

Active cell migration drives the unilateral movements of the anterior visceral endoderm

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Summary

The anterior visceral endoderm (AVE) of the mouse embryo is a specialised extra-embryonic tissue that is essential for anterior patterning of the embryo. It is characterised by the expression of anterior markers such as *Hex*, *Cerberus*-like and *Lhx1*. At pre-gastrula stages, cells of the AVE are initially located at the distal tip of the embryo, but they then move unilaterally to the future anterior. This movement is essential for converting the existing proximodistal axis into an anteroposterior axis. To investigate this process, we developed a culture system capable of imaging embryos in real time with single cell resolution. Our results show that AVE cells continuously change shape and project filopodial processes in their direction of motion, suggesting that they are actively migrating. Their proximal movement stops abruptly at the

junction of the epiblast and extra-embryonic ectoderm, whereupon they move laterally. Confocal microscope images show that AVE cells migrate as a single layer in direct contact with the epiblast, suggesting that this tissue might provide directional cues. Together, these results show that the anteroposterior axis is correctly positioned by the active movement of cells of the AVE in response to cues from their environment, and by a 'barrier' to their movement that provides an endpoint for this migration.

Supplemental data and movies available online

Key words: Mouse embryo, Anterior visceral endoderm, Patterning, Morphogenesis, Migration, Embryo culture, Time lapse imaging

Introduction

The early post-implantation mouse embryo consists of three tissues arranged like a cylinder, with extra-embryonic ectoderm positioned proximally, epiblast distally and visceral endoderm surrounding both (Fig. 1D). Cells of the epiblast give rise to the foetus whereas the other tissues contribute exclusively to extra-embryonic structures. The anterior visceral endoderm (AVE) is a specialised region of the visceral endoderm that, as much as a day before the formation of the primitive streak, expresses markers such as *Hex* (*Hhex* – Mouse Genome Informatics) (Thomas et al., 1998), *Hesx1* (Hermesz et al., 1996; Thomas and Beddington, 1996), *Lim1* (*Lhx1* – Mouse Genome Informatics) (Belo et al., 1997) and *Cerberus*-like (*cerberus 1* homolog, *Cer1* – Mouse Genome Informatics) (Belo et al., 1997; Shawlot et al., 1998).

The AVE plays an essential role in patterning the early embryo (Beddington and Robertson, 1999). This was first demonstrated by microsurgical removal of the AVE, which causes loss of forebrain markers such as *Hesx1* (Thomas and Beddington, 1996). In addition, chimera experiments indicate that genes such as *Hnf3b* (*Foxa2* – Mouse Genome

Informatics) (Dufort et al., 1998), *Otx2* (Rhinn et al., 1998), *Smad2* (*Madh2* – Mouse Genome Informatics) (Waldrip et al., 1998) and *Lim1* (Shawlot et al., 1999) are required specifically in the visceral endoderm for proper patterning of the epiblast (reviewed by Martinez-Barbera and Beddington, 2001). For example, embryos lacking *Lim1* activity in the visceral endoderm completely lack anterior structures (Shawlot et al., 1999). The AVE functions in part by repressing the expression of posterior genes in the anterior epiblast (Kimura et al., 2000; Perea-Gomez et al., 2001), thereby causing the primitive streak to form on the opposite side of the embryo (Perea-Gomez et al., 2002). Although transplantation of the AVE alone is not sufficient to induce ectopic neural structures, it is capable of doing so in combination with anterior epiblast tissue (Tam and Steiner, 1999).

DiI-labelling experiments show that the AVE moves unilaterally from its initial position at the distal tip of the egg cylinder to the future anterior of the embryo, thereby converting a proximodistal axis to an anteroposterior axis (Thomas et al., 1998). Embryos mutant for *cripto* or *Otx2* fail to effect this movement (Ding et al., 1998; Kimura et al., 2000;

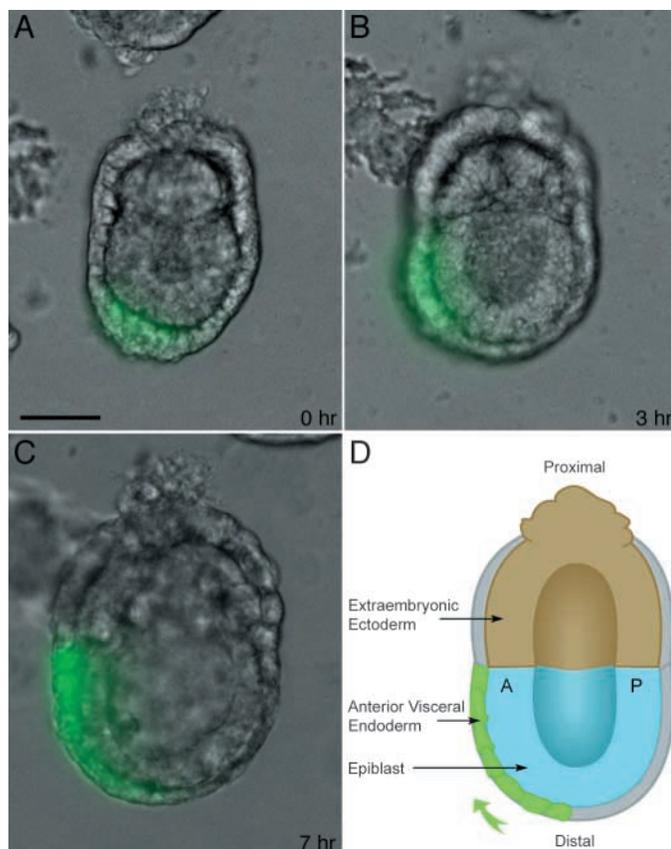


Fig. 1. Representative frames (A-C) from a movie of a cultured 5.5 dpc embryo (see Movie 1 at <http://dev.biologists.org/supplemental/>). (D) Drawing depicting the orientation of the embryo. The embryo was imaged every 15 minutes with phase-contrast and fluorescence optics. Time from the start of imaging is indicated in hours at the bottom right of each frame. EGFP fluorescence (green) marks the anterior visceral endoderm (AVE). The embryo develops normally and the AVE moves unilaterally to the extra-embryonic ectoderm in approximately 4 hours. It does not move beyond the epiblast for the remainder of the culture period. A, prospective anterior; P, prospective posterior. Scale bar in A: 50 μ m for A-D.

Perea-Gomez et al., 2001), and the epiblast becomes mispatterned such that the distal region adopts an anterior character and the proximal region a posterior character.

The central cells of the blastocoel floor of the *Xenopus* embryo (Jones et al., 1999) and the hypoblast of the chick embryo (Foley et al., 2000) are thought to correspond to the mouse AVE. The fates of these regions are similar to that of the AVE and, like the AVE, they express genes such as *Hex*. The regions also show functional similarities, capable of imposing anterior character on *Xenopus* ectoderm (Jones et al., 1999) and restricting the formation of multiple primitive streaks in chick (Bertocchini and Stern, 2002). Significantly, the anterior movement of AVE cells is conserved in the equivalent tissues in both species (Jones et al., 1999), again highlighting the significance of this process.

Although the unilateral movement of AVE cells is crucial for anterior patterning of the embryo, very little is known about how it takes place. One suggestion is that it involves different rates of proliferation of anterior and posterior cells of the

visceral endoderm or the polarised division of these cells (Lawson and Pedersen, 1987; Thomas et al., 1998). Another is that the AVE might be carried unilaterally as part of a global movement of the entire visceral endoderm (Weber et al., 1999). In contrast to these passive means of movement, it is also possible that AVE cells actively migrate to their anterior position, implying that they respond to, or are directed by, environmental cues. It is impossible to distinguish between these possibilities by studying fixed specimens, and we have therefore developed a system to observe AVE movement in real time.

Our results show that AVE cells actively migrate from the distal tip of the egg cylinder to presumptive anterior regions. Time-lapse imaging reveals that this migration comes to an abrupt halt at the junction of the epiblast with the extra-embryonic ectoderm. Cell tracking reveals that once they reach this border, cells spread laterally in both directions, with highly convoluted paths. Confocal microscopy shows that migrating AVE cells retain direct contact with the epiblast at all times. Together, our results show that the anterior movement of AVE cells is the result of active cell migration, perhaps in response to cues from the epiblast or the extracellular matrix surrounding it.

Materials and methods

Embryo dissections and culture

Embryos carrying the *Hex-GFP* transgene were obtained from *Hex-GFP* mice (Rodriguez et al., 2001) maintained on a mixed CBA/J and C57BL6 background. Mice were kept on a 10-hour light, 14-hour dark cycle, and noon of the day of finding a vaginal plug was designated 0.5 dpc. *Hex-GFP* embryos were dissected at 5.25–5.75 dpc in M2 medium using forceps as described (Beddington, 1987), and tungsten needles were used to reflect Reichert's membrane. Embryos were cultured in an FCS2 chamber (Bioptechs) with a 0.5 mm spacer to create a culture cavity. The spacer was placed orthogonally to the intended orientation, so as to cover the unused perfusion ducts. The FCS2 chamber is designed to maintain the embryos at any desired temperature. The culture medium consisted of 50% DMEM/50% rat serum pre-equilibrated for 2 hours at 37°C, in an atmosphere containing 5% CO₂. Rat serum was prepared as described (Beddington, 1987). Embryos were cultured for periods of up to 15 hours at a temperature of 37°C.

Time-lapse imaging of embryos

Phase contrast and epifluorescence digital time-lapse images were acquired using the Deltavision system from Applied Precision. Embryos were cultured directly on the stage of an Olympus IX70 inverted microscope and imaged using an Olympus 20 \times objective with a numerical aperture of 0.4. EGFP was excited using a standard 100 W mercury vapour lamp (Osram), with 490/20 excitation and 528/38 emission filters from Chroma. Exposure times were between 0.5 and 1.0 seconds per fluorescent image. Five images from different focal planes were usually captured at each time point. Fluorescent images from multiple focal planes were de-convolved and an extended-focus image was projected for each time point. When cultured embryos drifted in the field of view, projected images from different time points were manually set in register using Adobe Photoshop. QuickTime movies were compiled from individual still images using the Graphic Converter programme.

Quantitation of filopodial orientation

For the purpose of this study, filopodia were defined as cellular processes that were transient, were less than a fourth of a cell diameter

in width at their base, and had at least one side that formed an angle of less than 120° with respect to the tangent that passed through the base of the filopodium. The Velocity program (Improvisation) was used to outline 23 migrating AVE cells from seven embryos and then to calculate the centroids of each cell. The base and the tip of each filopodium on each cell were also marked, and their coordinates were determined. The length of each filopodium was calculated as the square root of $[(x_2-x_1)^2+(y_2-y_1)^2]$, where (x_1, y_1) and (x_2, y_2) are the co-ordinates of the base and the tip of the filopodium. The 'radius' of the cell was computed using the same formula, except that (x_1, y_1) and (x_2, y_2) were the coordinates of the base of the filopodium and the centroid of the cell. The length of the filopodium was expressed as a fraction of the 'radius' of the cell. The angle of the filopodium with respect to the proximodistal axis of the embryo was determined as the arc tangent of $[(y_2-y_1)/(x_2-x_1)]$, where (x_1, y_1) and (x_2, y_2) are the coordinates of the base and tip of the filopodium, respectively. The programme CricketGraph was used to plot the data as a polar graph.

Cell tracking

Cell tracking was performed using the tracking module of the Velocity programme (Improvisation). Eight cells were manually outlined using a graphics tablet and their centroids calculated at each time point. The path taken by cells was generated using Velocity. Data on the positions of the cells were imported into Microsoft Excel, where all further calculations were performed. The ratio of the movement of each cell was calculated as the absolute value of $(y_2-y_1)/(x_2-x_1)$ where (x_1, y_1) and (x_2, y_2) are the coordinates of the centroids of a cell at two consecutive time points. The ratios of all eight cells tracked were then averaged for each time point. The distance separating two cells was calculated as the square root of $[(x_2-x_1)^2+(y_2-y_1)^2]$ where (x_1, y_1) and (x_2, y_2) are the co-ordinates of the centroids of cells 1 and 2, respectively. The distance covered by a cell in each time interval was computed using the same formula, except that (x_1, y_1) and (x_2, y_2) were the coordinates of the same cell at two consecutive time points.

Phalloidin staining and confocal imaging of embryos

Embryos were fixed for 1 hour at 4°C in a solution of 4% paraformaldehyde in PBS, rinsed once at room temperature in PBT (0.1% Triton-100 in PBS) and then stained for 2 hours at 4°C in 0.5 $\mu\text{g/ml}$ TRITC-Phalloidin (Sigma) in PBT. They were then washed once at room temperature in PBT and mounted on a slide using DAPI-Vectashield mounting medium (Vector Laboratories). Confocal images of the embryo were captured on a Leica TCS-SP upright microscope and de-convolved using the Huygens programme from Scientific Volume Imaging. Confocal stacks were rendered as 3D volumes using Velocity (Improvisation).

Whole-mount in situ hybridisation

Embryos were dissected at roughly 5.75 dpc, after the AVE was likely to have moved anteriorly. Embryos were fixed in a solution of 4% paraformaldehyde in PBS and in situ hybridisation was carried out following standard procedures (Wilkinson, 1992). *Hex* (Thomas et al., 1998), and *Cer1* (Thomas et al., 1997) probes were as described.

Results

Pre-streak embryos imaged in static culture develop normally

Embryos at the early headfold stage and later can be maintained in rolling culture, under which conditions they show remarkably normal development (Tam, 1998). More recently it has become possible to maintain such embryos in static culture, making it possible to image their development in real time (Jones et al., 2002). Embryos at earlier, pre-streak stages also develop well in static culture, even progressing as

far as the primitive streak stage (Thomas et al., 1998). In order to image such embryos, we adapted a commercially available culture chamber intended for imaging living cells (see Materials and methods). Though DiI and other vital dyes have long been used to label cells during embryogenesis, there is evidence that in early mouse embryos the invasive labelling of cells perturbs their behaviour by delaying cell division (Piotrowska et al., 2001). Therefore, to visualise the cells of the AVE, we used transgenic mice that express enhanced green fluorescent protein (EGFP) under the control of *Hex* regulatory sequences (Rodriguez et al., 2001). These embryos recapitulate the early expression pattern of *Hex* (Thomas et al., 1998), initially in the distal visceral endoderm and later in the AVE.

Embryos were dissected and set up in culture at approximately 5.5 dpc (early egg cylinder stage), when *Hex* is expressed at the distal tip of the embryo. Phase-contrast and EGFP fluorescence images were captured every 10-15 minutes over a period of 8-10 hours. Of 32 embryos cultured under these conditions, 27 developed normally, as indicated by morphological criteria (expansion of pro-amniotic cavity into the extra-embryonic ectoderm and overall growth) and by the unilateral shift of the AVE from the distal tip of the egg cylinder to the prospective anterior (Fig. 1, and see Movie 1 at <http://dev.biologists.org/supplemental/>).

AVE cells migrate up to a proximal boundary

In 12 of the 27 embryos that developed normally, we were able to discern individual cells of the AVE in great detail as they moved to the prospective anterior of the embryo. AVE cells at the distal tip of the embryo are columnar, tightly clumped together and inactive (Figs 2, 5). As soon as movement is initiated, however, they undergo a dramatic change in morphology. In particular, they become squamous and motile, and they project filopodial processes, primarily in the direction of motion (Fig. 2, Fig. 3A-D,G, and Movies 3 and 5 at <http://dev.biologists.org/supplemental/>). Filopodia are frequently greater than one cell radius in length (Fig. 3G) and they often make contact with surrounding cells (Fig. 3A-D), allowing for the possibility of intercellular communication. AVE cells are occasionally seen to divide (Fig. 3E,F), but not in any consistent orientation. They show the hallmarks of migration, in that they translocate and project filopodia, predominantly in the direction of their motion.

AVE cells generally traverse the approximate $100\ \mu\text{m}$ from the distal tip of the egg cylinder to the prospective anterior in 4-5 hours (Figs 1, 2). On reaching the junction of the epiblast and the extra-embryonic ectoderm they come to an abrupt halt (Figs 1, 2), as if encountering a physical barrier (see Movies 2 and 4 at <http://dev.biologists.org/supplemental/>). The cells then start to spread laterally on the epiblast, they no longer send out filopodia and they become elongated, with their long axes parallel to the junction of the epiblast and extra-embryonic ectoderm (Fig. 2).

Tracking AVE cells

To compare the trajectories of individual AVE cells during migration, we computed the positions over time of AVE cells in representative embryos. Fig. 4 shows the tracks of eight cells in the embryo shown in Fig. 2 (see Movies 2 and 3 at <http://dev.biologists.org/supplemental/>). The tracks of AVE cells in two other embryos (Movies 4, 5, 6 and 7) are shown

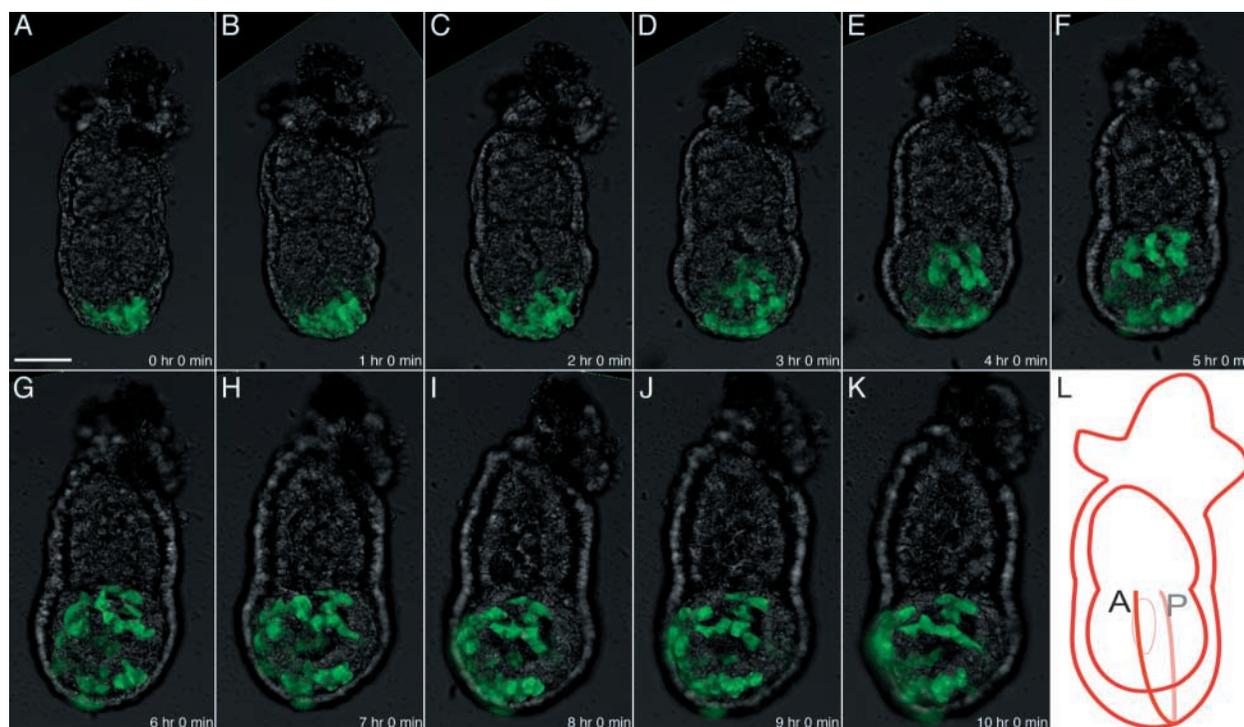


Fig. 2. Frontal view (A-K) of a developing 5.5 dpc embryo (see Movies 2 and 3). (L) Drawing depicting the orientation of the embryo, with anterior facing the reader. The embryo was imaged every 12 minutes with phase-contrast and fluorescence optics. In this figure the phase-contrast images have been made darker so that the fluorescent AVE cells can be seen more clearly. Time from the start of imaging is indicated in hours and minutes at the bottom right of each frame. AVE cells move proximally until they reach the junction of the epiblast with the extra-embryonic ectoderm and then start spreading laterally. Cells cover this distance in about 5 hours and project filopodia in the direction of motion (see Fig. 3). Note that fluorescent cells can be seen interspersed with non-fluorescent cells at time points later than 2 hours. Scale bar in A: 50 μ m for A-L.

in supplementary Fig. S1. The tracks of the eight cells in Fig. 4 show that not all reach the extra-embryonic ectoderm. The leading cells (cells 1, 2 and 3 in Fig. 4A) do reach the extra-embryonic ectoderm and start to spread laterally. The cells behind them, however (cells 4, 5, 6, 7 and 8 in Fig. 4A,B), stop migrating proximally and start spreading laterally before reaching the extra-embryonic ectoderm, presumably because they are obstructed by the leading cells. Cells 5 and 6, which are sisters, share a common track before they divide. They have separate tracks after division, but remain in contact with each other throughout their further migration (Fig. 4B). The same is observed for cells 4 and 7, which are also sister cells (Fig. 4A,B).

When migrating proximally, all eight cells show a rather direct trajectory, but when spreading laterally their trajectories become highly convoluted, albeit with a net lateral movement. This can be illustrated by calculating the ratios of the proximal movements of cells to their lateral movements at different times during development (Fig. 4C). Initially, the means of these values are greater than unity, indicating that AVE cells tend to move proximally. However, when the leading cells reach the boundary of the extra-embryonic ectoderm (red arrow, Fig. 4C) the means fall below one, indicating that although their tracks become convoluted the net displacement of AVE cells is lateral rather than proximal.

The tracks of all cells in Fig. 4 move to the left at the boundary. This is a result of the embryo ‘rolling’ during culture

(see Movie 2 at 7 hours of culture). To correct for this rolling, and to compare the lateral directions in which cells move at the boundary, we calculated the distances between cells over time, which should be less affected by the rolling. The separation between two representative pairs of cells is shown in Fig. 4D. Cells 1 and 3 move away from each other after reaching the boundary, whereas cells 2 and 4 move towards one another. This indicates that on reaching the boundary, the lateral movement of AVE cells does not occur in a coordinated manner, but that cells move independently of one another in either direction.

AVE cells migrate in direct contact with the epiblast

Our observations show that AVE cells migrate from the distal region of the embryo to the junction between the epiblast and the extra-embryonic ectoderm. Once AVE cells have migrated some distance proximally, EGFP-expressing cells can be seen intermingled with non-expressing cells (Figs 2, 3). To investigate the significance of this apparent mixing and to determine whether AVE cells migrate on top of more proximal visceral endoderm cells, we examined phalloidin-stained pre-streak *Hex-GFP* transgenic embryos by confocal microscopy.

The AVE is often discernible as a thickening of the visceral endoderm (Fig. 5A). Kimura and colleagues have suggested that the AVE is thicker than surrounding visceral endoderm because it consists of two cell layers that form a stratified cuboidal epithelium (Kimura et al., 2000). However, detailed

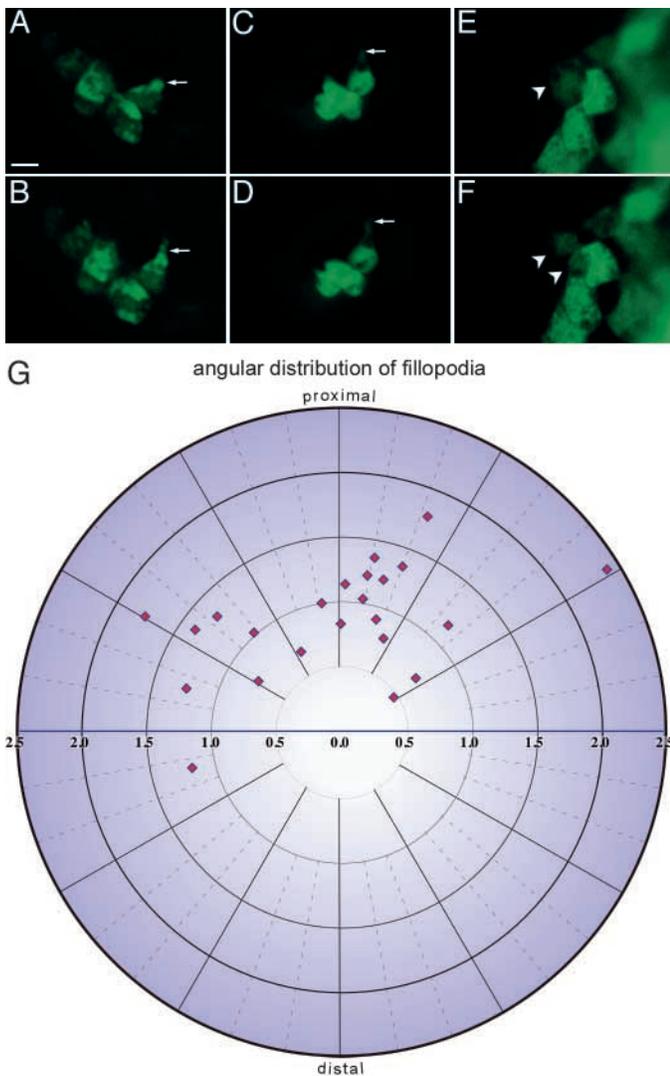


Fig. 3. Details of migrating AVE cells, showing filopodial processes. In all the panels, proximal is to the top and distal to the bottom. (A,B) Cells from one embryo, separated by an interval of 10 minutes. (C,D) Cells from a different embryo, separated by an interval of 12 minutes. Filopodia (marked with arrows) form primarily in the proximal direction (the direction of motion of the cells). (E,F) Panels separated by an interval of 10 minutes showing an AVE cell dividing (arrowhead). The orientation of the division of AVE cells is not consistently aligned to the direction of motion of the AVE, and divisions are not observed frequently enough to drive the movement of the AVE. Scale bar in A: 10 μ m for A-F. (G) A polar plot of the direction and length of 23 filopodia observed in seven embryos. The lengths of the filopodia are expressed as a fraction of the radius of the cell. Filopodia form predominantly in the proximal direction of the embryo and are often greater than one cell radius in length.

confocal imaging of six embryos shows that the AVE comprises a single layer of approximately 10–15 cells, their columnar nature accounting for the observed thickening (Fig. 5). The columnar cells of the AVE are polarised, their nuclei displaced towards the epiblast and showing stronger actin staining on the surface away from the epiblast (Fig. 5C).

As AVE cells migrate proximally, they lose their columnar

nature and become squamous. In confocal sections through nine embryos, migrating AVE cells are never seen on top of other visceral endoderm cells but are apposed to cells of the epiblast (Fig. 5D). This suggests that anterior migration occurs directly on the epiblast rather than on other cells of the visceral endoderm.

Significantly, we observe that even prior to migration, when AVE cells are at the distal tip of the embryo, they comprise a population of both *Hex-GFP* expressing and non-expressing cells (Fig. 5C), indicating that the salt-and-pepper appearance of migrating AVE cells is likely to be a consequence of the initial heterogeneity within the AVE. Although some intermingling with non-AVE cells may occur during migration, this is likely to be limited, because little cell mixing occurs in the visceral endoderm (Gardner and Cockroft, 1998). A similar salt-and-pepper pattern of expression is seen with endogenous *Hex* transcripts as well as other markers of the AVE, such as *Cerberus-like* (Fig. 6), suggesting that expression of the *Hex-GFP* transgene is a true reflection of *Hex* transcription and not a result of position effect variegation.

Discussion

We followed the behaviour of cells of the anterior visceral endoderm (AVE) of the mouse embryo as they moved towards the proximal anterior region of the embryo. Confocal microscopy revealed that the AVE consists initially of a single layer of cells that is tightly packed, polarised and columnar, causing a discernible thickening compared with the rest of the visceral endoderm (Fig. 5A–C). The expression of *Hex* at this stage, as revealed by a *Hex-GFP* transgene, is heterogeneous, with some cells expressing the gene and others not. This salt-and-pepper pattern is maintained within the AVE during migration, with GFP-expressing cells intermingled with non-expressing cells. In situ hybridisation experiments confirmed that endogenous *Hex* transcripts are also expressed in a heterogeneous manner. This suggests that the AVE comprises several different cell types that express different molecular markers. A detailed and high-resolution analysis of the expression domains of such AVE markers will be needed to resolve this issue.

Migration of AVE cells

As they begin to move proximally, cells of the AVE become squamous and their behaviour becomes highly dynamic; they change their shapes continuously and project filopodia in the direction in which they are moving. Our results suggest that most of the movement of AVE cells can be accounted for by migration. The increase in the surface area occupied by AVE cells as a result of their becoming squamous might contribute to their translocation to some extent, but is unlikely to be the primary motive force. For example, cells occasionally switch positions with respect to one another (cells 3 and 4 in Fig. 4A and Movie 3; cells 2 and 3 in supplementary Fig. S1, panel B, and Movie 7). This would not occur if cell movement were the result of passive expansion due to changes in cell shape. Cell division is also unlikely to contribute significantly to their movement because cleavage does not occur with a consistent orientation and because the movement is completed in four to five hours, during which time only one or two AVE cells are usually observed to divide. The sister cells in Fig. 4B, for

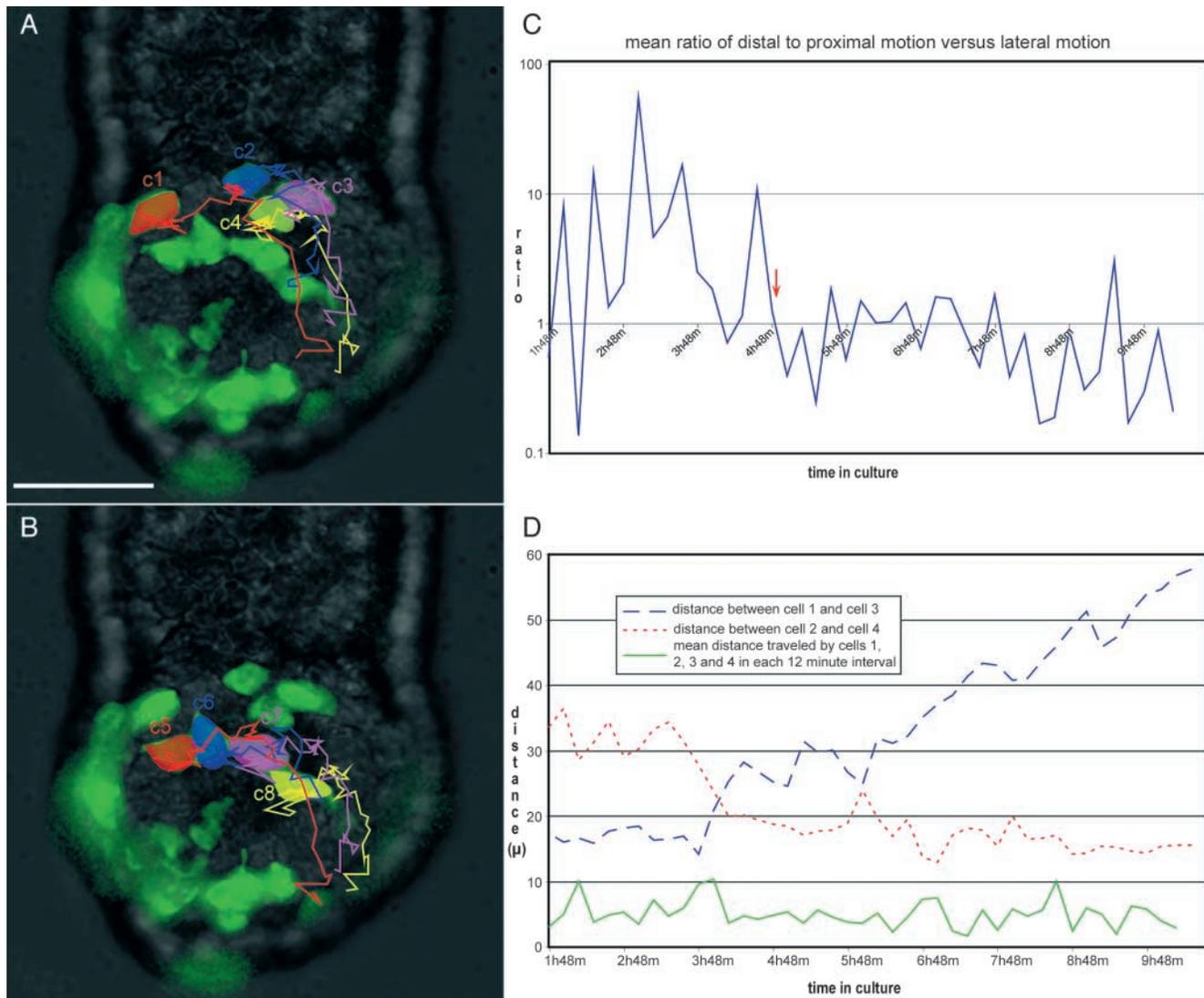


Fig. 4. Movement of individual cells of the AVE. (A,B) Tracks of eight cells overlaid on the last frame of the movie so as to show the paths they took to reach their final position (Fig. 2, Movies 2 and 3). Cells are labelled c1 to c8. Cells were tracked after 1 hour and 48 minutes had elapsed because they could not be reliably distinguished before this. Cell movements are rather direct during distal to proximal migration, but become highly convoluted once they start spreading laterally. Cells 4 and 7, and 5 and 6 are sister pairs, and hence share a common track prior to their division. (C) A quantitative measurement of cell behaviour was obtained by calculating, for each cell and at each time interval of 12 minutes, the ratio of its proximal displacement to its lateral displacement. The mean values for all eight cells were calculated for each time point, and are plotted against time in culture. Initially, the ratio tends to be substantially greater than unity, indicating that displacement is predominantly proximal. At approximately 5 hours (red arrow), when cells reach the boundary of epiblast and extra-embryonic ectoderm, the ratio decreases to less than one, indicating that motion is predominantly lateral. (D) Inspection of panels A and B suggests that cells move to the left on reaching the boundary between epiblast and extra-embryonic ectoderm. This is an artefact introduced by the fact that the embryo ‘rolled’ slightly to the left during culture (see Movie 2, at 7 hours of culture). The effects of rolling can be abrogated by calculating the separation between cells, and D plots the separation between two representative pairs of cells, as well as the average distance moved by the four cells during each 12-minute time interval. Cells 1 and 3 move apart during culture, whereas 2 and 4 come closer together, indicating that cells do not behave in a coordinated manner on reaching the extra-embryonic ectoderm. Though the distance covered by cells in each time interval varies widely, it does not show any trend over the course of culture, indicating that the cells do not slow down or speed up. Scale bar: 50 μm .

example, have migrated a substantial distance proximally before they divide. Global movement of the surrounding visceral endoderm is also unlikely to contribute significantly to AVE movement. Although most AVE cells move proximally, some invariably remain at the distal tip of the embryo (Figs 1, 2). This would not occur if the entire visceral endoderm were moving.

A barrier to migration

Our data show that AVE cells migrate proximally, to the boundary of the epiblast and extra-embryonic ectoderm, and then abruptly begin to move laterally. This boundary provides an endpoint to the proximal migration of AVE cells and positions them such that they can undertake their subsequent role of patterning the underlying epiblast. We

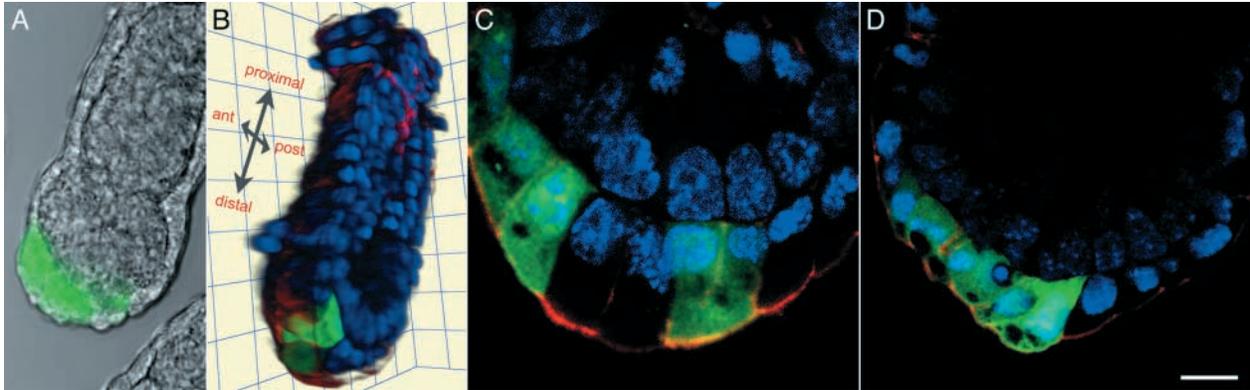


Fig. 5. Pre-streak stage mouse embryos imaged with epifluorescence and confocal microscopy. *Hex-GFP* expressed in the AVE is green, nuclei are blue (stained with DAPI) and cell borders are red (stained for actin with TRITC-Phalloidin). Each panel shows a different embryo, with anterior always to the left. (A) An epifluorescence and phase-contrast image of an embryo showing the AVE clearly discernible as a thickening of the visceral endoderm. (B) A 3D-volume rendering of a confocal image stack of an embryo at an equivalent stage, showing the plane at which the confocal sections in panels C and D were acquired. (C) A confocal section through the distal tip of an embryo, illustrating the columnar nature of the single layer of cells at the distal tip. The cells are clearly polarised, their nuclei closer to the epiblast. GFP-expressing and non-expressing cells can be seen intermingled in the AVE. (D) An embryo in which the AVE has started migrating proximally. AVE cells migrate in contact with the epiblast at all times, and are never seen on top of other visceral endoderm cells. Scale bar in D: 40 μm for A; 10 μm for C; 15 μm for D.

note that not all migrating AVE cells reach the boundary; rather, those that arrive first appear to prevent cells behind them from moving further proximally. It is possible that cells at the boundary become 'squashed' by later-arriving cells, causing them to become elongated along an axis parallel to the junction between the epiblast and extra-embryonic ectoderm.

In retrospect, the existence of this boundary can be inferred from previous cell lineage analyses. When pre-streak embryos are labelled in the region we now recognise as the AVE, and then cultured to early streak stages, labelled cells are observed to have moved proximally, but never beyond the epiblast (Lawson and Pedersen, 1987).

What prevents leading AVE cells from migrating beyond the

epiblast? One possibility is that migration is controlled by the underlying epiblast. Confocal images show that AVE cells migrate in direct contact with the epiblast. The functions of genes such as *cripto* and β -catenin, which are necessary for AVE migration, are required in the epiblast and not the visceral endoderm (Ding et al., 1998; Huelsken et al., 2000). If AVE cells require contact with the epiblast in order to migrate, this would explain why they do not migrate onto the extra-embryonic ectoderm.

Alternatively, signals from the extra-embryonic ectoderm (or the visceral endoderm overlying the extra-embryonic ectoderm) might actively repel AVE cells, preventing them from migrating beyond the epiblast. We note that later in development AVE cells do move beyond the epiblast; during gastrulation they are displaced onto the forming yolk sac by the anterior definitive endoderm (Lawson and Pedersen, 1987; Thomas and Beddington, 1996). This might occur because AVE cells have themselves changed, as suggested by the fact that they downregulate markers such as *Hex* (Thomas et al., 1998). Another possibility is that by these stages the extra-embryonic ectoderm has been displaced by the forming yolk sac, which does not repel AVE cells. Interestingly, in embryos mutant for *angiomin*, the AVE is not displaced onto the extra-embryonic region during gastrulation (Shimono and Behringer, 2003). Analysis of these mutant embryos should help resolve this issue. We hope to address these, and other questions, by continuing to observe mouse embryos in real time as they develop in culture.

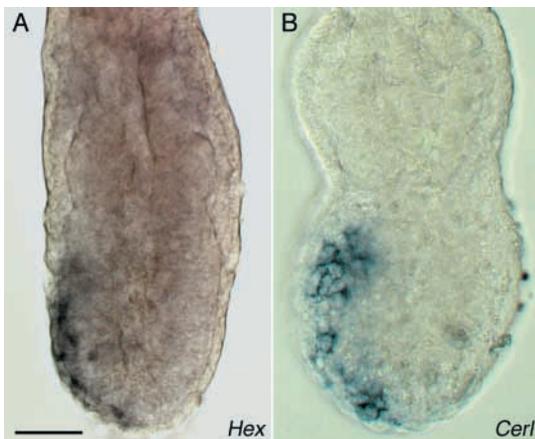


Fig. 6. Whole-mount in situ hybridisation of wild-type 5.5 dpc embryos showing expression of *Hex* (A) and *Cerl* (B). Note that not all AVE cells express these two markers of the anterior visceral endoderm. Scale bar: 50 μm .

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