

Cell movement during chick primitive streak formation

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Abstract

Gastrulation in amniotes begins with extensive re-arrangements of cells in the epiblast resulting in the formation of the primitive streak. We have developed a transfection method that enables us to transfect randomly distributed epiblast cells in the Stage XI–XIII chick blastoderms with GFP fusion proteins. This allows us to use time-lapse microscopy for detailed analysis of the movements and proliferation of epiblast cells during streak formation. Cells in the posterior two thirds of the embryo move in two striking counter-rotating flows that meet at the site of streak formation at the posterior end of the embryo. Cells divide during this rotational movement with a cell cycle time of 6–7 h. Daughter cells remain together, forming small clusters and as result of the flow patterns line up in the streak. Expression of the cyclin-dependent kinase inhibitor, P21/Waf inhibits cell division and severely limits embryo growth, but does not inhibit streak formation or associated flows. To investigate the role of cell–cell intercalation in streak formation we have inhibited the Wnt planar-polarity signalling pathway by expression of a dominant negative Wnt11 and a Dishevelled mutant Xdd1. Both treatments do not result in an inhibition of streak formation, but both severely affect extension of the embryo in later development. Likewise inhibition of myosin II which has been shown to drive cell–cell intercalation during *Drosophila* germ band extension, has no effect on streak formation, but also effectively blocks elongation after regression has started. These experiments make it unlikely that streak formation involves known cell–cell intercalation mechanisms. Expression of a dominant negative FGFR1c receptor construct as well as the soluble extracellular domain of the FGFR1c receptor both effectively block the cell movements associated with streak formation and mesoderm differentiation, showing the importance of FGF signalling in these processes.

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Introduction

The formation of the primitive streak, a condensation of cells in the epiblast, is the first visible sign of gastrulation in the chick embryo (Eyal-Giladi and Kochav Stage XIII and HH stage 2 (Eyal-Giladi and Kochav, 1976; Hamburger and Hamilton, 1992). Fate-mapping experiments show that the primitive streak derives mainly from epiblast cells overlying Koller's sickle

(Bachvarova et al., 1998; Callebaut, 2005; Lawson and Schoenwolf, 2001a). Early time lapse observation found directed movements into the forming primitive-streak (Bortier et al., 1996; Graeper, 1929; Vakaet, 1970). During these movements cells from the lateral posterior marginal zone move towards the posterior centre of the marginal zone where they join up, change direction and extend anteriorly in so called polonaise movements (Graeper, 1929). More recent quantitative velocity field analysis, analysis of time-lapse sequences of bright field images of streak formation and DiI labeling experiments, showed the existence of two counter-rotating cell flow vortices in the epiblast that merged at the site of streak formation during streak initiation (Cui et al., 2005). It was shown that all cells move actively, but the underlying mechanism remained elusive. Hatada and Stern mapped the fate of the cells in the epiblast in detail (Hatada and Stern, 1994). The dynamic transformations of

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the different cell fate domains at the early stages of development are in good agreement with the observed cell flow patterns, suggesting that these flows are a major organising principle at these pre-streak and early stages of development.

Cells overlying Koller Sickle express many genes important in later development, e.g., signalling molecules of the FGF and TGF- β families of growth factors, members of the Wnt family of signalling molecules and cell-type-specific transcription factors such as Brachyury and Gooseoid (Chapman et al., 2002, 2004; Lawson et al., 2001b). During streak formation, the expression domain of these genes co-ordinately transforms from a sickle shaped expression domain via a triangular domain, into an elongated streak stretching posterior–anteriorly along the midline. Expression of the TGF- β family member Vg1 in a region corresponding to Koller's sickle in the posterior marginal zone (PMZ) and Wnt8C in a circular domain in the marginal zone and *area opaca* seem to induce the primitive streak (Bachvarova et al., 1998; Eyal-Giladi and Khaner, 1989). Mis-expression of Vg1 leads to ectopic streak formation (Bertocchini et al., 2004; Shah et al., 1997; Skromne and Stern, 2001, 2002). These signals then induce Nodal required for streak formation and Chordin, an inhibitor of BMP signalling pathway. BMP signalling needs to be repressed for streak formation to occur (Streit et al., 1998). Hypoblast secretion of a nodal antagonist is necessary to prevent ectopic streak formation (Bertocchini and Stern, 2002). Wnt8C expression then restricts to the posterior streak and the migrating mesoderm cells leaving the streak. FGF signalling has been shown to act in a parallel pathway to the nodal signalling pathway and may act as a competence factor for mesoderm induction as is the case in frogs and fish (Bertocchini et al., 2004; Bottcher and Niehrs, 2005). FGF activates the expression of Brachyury in the streak, which may directly or indirectly be involved in the control of E and N cadherin expression (Batlle et al., 2000; Ip and Gridley, 2002; Nelson and Nusse, 2004; Nieto et al., 1994).

We know little about the cellular mechanisms responsible for streak formation nor which signals control them. Gastrulation has been most thoroughly investigated in frogs and fish (Keller et al., 2003). The streak equivalent in frogs, the *blastopore* (Arendt and Nubler-Jung, 1999; Eyal-Giladi, 1997) forms through vegetal rotation and the formation of bottle cells (Winklbauer and Schurfeld, 1999), the molecular bases of which are unknown. After involution has started frog embryos elongate through convergent extension (CE), during which cells in the mesoderm and overlying forming neuralplate move towards the midline.

These movements are due to medio-lateral intercalation of cells in the mesoderm and dorsally biased monopolar intercalation in the neuralplate (Ezin et al., 2003; Keller et al., 2000). It has been suggested that streak formation in the chick embryo also involves cell–cell intercalation, based on Dil labelling experiments where the fate of small groups of cells in different lateral positions of the streak were followed, and where it was found that these cells became intermixed with neighbouring cells over time (Lawson and Schoenwolf, 2001a,b). In frogs and fish convergent extension observed during gastrulation may occur under the control of the Wnt planar-polarity signalling

pathway, originally discovered in *Drosophila* (Adler and Lee, 2001; Keller, 2002; Ma et al., 2003; Myers et al., 2002). In this pathway Wnt11 and Wnt5 signal through Frizzled receptors to Dishevelled, which signals through the formin Daam1 to members of the Rho-family of small GTPases and their downstream effectors such as Rho kinase (Habas et al., 2001, 2003; Keller, 2002; Marlow et al., 2002; Wallingford et al., 2002). Studies of *Xenopus* CE have indicated the importance of the atypical protein kinase C δ which translocates to the membrane in response to Frizzled signalling, an event that has been shown to be required for Dishevelled translocation and Jun terminal kinase activation by Frizzled (Kinoshita et al., 2003). In the chick, Wnt11 and Wnt5 are expressed during pre-streak stages, as are some of the frizzled receptors (Chapman et al., 2004; Skromne and Stern, 2001), suggesting that they may also be involved in controlling convergent extension at this stage of development. Less is known about the expression of dishevelled and other components of this pathway (Chapman et al., 2004; Skromne and Stern, 2001).

Another mechanism proposed to be involved or drive streak formation is that of oriented cell division, in which daughter cells line up in the direction of streak extension (Wei and Mikawa, 2000). However, culturing of pre-streak embryos in the presence of the DNA-polymerase-inhibitor Aphidicolin, which effectively blocks cell division did not inhibit streak formation, though it did disrupt later gastrulation (Cui et al., 2005).

Finally, purely on theoretical grounds, it has been proposed that streak formation might involve chemotaxis (Mikawa et al., 2004; Painter et al., 2000). Cells in the epiblast could secrete an attractant for streak cells or a combination of attractants and repellents as proposed for the migration of mesoderm cells away from the streak and back towards the midline (Yang et al., 2002). However no experiments so far support this mechanism or have identified potential signalling molecules.

To investigate the cellular mechanisms of streak formation we developed an electroporation based transfection protocol that allows us to transfect very young chick embryos blastoderms with DNA expression constructs that direct the expression of GFP or GFP fusion proteins. Expression of GFP driven by the strong CMV promoter becomes visible 2–3 h after transfection. Analysis shows that predominantly randomly scattered cells in the epiblast are labelled using this procedure. These experiments allow us to analyse the above described large-scale circular cell flow patterns at the individual cell level under normal and experimentally perturbed conditions. These experiments also allow us to follow cell division during streak formation.

Materials and methods

Embryo culture and electroporation protocol

Fertile white leghorn eggs (High Sex \times Rhode Island Red; Winter Farm, Thirplow, Herts, UK) were incubated for 1–4 h at 38°C in a humid incubator before transfection and culturing. Embryos were cultured using the Easy Culture (EC) technique (Chapman et al., 2001), with the hypoblast side pointing up. To transfect the embryos we carefully pipette 1.0 μ l DNA (from a Qiagen plasmid Midi Prep, 5–10 μ g/ μ l, dissolved in PBS) onto the embryo. At these early stages

of development the hypoblast is not yet completely sealed, thus the DNA has direct access to the epiblast. The embryo is then moved from EC culture to the electroporator consisting of two square (0.16 cm²) platinum electrodes. The bottom electrode (anode) is fixed in the dish and pre-wetted by 0.15 M NaCl, the upper electrode (cathode) also pre-wetted with 0.15 M NaCl is positioned 2 mm above the embryo using a micromanipulator. The embryos are transfected with one square 50 ms pulse of 15 V and with the anode as the ventral electrode using a custom made “electroporator” (Momose et al., 1999). After transfection, the embryos were transferred back onto the agar plates and incubated at 37°C in a humidified incubator. Expression of GFP expression constructs was readily detectable 2 to 4 h cells after transfection, while the expression of GFP fusion constructs normally took longer (4–6 h) most likely due to the lower transfection efficiency of larger constructs. We then transferred the embryos to a custom-made air-heated microscope incubation chamber, kept at 38°C and 90% humidity, and imaged development for the next 15–20 h (Yang et al., 2002).

For visualisation of GFP fluorescence, some embryos were fixed at different times after transfection in 4% paraformaldehyde in PBS (pH 7.4) and processed for cryostat sectioning.

Embryos and sections were photographed using a Nikon fluorescence dissecting microscope equipped with a Nikon DXM1200 digital camera. Confocal microscopy was performed with a Leica SP2 confocal microscope.

Constructs and chemicals

pEGFP-N1 (Clontech) was used as the GFP expression vector. The p21CIP/WAF expression construct (pcDNA3.1) and was kindly provided by Dr. Kathryn Ball, Dundee, which was also co-transfected with pEGFP-N1. The *Xenopus* dnWnt1 expression construct (Hoppler et al., 1996; Tada and Smith, 2000), was co-transfected with the pEGFP-N1 expression vector. Dr. Sergei Sokol provided the Xdd1 (a *Xenopus* Dishevelled mutant lacking the PDZ Domain) plasmid (Rothbacher et al., 2000; Sokol, 1996) was also co-transfected with pEGFP-N1.

The dnFGFR1-GFP was described in detail previously (Yang et al., 2002). The FGFR1-Fc expression vector was made by PCR amplifying chick FGFR1c extracellular domain with *EcoRI*-forward (GACTGAATTCATGTTTACCTG-GAGG TGCCTCATCC) and *Bgl*III reverse primers (GATCAGATCTTACCTG-TCTGCGCAGTGGGATGCTC) using pFGFR1c-YFP plasmid the PCR fragment (1287 bp) was inserted into pFUSE-hFc1 expression plasmid (Invivogen).

In situ hybridisation with the Brachyury probe (Smith et al., 1991) was performed according to standard published procedures (Wilkinson and Nieto, 1993).

Phalloidin staining was performed by fixing embryos overnight in a solution of 4% paraformaldehyde in PBS (pH 7.4), followed by 2 washes in PBS, permeabilised, in PBS containing 0.1 Triton X100 for 20 min and incubated in rhodamine phalloidin (100 µg/ml) in PBS for 20–40 min. Blebbistatin was obtained from Calbiochem and dissolved to 100 mM in 90% DMSO. Rhodamine phalloidin was obtained from Sigma and dissolved DMSO to 10 mM.

Imaging and analysis

Brightfield and fluorescence images were taken on a Zeiss Axiovert 135TV inverted microscope, using a Hamamatsu ORCA-II-ER CCD Camera and fitted with a 37°C purpose built stage incubator, every 3 min as detailed in (Yang et al., 2002). We used routines in the Optimas VI (Media Cybernetics) macro language or macros for Image J to reconstruct cell movement trajectories from successive time-lapse images (Image J plugins will be made available on request). We analysed cell division manually by tracking individual GFP-expressing cells and measuring the time interval between successive divisions to the nearest 3 min.

Results

Cell movement during primitive streak initiation involves two counter-rotating vortices

Previously we have shown using optical flow image processing techniques and by following the trajectories of multiple

groups of DiI labelled cells in prestreak chick embryos that the tissue movements occurring during streak formation are detectable from pre-streak stages onwards and are organised in two counter rotating cell flow patterns that meet at the site of streak formation (Cui et al., 2005). To be able to analyse these cell movements at the individual cell level, we developed an electroporation protocol that allowed us to transfect scattered cells in the early chick-embryo epiblast cells with GFP expression constructs. Our electroporation protocol typically results in the transfection of several hundred to thousands of cells in the *area pellucida* and *area opaca* in a mosaic pattern (Figs. 1A, B, C). Sectioning of the early transfected embryos confirmed that the majority of the cells (>90%) transfected were found in the epiblast. (Figs. 1D, E). GFP fluorescence of expression constructs under the control of the CMV promoter becomes visible 2–4 h after transfection and the fluorescence intensity/cell normally increases during the next 5–10 h of incubation. This labelling method allowed us to track the movement and division of individual cells for up to 24 h of development. Every track shown in Figs. 2C, F, I is the movement trajectory of an individual GFP-expressing cell, over a 4-h period. These cell tracking experiments confirmed the existence of the two counter-rotating cell flows in the epiblast, which merged at the site for the future streak primitive streak initiation (Fig. 2). The flows become visible well before any optically-dense structure in the streak are detected. Cells overlaying Koller’s sickle move into the streak and are being replaced by cells from more antero-lateral positions. Cells just anterior to the Sickle move forward and sideways into the area destined to become the neural plate (Hatada and Stern, 1994). The cells in the centres of both counter-rotating flows move relatively little. These centres do not coincide with any known morphological structures, suggesting that they are not special signalling centres. Speeds of cell movement vary from 0 to 2 µm/min, with the highest speeds at the periphery of the vortices. These data confirm our previous observations using DiI labelling (Cui et al., 2005), with better spatial coverage and at cellular resolution.

In some embryos transfected very early in their development cells initially moved more or less iso-tropically outward (Fig. 2C), as cell division increased the surface area of the epiblast. However, after a few hours, the movements suddenly (within 20 min) changed geometry into the rotational flow (Figs. 2F, I). The change in the flow is essentially simultaneous everywhere and does not appear to be initiated from a particular point in the embryo. Longer cell tracking showed that cells trace rather parallel paths, with some variation in azimuthal velocity. There is relatively little evidence for mixing of cells in the two streams after they merge, since the tracks of individual cells very rarely appear to cross and or merge. It can also be seen that the tracks of cells that start close together are still found close together after 4 h at the end of the track showing highly correlated spatial movement.

Cell division during primitive streak initiation

Observation of the GFP labelled cells showed that they divide while migrating. We investigated the relationship between cell division, movement and streak formation. Pre-

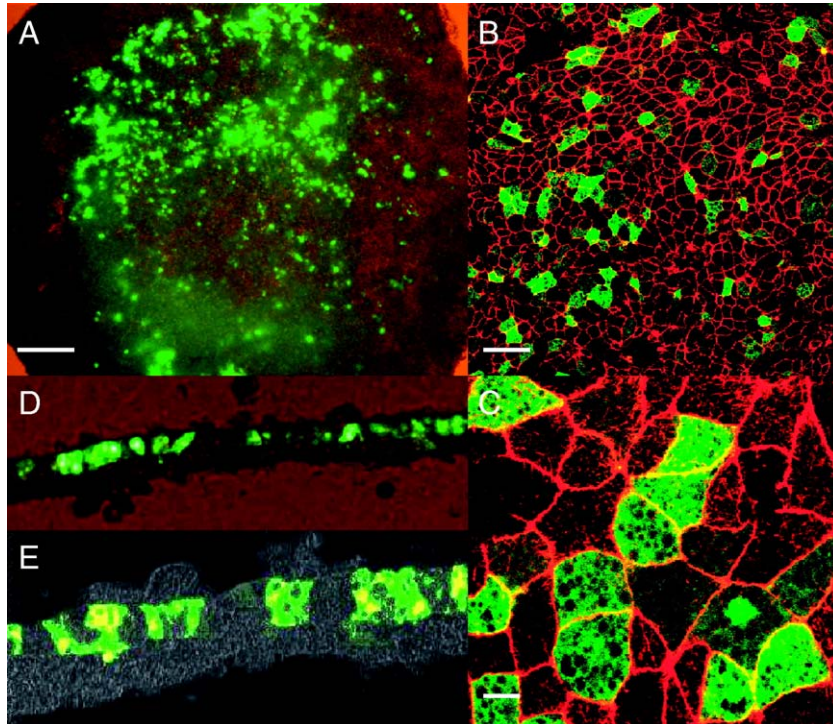


Fig. 1. Transfection of cells in the early chick blastoderm results in predominant transfection of the epiblast. (A) Low magnification image of an embryo 5 h after the start of development and 3 h after transfection with pEGFP-N1 (Size bar 0.5 mm). (B) Confocal image of a section of the embryo shown in panel A where the actin cytoskeleton has been stained with rhodamine phalloid (Size bar 50 μ m). (C) Higher magnification image of same embryo shown in panels A, B. (Size bar 10 μ m). (D) Section through the embryo shown in panels A–C. (E) Confocal image of section of embryo shown in panels A–D showing clear staining of cells in the upper epiblast and no labeled cells in the lower hypoblast layer. All embryos in this and subsequent figures are shown with the epiblast side on top. The embryo is oriented along the anterior–posterior axis with Koller's sickle (only faintly visible) at the bottom of the image (A).

viously, we showed that during pre-streak stages, cells in S-phase distribute more or less randomly in the epiblast (Cui et al., 2005). Culturing the embryos in the presence of the DNA-polymerase inhibitor aphidicolin blocked streak elongation but not streak formation (Cui et al., 2005). We have now followed the division of individual cells during streak formation. Cells in the outer periphery divided 2–3 times during the 20 h of observation, the average cell cycle time was 378 ± 30 min per division ($N = 34$). If all cells in the epiblast divide at this rate and the epiblast is essentially two-dimensional, the area of epiblast would increase roughly 4-fold or the embryo radius 2-fold in 12 h, roughly in agreement with experimental observations during this stage of development. We do not know whether all cells have similar cycle times, but we observe similar timed divisions everywhere in the anterior, posterior, medial and lateral epiblast. Furthermore, these experiments showed that cells located in the streak typically divided only once, but no more than two times during streak extension. This is in agreement with the observation that the cell cycle of these cells is around 6.5 h, i.e., too slow to produce long lines of daughter cells necessary to account for streak formation as has been suggested previously (Wei and Mikawa, 2000).

To further investigate the need for cell division during streak formation, we expressed the cyclin-dependent-kinase (CDK) inhibitor p21Cip/Waf (Ball et al., 1997), which blocks the cell cycle in S phase, in the epiblast before streak formation. We confirmed the action of p21Cip/Waf by the absence of BrdU

incorporation of P21-expressing cells (data not shown). Expression of p21Cip/Waf in a mosaic of cells in the epiblast, resulted in many of the p21Cip/Waf-expressing cells to move into the forming streak (Fig. 3D). Although transfection greatly reduced overall embryo size (compare Figs. 3A and C), it did not noticeably alter streak formation. These observations strongly support our previous findings that cell division is not the driving force for streak formation and elongation (Cui et al., 2005).

Inhibition of the Wnt planar-polarity signalling pathway does not inhibit streak formation

The transformation of the crescent-shaped Koller's sickle into the perpendicular, elongated streak might suggest that the streak forms as a result of local cell–cell intercalation. Based on observation of dispersal of DiI-labelled cells during the formation of the primitive streak the suggestion was made that streak formation results from intercalation of cells in Koller's sickle to form the streak (Lawson and Schoenwolf, 2001a).

It has been suggested that intercalation of cells during gastrulation is under the control of the Wnt mediated planar polarity pathway. Ligands specific to the planar-polarity pathway, e.g., Wnt5 and Wnt11 are expressed, Wnt 5A in the outer area opaca and Wnt 11 throughout the epiblast and hypoblast of the *area pellucida* in pre streak embryos (Chapman et al., 2004; Skromne and Stern, 2001). To

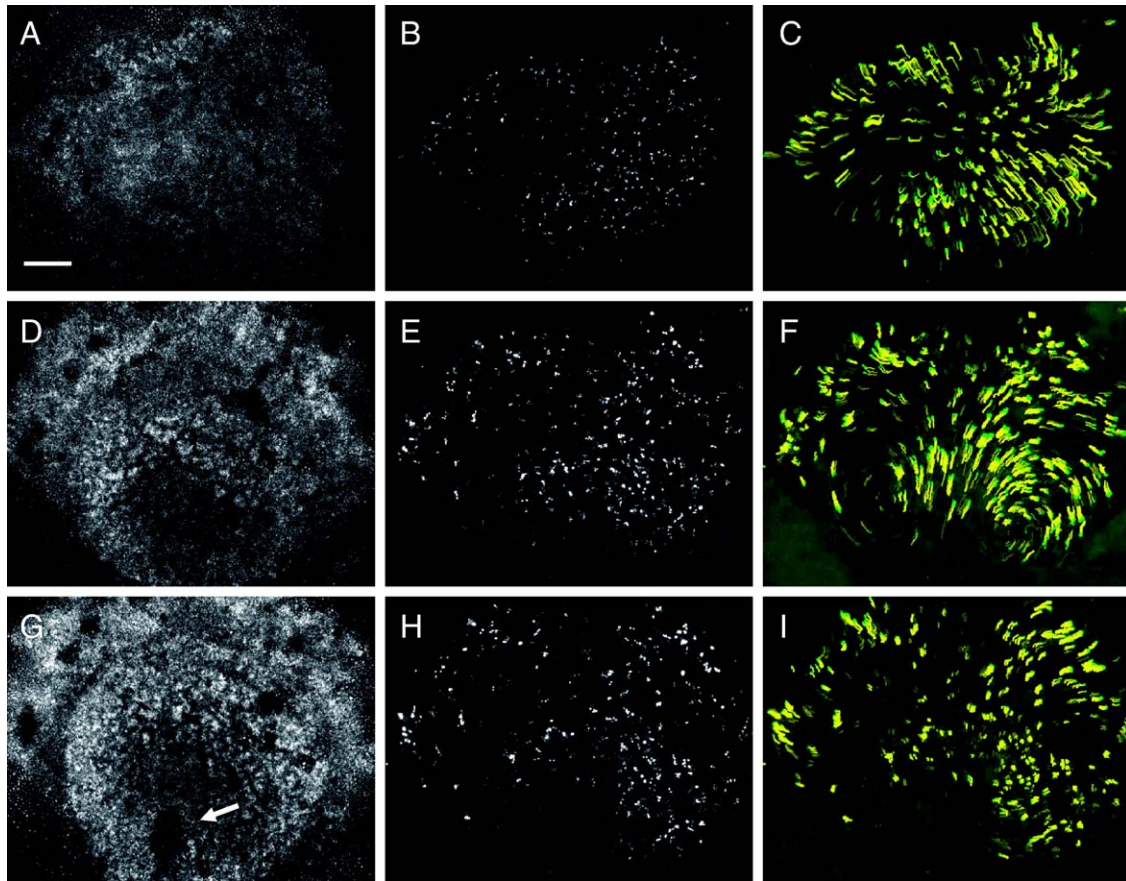


Fig. 2. Transfected embryos and tracks of GFP labelled cells during streak formation. We transfected a pre-streak embryo with a GFP expression vector pEGFPN1 and tracked the movement of individual GFP-expressing cells as described in Materials and methods. (A–C) Bright-field image, fluorescence image and cell trace of the same embryo. The cell trace is calculated over a period of 4 h. The green part indicates the movement during the last hour. The images shown are taken 7 h after the start of development, 4 h after transfection. (Size bar in panel A is 0.5 mm). (D–F) The same images of the same embryo 15 h after the start of the experiment. (G–I) The same images of the same embryo 18 h after the start of the experiment. All images of embryos are taken looking upon the epiblast in this and all following figures. (For additional information on the dynamics of the process see movie 1). The embryos are oriented with the tip of the streak pointing to the top. The white arrow in panel G points towards the tip of the forming primitive streak.

investigate the possible involvement of the planar polarity signalling pathway, we expressed a *Xenopus* dominant-negative Wnt11 construct, that effectively inhibits convergent extension in *Xenopus* (Tada and Smith, 2000), in the early chick epiblast and investigated the effects on cell movement and streak formation. The primitive streak initiated normally in almost all cases (9/10) and the initial vortices of cell movement in the epiblast were normal (Figs. 4C, F). However, although the streak formed and elongated, it extended primarily in a posterior direction, resulting in massive posterior-directed cell movements normally not observed in control embryos (Fig. 4I). These data suggest that Wnt11 signalling, while not absolutely necessary for primitive streak initiation and elongation, could be involved in anterior streak extension. Anterior and posterior streak extensions are experimentally separable, as we have shown previously using local inhibition of actin-polymerisation by applying beads soaked in the inhibitor Latrunculin A (Cui et al., 2005).

Convergent extension could depend on extracellular signals other than Wnt11, therefore we looked for involvement of planar-polarity signalling by blocking planar polarity signalling

through downstream intracellular components. A dishevelled construct lacking the an N-terminal Dix (Dishevelled and Axin) domain has been shown to block the planar polarity pathway and convergent extension in frog embryos (Rothbacher et al., 2000; Sokol, 1996; Wallingford et al., 2000). Pre-streak embryos expressing the Xdd1 construct (Figs. 5C, D) formed normal streaks that were indistinguishable from control embryos (Figs. 5A, B). However, their later development was abnormal due to problems with regression, which resulted in defective elongation of the embryonic axis (Figs. 5G, H) when compared to control embryos (Figs. 5E, F), suggesting that the construct was active, but that signalling through the Wnt planar polarity pathway is not essential for streak formation.

Finally we performed experiments with the Rho kinase inhibitor Y27632. The results showed that streaks formed (13/15 embryos, data not shown). However, later in development, major defects in heart formation and primitive-streak regression were observed in agreement with earlier findings (Marlow et al., 2002; Wei et al., 2001).

Recently, it has been shown that planar cell intercalation occurring during germband extension in *Drosophila*, is

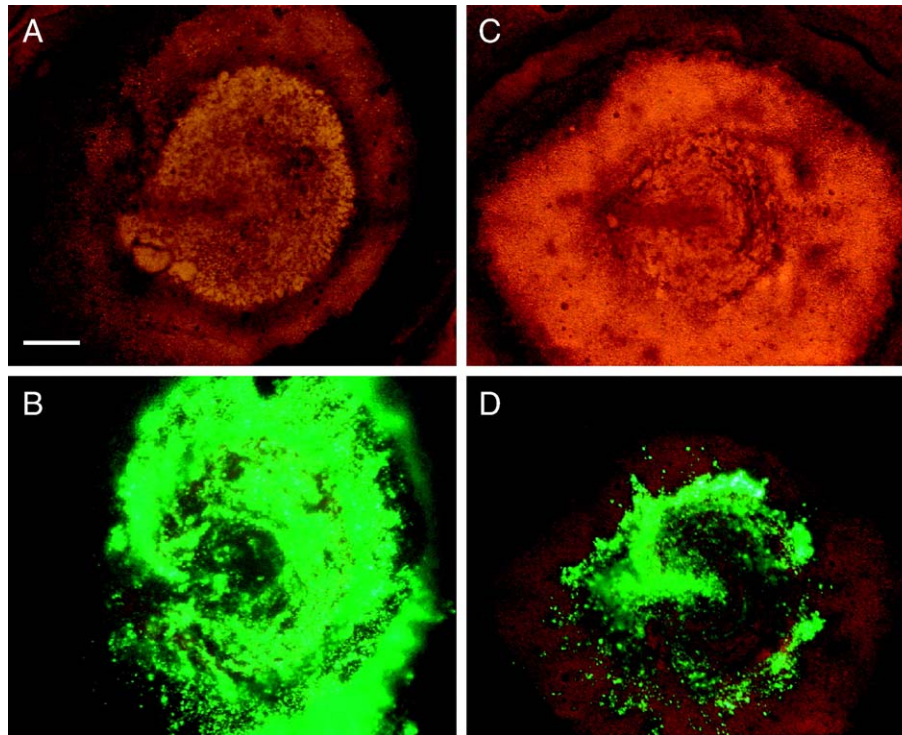


Fig. 3. Expression of p21Cip/Waf does not inhibit streak formation. (A) Bright-field image of a control embryo 18 h after electroporation with a pEGFP-N1 expression construct at Stage XIII. (Size bar 0.5 mm). (B) Merged fluorescence/bright-field image of the same control embryo showing the distribution of GFP transfected cells. (C) Bright-field image of an embryo expressing the CDK inhibitor p21Cip/Waf, also 18 h after transfection of a stage XIII embryo. (D) Merged fluorescence/bright-field image of the same embryo shown in panel C. Expression of p21Cip/Waf substantially reduces embryo growth, compare control (A) to p21Cip/Waf-expressing embryo (C), but does not inhibit streak formation (image width 4 mm). The embryos are oriented with the tip of the streak pointing to the right.

controlled by remodelling of cell–cell junctions and that this remodelling is dependent on the non-muscle myosinII (zipper) (Bertet et al., 2004). To test whether a myosin II-dependent cell intercalation mechanism could underlie streak elongation we investigated the effect of the myosinII specific inhibitor blebbistatin, which specifically blocks Myosin II motor function (Limouze et al., 2004; Ramamurthy et al., 2004; Straight et al., 2003), on streak formation. In these experiments un-incubated embryos were cultured in EC culture on plates containing 5 μ M blebbistatin for 48 h. Their development was compared to control embryos. There was no detectable effect on streak formation and elongation up to stage HH4 (Figs. 6A, C). The timing and morphological appearance of the streak was indistinguishable from that in control embryos. We noted however a very striking effect on the elongation of the embryo after the regression of the streak had started (Figs. 6B, D). The embryos did not elongate properly resulting in very short streaks and tightly condensed and too few somites as well as excessive large heads (Fig. 6B). These results show that the inhibitor worked effectively at the concentration used and that myosinII motor function is not critically important for the processes underlying streak extension. These experiments thus rule out a Myosin II-dependent cell intercalation mechanism, shown to underlie germband extension in *Drosophila*, as a mechanism for streak formation in the chick embryo. The results also show that Myosin II is required for elongation of the embryo during the regression phase of the streak, possibly through an effect on convergent extension. The role of Myosin

II in elongation of the embryo remains to be established by detailed observation of the cell behaviours at high magnification during regression. Furthermore it remains to be established in which tissues myosin II function is required in future experiments.

Taken together, absence of dramatic effects of expression of a dominant negative Wnt11, a dominant negative dishevelled, and the use of the Rho kinase inhibitor Y27632 and the myosinII inhibitor blebbistatin on streak formation make it unlikely that the planar polarity signalling pathway controls the early phase of streak formation. The effects of these treatments on later development show, however, clearly that the planar-polarity signalling pathway is important in later development during axis elongation as is the case in other vertebrate embryos (Keller, 2002; Keller et al., 2003; Wallingford et al., 2002).

FGF signalling is required for primitive-streak formation

The molecular signals controlling cell movement during primitive-streak initiation are so far unresolved. The FGF-family of signalling molecules are candidates for movement regulation during gastrulation since they have been shown to be involved in the control of cell movement during the later stages of gastrulation in the chick embryo (Yang et al., 2002). Furthermore, FGF receptors, especially FGFR1, express strongly in the early epiblast and several FGFs are expressed during early development (Karabagli et al., 2002a; Walshe and

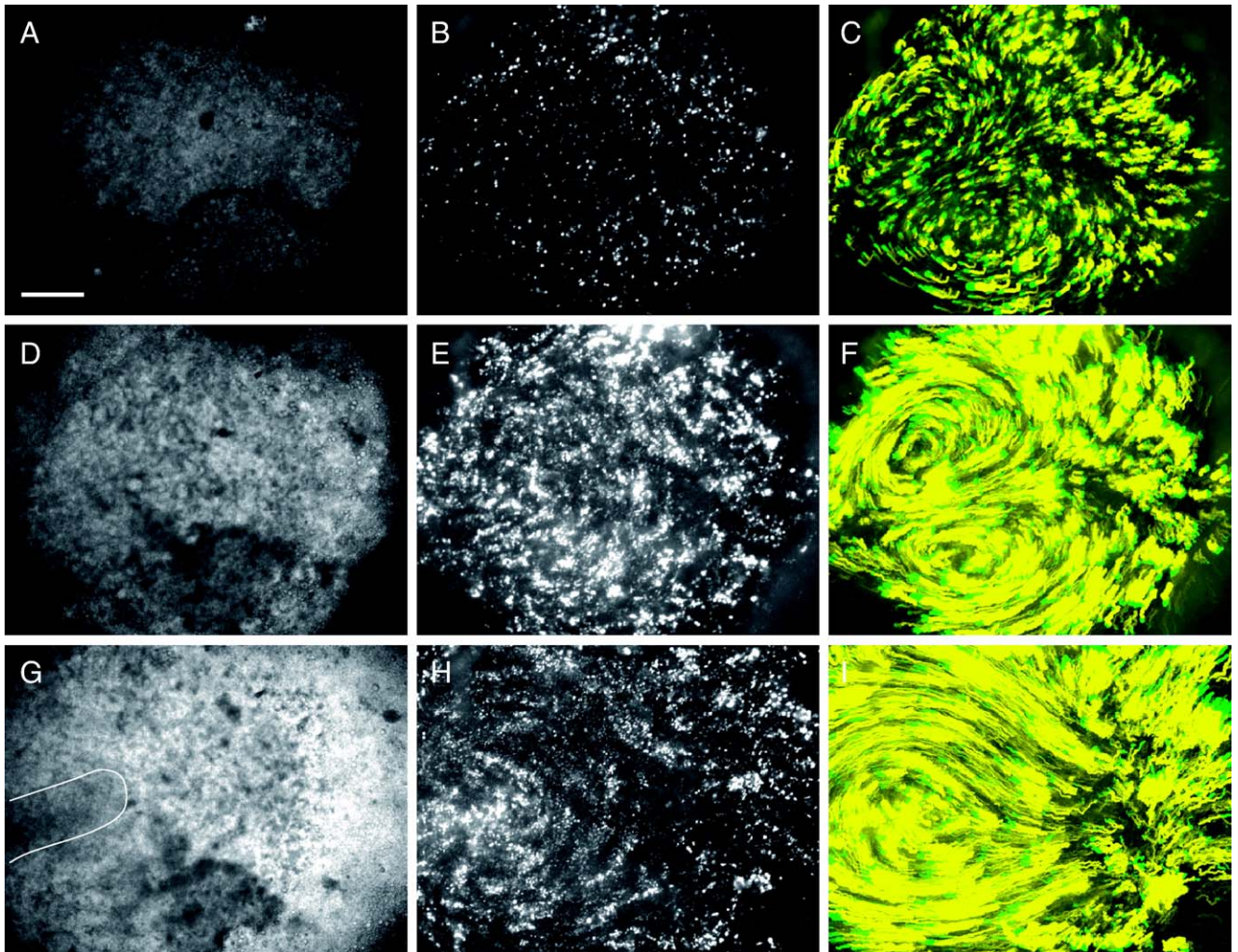


Fig. 4. Expression of dominant-negative Wnt11 does not inhibit streak formation, but inhibits the anterior extension of the primitive streak. (A–C) Bright-field image, fluorescence image and cell tracks of cells expressing a dominant negative Wnt11 construct. Tracks are calculated over 4 h, 4 h after transfection at stage XIII. (Size bar in panel A is 0.5 mm). (D–I) Same images after 9 and 18 h from the beginning of the recording respectively. Long before streak formation becomes visible the initial characteristic counter-rotating vortices are visible. However, once the streak forms (G–I), it does not extend anteriorly, the streak elongates by adding more cells to its posterior end. This change in behaviour is reflected by the cell tracks pointing backwards towards the bottom of the streak (I), where they appear to join in the streak. The movement of the cells over the last hour is shown in green. (see movie 2). The white line in panel G indicates the position of the streak with the tip of the streak pointing slightly up and to the right.

Mason, 2000) (Chapman et al., 2002; Karabagli et al., 2002b). To investigate the role of FGFs during streak initiation, we transfected the blastoderm with a dominant-negative FGFR1c construct (Yang et al., 2002). Expression of this construct in the epiblast before streak initiation resulted in highly abnormal development and prevented the formation of a normal streak (Figs. 7A–F). Vortices never formed, we only observed some radial outward movement due to cell division in the epiblast. To investigate the requirement for FGFs further we expressed a soluble FGFR1-Fc expression construct. These secreted fragments, dimerise and soak up extracellular FGF ligands, according to their binding specificity (Ornitz et al., 1996). Expression of these Fc-fragments in the epiblast of prestreak stage embryos resulted in very dramatic effects on development. It resulted in total inhibition of streak formation (Figs. 7J, K) and complete inhibition of FGF signalling as judged by the almost complete inhibition of expression of the FGF target gene

Brachyury (Smith et al., 1991) (Fig. 7L). These results show that FGF signalling is critical for streak formation and mesoderm differentiation.

Discussion

Patterns of epiblast movement

Formation of the primitive streak involves large-scale tissue remodelling. Two counter-rotating vortices meet and merge at the posterior pole of the embryo, starting well before the streak becomes visible as a condensation of cells at the ventral midline (Fig. 2). These movements translocate the epiblast cells overlying Koller's sickle destined to become the mesoderm and endoderm into the central midline of the embryo, where they will form the primitive streak. The flow patterns are very reproducible from embryo to embryo (compare Figs. 2C, F, 3C,

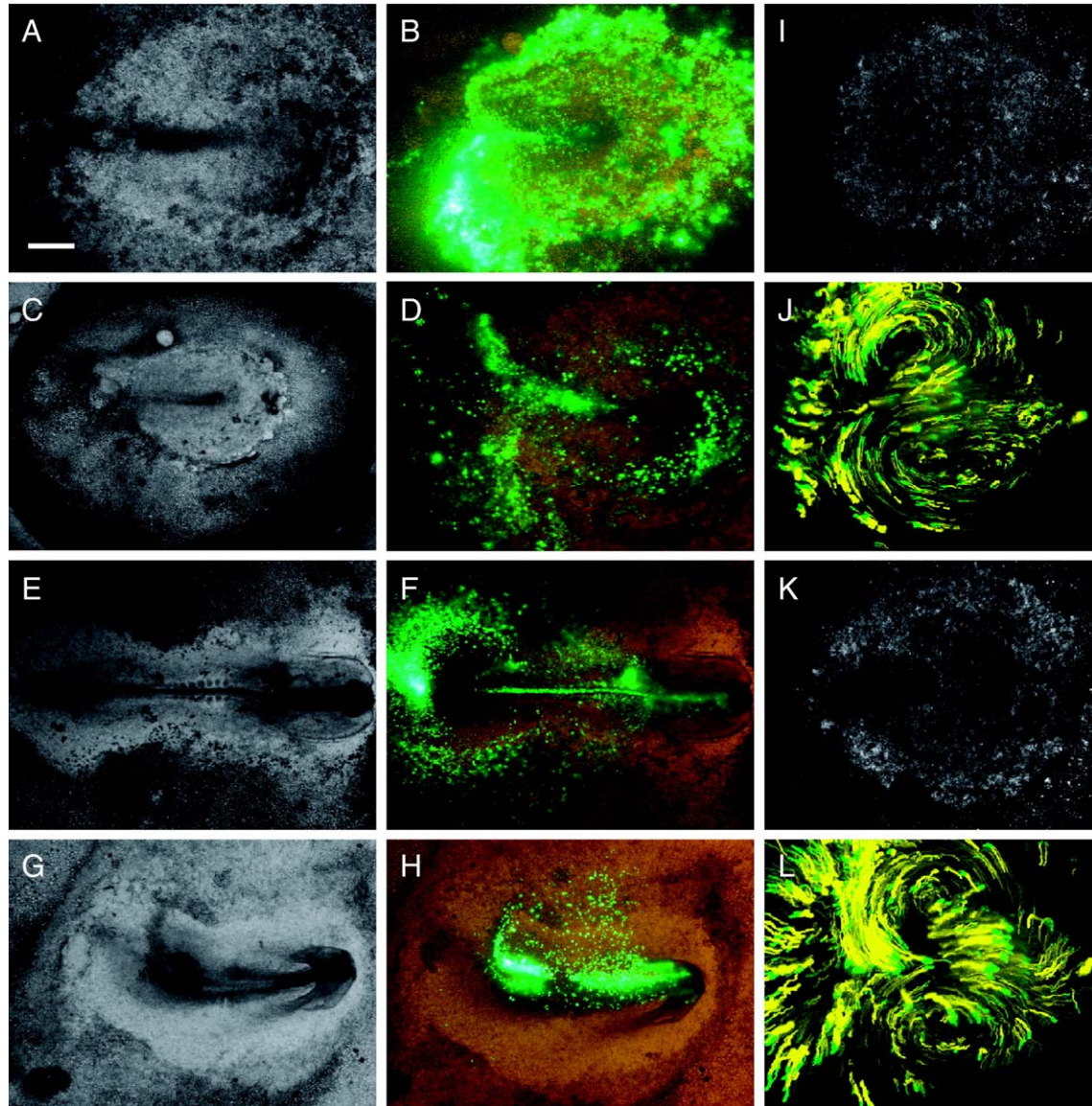


Fig. 5. Expression of Xdd1 does not inhibit the primitive-streak formation. (A, B) Fluorescence and bright-field images of an embryo transfected with a GFP expression construct at stage XIII and photographed after 18 h of development (Size bar is 0.5 mm). (C, D) Fluorescence and bright-field images of an embryo transfected with an Xdd1 expression construct at stage XIII and photographed after 18 h of development. (E, F) Fluorescence and bright-field images of an embryo transfected with a GFP expression construct at stage HH3 and photographed after 18 h of development. (G, H) Fluorescence and bright-field images of an embryo transfected with an Xdd1 expression construct at stage HH3 and photographed after 18 h of development. (I, J) Bright field image and cell track (4 h) of an embryo transfected with Xdd1/pEGFP-N1. (K, L) Bright field image and cell track (4 h) of an embryo transfected with Xdd1/pEGFP-N1 6 h after the images shown in panels I, J (see movie 3 for dynamics). Expression of the dishevelled mutant Xdd1 lacking the Dix domain does not inhibit streak formation (A, C). However, later development is severely abnormal suggesting that Xdd1 does interfere with planar-polarity signalling during later development (E, G). The embryos are oriented with the tip of the streak/heads pointing to the right.

4J). It seems plausible that the rotational flows constitute a centring mechanism that enables the streak to form precisely in the midline of the embryo. The observations on these large scale cell flows complement and extend earlier observations made of the flows of groups of cells during streak formation using carbon particle and DiI marking techniques (Cui et al., 2005; Graeper, 1929; Lawson and Schoenwolf, 2001a; Spratt, 1946; Vakaet, 1970) at the single cell level.

So far, the mechanisms underlying the vortex movement during streak formation are unresolved, but through the development of the electroporation techniques described in

this paper, it has now become possible to track the long-term movement of individual epiblast cells and interfere with possible signalling pathways that control their behaviour through the expression of dominant active and negative components of these pathways as well as analyse the effects of small molecule chemical inhibitors on these movements. We have now started to use this approach to investigate the mechanisms underlying streak formation. To start we have concentrated on two previously proposed mechanisms, oriented cell divisions and cell–cell intercalation and our results show that they are not the main mechanisms underlying streak formation.

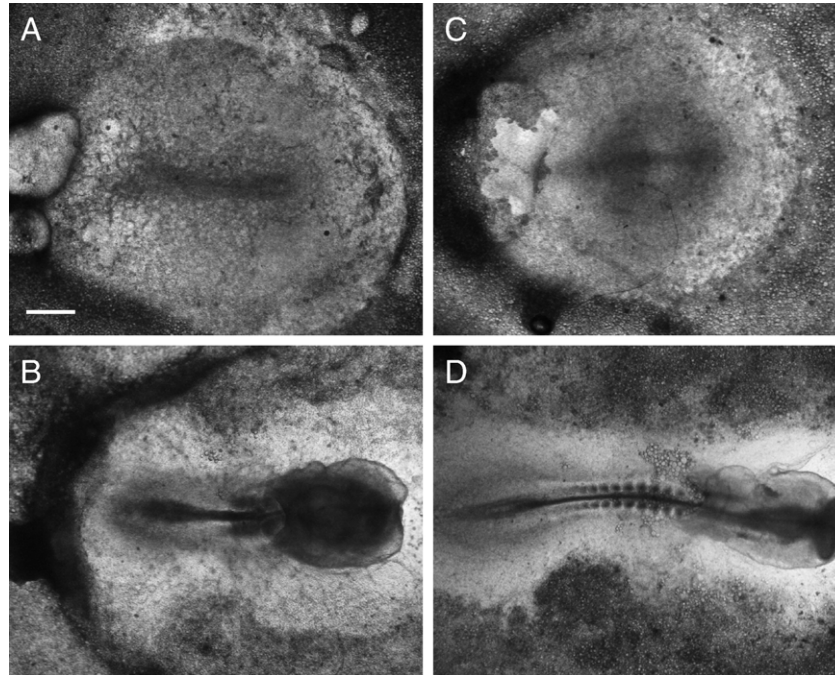


Fig. 6. Blebbistatin does not inhibit streak formation, but does inhibit regression. (A, B) Un-incubated embryos were put in EC culture on agar–albumin plates containing 5 μ M blebbistatin and photographed after 24 (A) and 48 h (B) respectively. (Size bar in panel A = 0.5 mm). (C, D) Un-incubated embryos were put in EC culture on agar–albumin plates. The embryos were photographed after 24 h (C) and 48 h (D), respectively. Streak formation (A, C) is not affected by blebbistatin at the concentration used, but that later development (B, D) is severely disturbed, there is a strong defect in the elongation of the embryo resulting in few compacted somites and large heads. These results are typical for results obtained in all embryos (15) investigated. The embryos are oriented with the tip of the streak (A, C) and heads (B, D) pointing to the right.

Wei and Mikawa suggested that oriented cell division might drive streak formation (Wei and Mikawa, 2000). The division time of cells moving in the streak is too long to account for extension, since the average cell cycle time is 6.5 h, while streak extension only takes around 12 h. Oriented cell division in the direction of streak elongation could at most elongate the streak 4-fold in the time available, which is not sufficient to achieve the observed 10- to 15-fold increase in length. Experiments where cell cycle progression was inhibited by expression of the CDK inhibitor p21Cip/Waf in over 50% epiblast cells resulting in at least a 50% reduction in embryo size (Figs. 3A, C) show that normal streaks still form (Fig. 3). These observations are complemented by our earlier findings that when we inhibit cell-cycle progression with a small molecule DNA polymerase inhibitor Aphidicolin, streak formation is initiated and the associated cell flow patterns start to develop normally (Cui et al., 2005). The vortex flows make it easy to understand how daughter cells will align along the flowlines in the direction of the streak extension, resulting in the observed grouping of daughter cells and even grand daughters in the direction of streak elongation (Wei and Mikawa, 2000).

Signalling through the planar polarity pathway does not drive streak formation

It has been suggested based on experiments where the epiblast overlying Koller's sickle was injected with two distinct fluorophores, that the formation of the streak is the result of

medio-lateral intercalation of cells in Koller's sickle to form the streak (Keller et al., 2003; Lawson et al., 2001a). Our more detailed cell tracking data, however, show that daughter cells, stay close together, compared to the overall length of their movement trajectories and that siblings align along the flow lines, but do not reveal any evidence for systematic intercalation, which would be visible as merging or even crossing cell tracks in our experiments. Therefore, the cell track data do not strongly support the involvement of cell–cell intercalation in streak formation.

Furthermore, although a cell–cell intercalation mechanism can easily give rise to tissues contraction along one axis and elongation along an axis at 90° angles, it is difficult to imagine how cell–cell intercalation can produce circular tissue flow patterns as observed in the experiments described here (Figs. 2, 4, 5). In agreement with these findings, we could not uncover a major role for the planar polarity signalling pathway in streak formation since neither expression of a dominant negative Wnt11 construct, nor that of a planar polarity specific dishevelled construct Xdd1 nor blocking of a downstream Rho kinase seemed to show any major effects on streak formation, although these treatments all more or less severely impeded development during the elongation phase of the embryo after streak regression had started, indicating that they were affecting this pathway. These experiments thus indicate that the Wnt planar-polarity signalling pathway is not critical for streak formation, but is important in later development during axis elongation, as is the case in other vertebrate embryos (Keller, 2002; Keller et al., 2003; Wallingford et al., 2002).

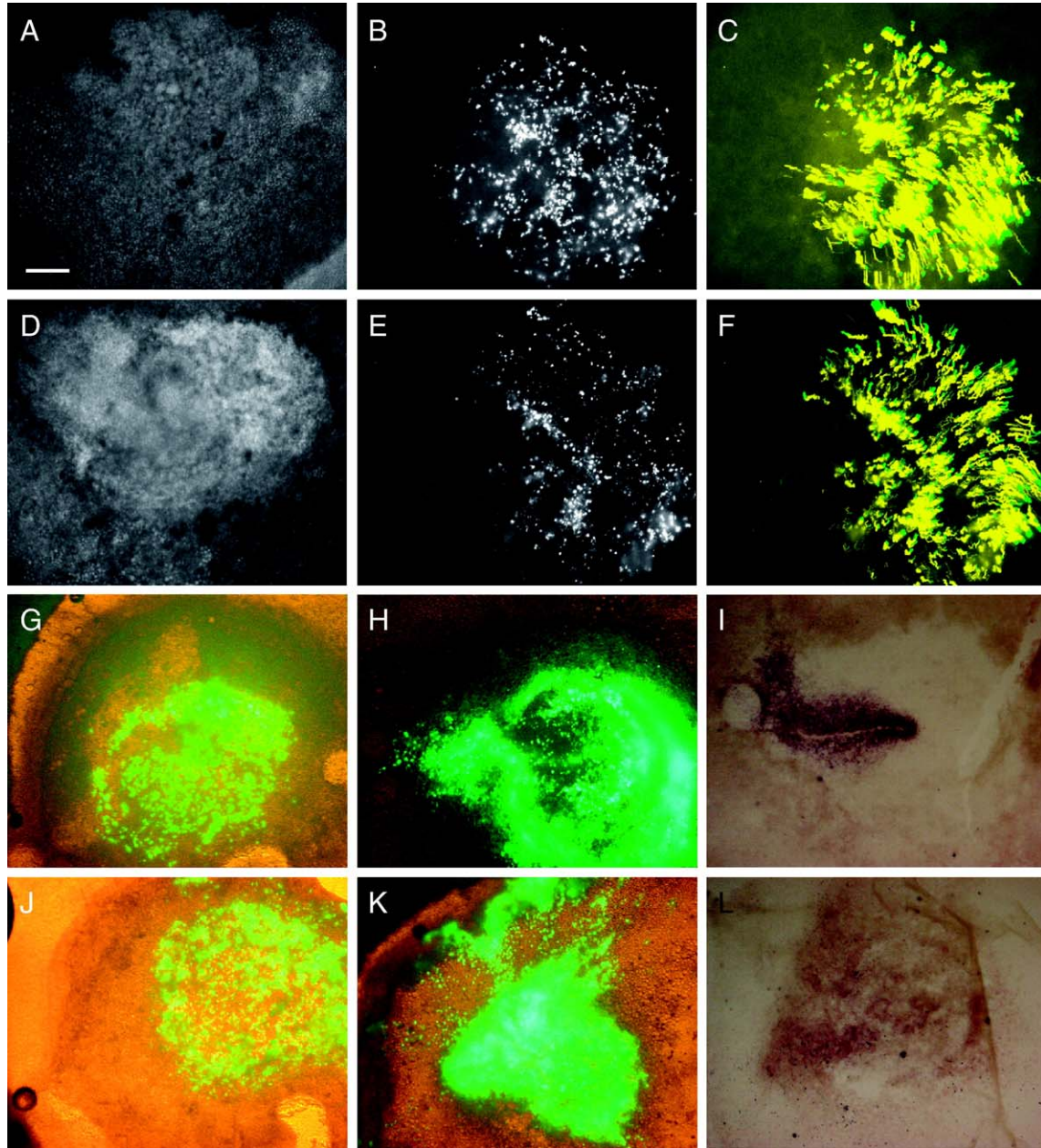


Fig. 7. Inhibition of primitive-streak formation by a dominant-negative FGFR1c construct and a FGFR1c-Fc fragment. (A–C) Bright-field image, fluorescence image and cell tracks calculated during 4 h of development, beginning 5 h after the transfection of a stage XIII embryo. (see movie 4). (D–F) Same images as in (A–C) of the same embryo, 6 h later. (Size bar in panel A is 0.5 mm). Expressing a GFP tagged dnFGFR1 receptor inhibits primitive streak and vortices in the epiblast. The colour coding of the tracks is as described in Fig. 2. Images are 3.3 mm wide. (G–I) Embryo transfected after 2.5 h incubation with pEGFP-N1. The images were taken 5 h after transfection, (G) 21.5 h later (I) Brachyury expression in the same embryo shown in panel H as detected by in situ hybridisation with a dioxygenin labelled RNA probe. (J–L) Embryo transfected after 2.5 h incubation with a FGFR1c-Fc. The images were taken 5 h after transfection (J) 21.5 h later (K). (L) Brachyury expression in the same embryo shown in panel K as detected by in situ hybridisation with a dioxygenin labelled RNA probe, not the absence of Brachyury expression. Note the lack of expression in FGFR1c-Fc-expressing embryos. We only observed 3 small Brachyury-expressing streaks in 12 transfected embryos, while we observed 12 Brachyury-expressing streaks in 13 control embryos transfected with pEGFPN1. The embryos are oriented with the tip of the

Germband extension in *Drosophila*, depends on local cell–cell intercalation, which requires the non-muscle myosinII. However the myosin II specific inhibitor blebbistatin had no major effect on streak formation, but severely affected streak regression. This clearly shows that streak formation does not depend on MyosinII-dependent junctional remodelling as proposed for *Drosophila* (Bertet et al., 2004).

Final evidence for the existence of appreciable cell–cell intercalation during streak formation has to come from studies performed at much higher magnification where it will be possible to follow the behaviour of individual cells for longer periods of time. This also will allow the investigation of the polarisation of the cells during streak formation which could also be indicative of the underlying mechanisms as has been

shown so elegantly in Frogs and Fish embryos (Keller, 2005; Keller et al., 2003; Sepich and Solnica-Krezel, 2005; Ulrich et al., 2005).

FGF signalling and the control of streak formation

Since neither cell division nor intercalation appears to be the major mechanisms driving streak formation we have started to consider alternative mechanisms. The tissue flow movements appear to start everywhere simultaneously, within the limits of the experimental observations. Applying an actin polymerisation inhibitor suggested that all cells in the forming streak move actively (Cui et al., 2005). Since the cells move in a highly coordinated fashion this implies that all cells respond to signals instructing them where to go. It appears feasible that some of the cells are guided by extracellular signalling molecules present in the form of gradients, which the cells sensitive to these factors can follow. It has been proposed on theoretical grounds that long-range chemo-attractants or chemo-repellents to which all or some epiblast cells respond could also coordinate cell movement during streak formation (Cui et al., 2005; Mikawa et al., 2004; Painter et al., 2000). Since many FGFs are expressed in the streak and they are known as potent attractants we have started to investigate whether FGFs guide streak formation. When we express a dominant-negative FGFR1c construct at sufficiently high levels it inhibits streak formation (Figs. 7A–F) as does taking away FGF ligands through expression of the FGFR1-Fc construct. However, these treatments also resulted in strongly reduced expression of the FGF target gene *Brachyury* in agreement with findings in chick and other organisms (Amaya et al., 1991; Bertocchini et al., 2004; Bottcher and Niehrs, 2005). Therefore, we can at this moment not discriminate between the possibilities that FGFs act as instructive cues that guide the movement of cells during streak formation or that as a result of inhibition of FGF signalling cells don't express receptors for other so far unknown guidance molecules. It is interesting to note that it has recently been shown in *Xenopus* that FGF directs the expression of the Neurotrophin related receptors NHR1a,b, which appear to be involved in the control of convergent extension movements in Keller explants (Chung et al., 2005; Sasai et al., 2004). The signalling pathways downstream of these receptors are still poorly understood, but they seem to exert their effects through activation of members of the Rho and Rac family of small G proteins. That manipulation of the signalling pathways downstream of the FGF receptor, after their divergence into distinct pathways leading to differentiation and movement may be possible, is suggested by findings in *Xenopus*, that show that overexpression of *Sprouty2* inhibits FGF-dependent signalling to PKC δ and Ca $^{++}$ and results in a dramatic inhibition of convergent extension, without affecting mesoderm differentiation. The FGF-dependent Map-kinase signalling that appears to control mesoderm differentiation can be selectively inhibited through expression of the *Sprouty*-related (*Spred*) proteins (Sivak et al., 2005). It will be interesting to see whether chick *Sprouty* and *Spred* homologues can be used to dissect the role of FGF mediated signalling pathways in the control of cell movement during primitive-streak formation in the chick embryo.

Finally, during their movement, the cells in the epiblast continually have to make and break contacts with other cells, which will require the control of turnover of adhesion molecules, as during germ-band extension in *Drosophila* (Bertet et al., 2004) and shield formation in zebrafish (Montero et al., 2005). It has been shown that FGF signalling is also very important in signalling and regulation of cell–cell adhesion via cadherins (Ciruna and Rossant, 2001; Nelson and Nusse, 2004) and FGF signaling could, through this mechanism, affect epiblast movement during streak formation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.451.

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