As the neural keel narrows and deepens, dividing cells also become restricted to the very midline (Fig. 7, insets). As has already been reported (Kimmel et al., 1994; Papan and Campos-Ortega, 1994), these cells first round to their apical (medial) side then, following cytokinesis, the medial-most

daughter crosses to the contralateral side of the embryo. This behaviour may reflect the maturation of neuroepithelial morphology in which divisions are located apically – cell apices being at the midline. Non-dividing cells are never seen to cross the midline of the neural keel. We are still unsure if the medial daughters of ML divisions in the lateral neural plate also migrate contralaterally in the same way, but suspect that they do not.

In the lateral regions of the embryo, the orientation of cell division changes from AP to become randomly oriented during neurulation (Fig. 3). This occurs as the border of the neural plate moves dorsally across the lateral region, leaving cells of the non-neural ectoderm on the lateral surface. During the course of our observations (to the 6-somite stage), we did not see a realignment of divisions to a preferred direction. On the ventral side, statistically significant AP alignment is lost by 90% epiboly, earlier than on the dorsal surface but divisions then become realigned in an ML direction. This happens at approximately the same time as realignment on the dorsal side (Fig. 3).

Relationship between cell movement and division

We asked if directed motions within the epiblast might be responsible for orienting cell divisions. The results of a statistical test for the alignment of cell division with the mean direction of movement are shown in Figs 3 and 4. During epiboly, we see that divisions and movement are both strongly aligned but in only four experimental windows, around the equator, do the mean orientations of movement and division not differ significantly (P<0.05). These are lateral and ventral regions in the epiblast before strong dorsalward convergence has begun. Elsewhere, and especially on the dorsal surface, there is always an element of convergence, which grows in strength and spreads laterally as gastrulation proceeds. The result is that the predominant AP direction of division remains independent of the degree of convergence, which changes enormously with location and time. Along the dorsal midline between the equator and the animal pole, a similar dissociation is seen: the direction of cell movement changes dramatically but the orientation of division much less-so (Fig. 4). In the four most-equatorial regions measured, divisions were strongly aligned close to or parallel to the AP axis. In none of these regions do movements and divisions align according to our statistical criteria and only where the influence of epiboly is weakest, close to the animal pole, do they approximate each other.

Cell movements and divisions are both strongly aligned to the ML axis during neurulation (in the period of 3 to 6 somites) and cannot be distinguished. However, preceding this period, there is a clear temporal dissociation between the attainment

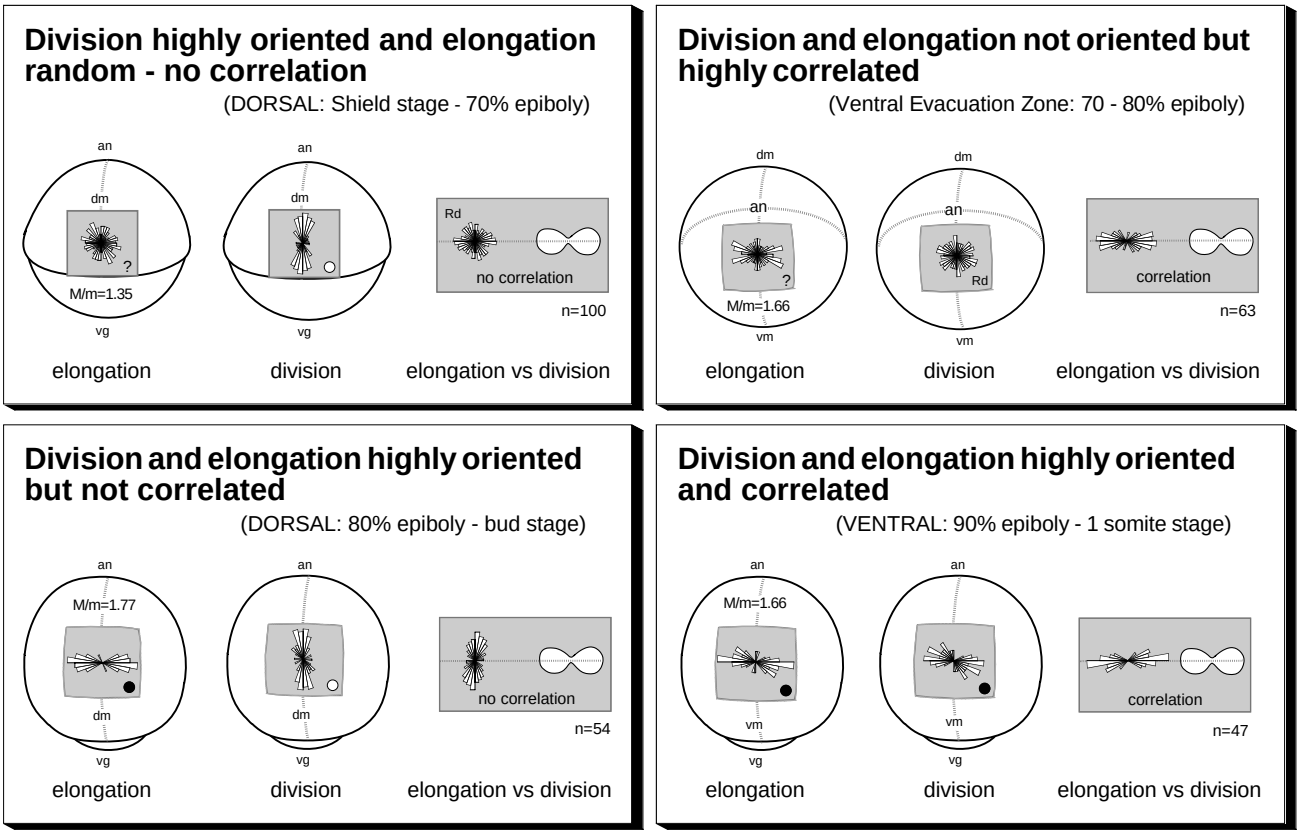

Fig. 8. Patterns of correspondence between the orientation of cell elongation, division and the embryonic axes. Four different patterns of correlation were observed according to location and stage of development. The extent of cell elongation (defined as the ratio M/m of the major and minor dimensions) and the orientation of elongation were determined in the last frame of interphase preceding division. Left schematic: the orientation of elongation plotted relative to the axes of the embryo. Middle schematic: the orientation of division plotted relative to the axes of the embryo. Right schematic: the direction of elongation is replotted, relative to the direction in which the cells will divide, which are normalised to horizontal. For discussion of the results see the text. Abbreviations and symbols as in Fig. 3.

of strong mediolateral cell movements that precede ML reorientation of division by about 1 hour (Fig. 3). This again implies that the direction of cell movement is not the primary determinant of the direction of cell division.

Correspondence between cell shape, cell division and the embryonic axes

We have also investigated the relationship between cell shape and the orientation of division. The extent and orientation of cell elongation were measured immediately before mitosis and during cytokinesis (Fig. 8) and data were compared using non-parametric statistical tests. We found four patterns of correlation during gastrulation.

Division highly oriented (to the AP axis) while elongation is random relative to the embryonic axes. This is seen at the equator of the early gastrula (shield to 70% epiboly), at all dorsoventral locations (only the dorsal surface is shown in Fig. 8). Cells here are only slightly elongated.

Division and elongation not oriented to the embryonic axes, but highly correlated. This is seen in two regions, the VEZ (shown in Fig. 8) and the animal pole at about 75% epiboly. Cells show only moderate elongation at the animal pole but become flatter and strongly elongated at the VEZ as they emigrate laterally. Elongation is poorly oriented, but cells are highly likely to divide in the direction of elongation.

Division and elongation both highly oriented but not correlated. This is seen at the dorsal equator between 75% and 95% epiboly. Cells become very elongated in the ML direction as they undergo active ML intercalation and convergence. Division, however, is aligned to the AP axis of the embryo.

Division and elongation both highly oriented and correlated. This is seen for cells dividing at the ventral equator between the stages of 90% epiboly and 1-somite. Cells are flat and very elongated and remain so throughout mitosis. They divide parallel to the direction of elongation. This might be a special case of the second pattern in which cell elongation is additionally aligned to an embryonic axis.

In summary, there is a tendency for cells in some regions to divide according to their longest axis. It is not, however, an universal rule since cells on the dorsal face divide with no regard to or even orthogonal to their major axis (and their direction of movement). This clearly shows that mechanisms other than cell elongation are operating to determine the plane of division for many cells of the embryo.

DISCUSSION

This paper analysed the geometrical pattern of cell division during gastrulation and neurulation, which we find to be unexpectedly stereotyped and ordered (Fig. 9). Kimmel et al. (1994) showed that cells in lineages contributing to the hindbrain and trunk spinal cord divided in these same orientations. We can confirm and extend in our study that this is a widespread phenomenon, which we believe is unlikely to be accounted for by a simple cell-cycle-dependent mechanism. Rather, alignments appear to reflect the development of structure and organisation within the embryo and, perhaps, the need of cells within assemblies to divide in a predictable manner. We will discuss in more detail possible mechanisms and functions of these behaviours.

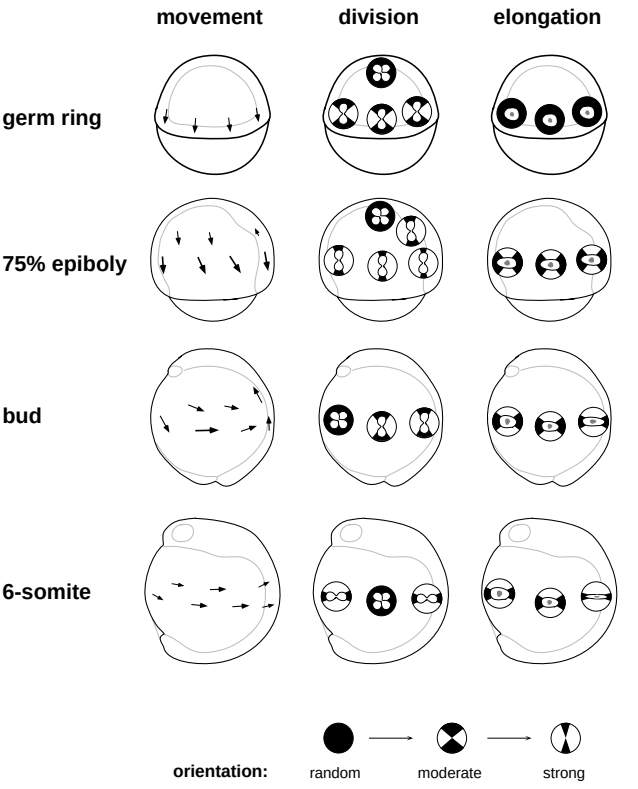

Fig. 9. Summary of the changes in cell movement, division and elongation during gastrulation and the beginning of neurulation. For details see text.

We have seen that the passage from blastula to gastrula in the zebrafish is signalled by a major transformation in the behaviour of deep cells in which they change from dissociated cells with small and incoherent movements into organised sheets of cells that move as a coherent group. Movements in zebrafish resemble those previously described for other, larger, teleosts (Lesseps et al., 1975, 1979; Trinkaus, 1992; Trinkaus and Lentz, 1967; Trinkaus et al., 1992); the major discrepancy, that cells move as sheets in zebrafish rather than clusters or individuals, may just reflect its relatively high cell density.

How is the orientation of cell division controlled?

We have shown that cells at the surface of the epiblast begin to align their divisions at the same time that they coalesce to form a sheet and may depend upon this cohesion to become aligned. We have investigated the relationship between division and the two most striking cellular morphological processes shaping the embryo at this time: cell movement and cell shape. Either of these could provide directional information and thereby the means of aligning mitotic spindles.

It has been argued on theoretical grounds and supported by experimental evidence (Honda and Yamanaka, 1984) that cell shape may determine the direction of cell division. Indeed, we find regions in the zebrafish embryo where cells are bisected along their longest axis. This includes regions where cells are elongated randomly and others where they are also aligned relative to the embryo. In fact, this may be a default mechanism and a means of maintaining compact cells within a growing epithelium (Honda, 1983). However, this rule does

not hold for much of the epiblast, in particular, in the development of AP-aligned divisions, around the equator and along the dorsal axis. Most strikingly, on the dorsal and lateral faces of the gastrula, despite clearly ML-polarised movement and elongation during planar intercalation, divisions remain strongly AP-aligned. This shows that neither the net direction of cell movement nor the direction of cell elongation are the direct determinants of cleavage orientation for a large number of cells in the embryo.

That there may be several mechanisms controlling the orientation of division is also seen during neurulation when divisions on both the dorsal (see also Kimmel et al., 1994; Papan and Campos-Ortega, 1994) and ventral surface become realigned to ML. Despite almost coincidental appearance of this behaviour on the two opposing faces of the embryo, their circumstances differ. During gastrulation and neurulation, the ventral epiblast has transformed into a thin, extended layer in which cells are stretched in the ML direction. We suggest that these cells simply cleave perpendicular to the direction of greatest elongation. On the dorsal surface, cells have been moving and extending ML for some time while continuing to divide AP, long before divisions become ML. Reorientation here is better-correlated with the formation of the neural plate and only then are divisions coincident with the direction of movement and the progressive change in cell organisation – parallel to the future dorsoventral axis of the neural tube. ML divisions later become restricted to the midline in the neural keel to segregate daughter cells bilaterally (Kimmel et al., 1994; Papan and Campos-Ortega, 1994; this study). These midline divisions are therefore aligned parallel to the apicobasal axis of the neuroepithelium. As the nerve rod cavitates to make the neural tube, cell divisions again become planar with the apical surface of the neuroepithelium (data not shown). Cells in the region of the embryo destined to become the neural tube have therefore undergone several orthogonal changes in preferred direction of division: planar-AP, planar-ML, apicobasal and finally planar in the neural tube (Fig. 10). These divisions are clearly non-random and appear most-closely associated with changes in the structure of the tissue as it undergoes its morphogenetic maturation into a neural tube.

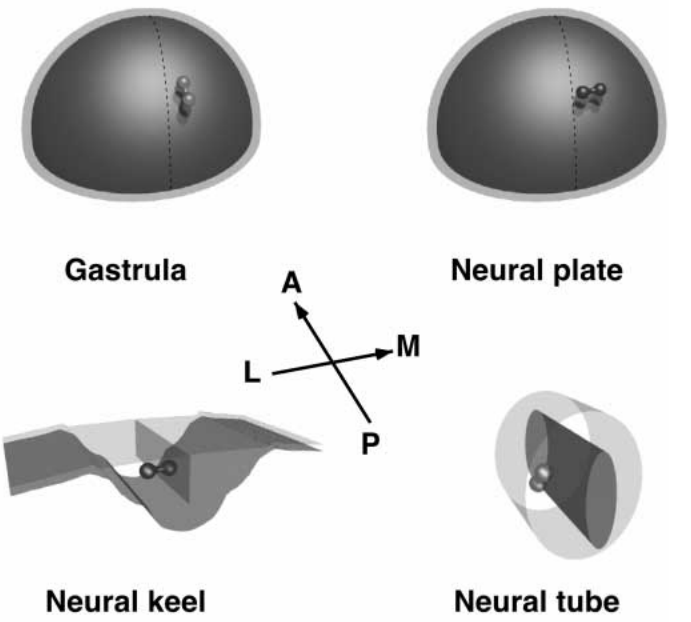

Fig. 10. The orientation of cell division for those cells that will contribute to the CNS will tend towards several mutually orthogonal alignments during the course of gastrulation and neurulation. Anterior-posterior alignment in the gastrula gives way to mediolateral at the neural plate stage, followed by apicobasal – relative to the neuroepithelium – in the neural keel and neural rod. Finally, after cavitation of the neural rod, divisions are again radial to the neuroepithelium in most instances (original data not shown).

Do mechanical forces align mitotic spindles in vivo?

We have considered whether mechanical factors may align divisions during gastrulation. Epiboly must produce a persistent vegetalward tension within the epiblast and EVL (Trinkaus, 1984b; Weliky and Oster, 1990), propagating from the border of the EVL with the YSL. Motion of deep cells, assembled into a sheet, appears passive implying that they must experience a component of force in that direction. The finding that the direction and degree of alignment of divisions is graded towards the animal pole, in a way expected for tension, especially within the EVL, is consistent with this model. However, tension is not sufficient to shape these cells to any measurable degree, in fact deep cells are only modestly elongated with their principal axis oriented randomly while their divisions are strongly AP-oriented. Also, a mechanical model would require that only the purely vegetal component of tension due to epiboly be responsible for the alignment of division; cell divisions are apparently impervious to the degree of ML convergence, which is a major component of net cell movement. Cells may dissociate these two components if they could respond specifically, for instance, to tension within the overlying EVL with which the outer cells of the epiblast are in intimate contact (Trinkaus, 1984b). Mechanical information could be acting upon the cytoskeleton to align the mitotic spindle (Maniotis et al., 1997). An important, but as-yet-unanswered, question is whether dividing cells beneath the surface layer also align in the AP direction.

Cell orientation could require non-mechanical signals. Information can propagate via cell-cell interactions and by the diffusion of molecules. It is known that, at the time that we see extensive alignments of cell behaviour, positional identity along the anteroposterior and mediolateral axes of the embryo is being established (Sasai and de Robertis, 1997). It is possible that such information could determine orientation as well as position within the embryo, and so may also align the mitotic spindle. Indeed, the propagation of directed cell behaviour leading to morphogenic segregation of the mesoderm has been seen in *Xenopus* (Domingo and Keller, 1995). Further, polarised cell-cell interactions are also postulated for other epithelia in which cells are oriented within the plane – such as the insect cuticle (Alder, 1992). Therefore, we cannot exclude that oriented divisions reflect the propagation of orientational or directional information within the embryonic ectoderm that is potentially available during pattern formation.

Are cell divisions involved in morphogenesis?

It has been suggested that cell division may actually provide

a morphogenetic force during embryonic growth (Sausedo et al., 1997; Schoenwolf and Alvarez, 1989; Tuckett and Morriss-Kay, 1985). This idea can be traced back to His (1874) who suggested that differential growth could provide a morphogenetic force but subsequent studies in amphibia have diminished the relevance of this idea in early development (Holtfreter, 1943). Simply in terms of geometry, the division of spherical cells within a plane does lead to an increase in area and if aligned in one direction, as seen here, may contribute to force production. Indeed, we often observe the displacement of neighbouring cells by the poleward-extensions of anaphase and cytokinesis (unpublished observations). In the absence of cell rearrangement or shape change, two rounds of axial cell division could lead to a 2.5- to 4-fold increase in length, depending upon the extent of cell growth between divisions. However, persistence of gastrulation (Kessel, 1960) and neurulation (Harris and Hartenstein, 1991) after mitoses have been blocked implies that cell division may not be an essential mechanical force for early morphogenesis, and the forces generated by cell movement greatly exceed those of cell division. Division may none-the-less contribute to morphogenesis. It cannot be excluded, for instance, that cell divisions are aligned to not oppose morphogenesis; inhibition of division does not address this point. Alignment of divisions to the axes of the developing neural tube has been seen in other vertebrate species (Sausedo et al., 1997; Schoenwolf and Alvarez, 1989; Tuckett and Morriss-Kay, 1985) and alignment appears correlated with morphogenesis. However, as we have shown in zebrafish, divisions do not precisely match the net direction of cell movement. The behaviour of the mitotic spindle in dividing cells of later neuroepithelia again suggests that orientation in both the apicobasal direction and within the plane of the epithelium are determined (Adams, 1996). It is well established that the orientation of the mitotic spindle dictates the plane of cleavage (reviewed Rappaport, 1996), it therefore remains to establish which factors – intrinsic or extrinsic – control the behaviour of the spindle to understand how these divisions come to show such a surprising degree of order. Morphogenesis could therefore provide a reason, if not the mechanism, for oriented divisions.

In conclusion, the orientation of cell divisions may be an indicator of cellular interaction and organisation during the early development of the zebrafish. It is still too early to know its developmental role or the means by which it is achieved. Nevertheless, such highly stereotyped behaviour does indicate that cells of the embryo must receive and respond to signals that convey directional information at a time when important developmental decisions that pattern the growing embryo are being made.

This work was supported by The MRC Research Centre for Cognitive Neuroscience, Oxford University. M. L. C. was supported by a CONICYT-British Council Fellowship (Chile). We wish to thank Stephen Massey and Dr Julian Lewis for generously providing us with zebrafish embryos, Professor Colin Blakemore for providing facilities and support, Dr Mario Cortina-Borja for advice on statistical analyses and Mr William Hinkes for his expert technical assistance. We also wish to thank Drs Dennis Bray, Jonathan Slack, Aurora Lombardo and Steve Wilson for critically reading versions of this manuscript. M. L. C. wishes to address special thanks to Drs Rosa Devés, Jorge Mpodozis and Francisco Aboitiz for their encouragement.

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