

Cell Movement Patterns during Gastrulation in the Chick Are Controlled by Positive and Negative Chemotaxis Mediated by FGF4 and FGF8

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Summary

During gastrulation in amniotes, epiblast cells ingress through the primitive streak and migrate away to form endodermal, mesodermal, and extraembryonic structures. Here we analyze the detailed movement trajectories of cells emerging at different anterior-posterior positions from the primitive streak, using *in vivo* imaging of the movement of GFP-tagged streak cells. Cells emerging at different anterior-posterior positions from the streak show characteristic cell migration patterns, in response to guidance signals from neighboring tissues. Streak cells are attracted by sources of FGF4 and repelled by sources of FGF8. The observed movement patterns of anterior streak cells can be explained by an FGF8-mediated chemorepulsion of cells away from the streak followed by chemoattraction toward an FGF4 signal produced by the forming notochord.

Introduction

Gastrulation, the process that establishes the three-layered body plan of the embryo, is one of the main morphogenetic events that shape the early embryo. It involves the coordinated movement of hundreds to thousands of cells. Through the pioneering studies of Keller and coworkers, much has been learned about the cellular basis of gastrulation in amphibians, especially *Xenopus* (Keller et al., 2000; Keller and Winklbauer, 1992). *Xenopus* gastrulation is driven by radial intercalation of cells in the animal cap, which underlies the epiboly of the ectoderm and convergence and extension of the mesoderm and overlying neuroectoderm, resulting in the elongation of the embryo. Wnt signaling pathways have been shown to be involved in controlling various aspects of cell movement during gastrulation (Heisenberg et al., 2000; Wallingford and Harland, 2001; Wallingford et al., 2000; Winklbauer et al., 2001), but the precise mechanisms by which these signals control cell movement are still unknown. During *Xenopus* gastrulation all cells are embedded in epithelial sheets, and, therefore, gastrulation is the result of a rearrangement of cells within these sheets. In avian and mammalian embryos, the process, although similar in principle, is different at the cellular

level. The early chick embryo consists of two layers, the epiblast and hypoblast. The epiblast gives rise to extraembryonic structures and all the structures of the embryo proper, while the hypoblast gives rise to extraembryonic structures only. Gastrulation starts with the formation of the primitive streak, which forms as a thickening of the epiblast at the posterior margin. The initial formation and anterior extension of the streak may involve the convergent extension of epiblast cells overlaying Koller's sickle (Keller et al., 2000; Lawson and Schoenwolf, 2001; Lawson et al., 2001b). The anteriormost part of the streak is known as Hensen's node and acts as the avian organizer (Boettger et al., 2001; Joubin and Stern, 1999, 2001). When the streak is reaching half-maximal extension, epiblast cells in the streak undergo an epithelial to mesenchymal transition and start to move as individual cells into the space between the epiblast and the hypoblast to form axial and lateral mesoderm as well as definitive endoderm (Lawson and Schoenwolf, 2001). Extensive knowledge of the fate of the cells at different stages during development has been gained through fate-mapping studies, with radioactive tracers, fluorescent labels, and chick-quail chimeras (Garcia-Martinez and Schoenwolf, 1992; Hatada and Stern, 1994; Le Douarin et al., 1996; Nicolet, 1965, 1970; Psychoyos and Stern, 1996; Schoenwolf et al., 1992; Teillet et al., 1999); however, there exists much less insight into the cellular behavior during gastrulation in avian and mammalian embryos than in *Xenopus* and fish. Furthermore, little is known about which signals guide the movement of the epiblast cells and the endoderm and mesodermal cells at different stages of gastrulation.

Chemotactic signals coordinate cell movement in a variety of developmental systems and contexts. In many cases these signals are members of the FGF family of growth factors. FGF's have been shown to be able to act as chemoattractants during development of the tracheal system in *Drosophila*, the migration of sex myoblast cells during *C. elegans* gonad development, and limb development in the chick (Branda and Stern, 1998; Chen and Stern, 1998; Dossenbach et al., 2001; Li and Muneoka, 1999; Montell, 1999). Furthermore, in *Drosophila*, FGF signaling has been implicated in controlling gastrulation movements, especially the spreading of the mesoderm cells over the ectoderm (Wilson and Leptin, 2000), and, in *Xenopus*, there is considerable evidence that FGF is involved in controlling the convergent extension movements of the mesoderm (Amaya et al., 1991; Kroll and Amaya, 1996; Nutt et al., 2001). The phenotypes of knockout mice provide strong evidence that FGFs are involved in controlling cell movement during gastrulation, since FGF8 and FGFR1 mutants show severe defects in cell movement at the primitive streak stages (Ciruna and Rossant, 2001; Ciruna et al., 1997; Rossant et al., 1997; Sun et al., 1999). It is, however, not yet clear whether these effects are due to FGFs directly controlling movement or whether FGFs mediate their effects indirectly via effects on expression of target genes, which then control cell movement.

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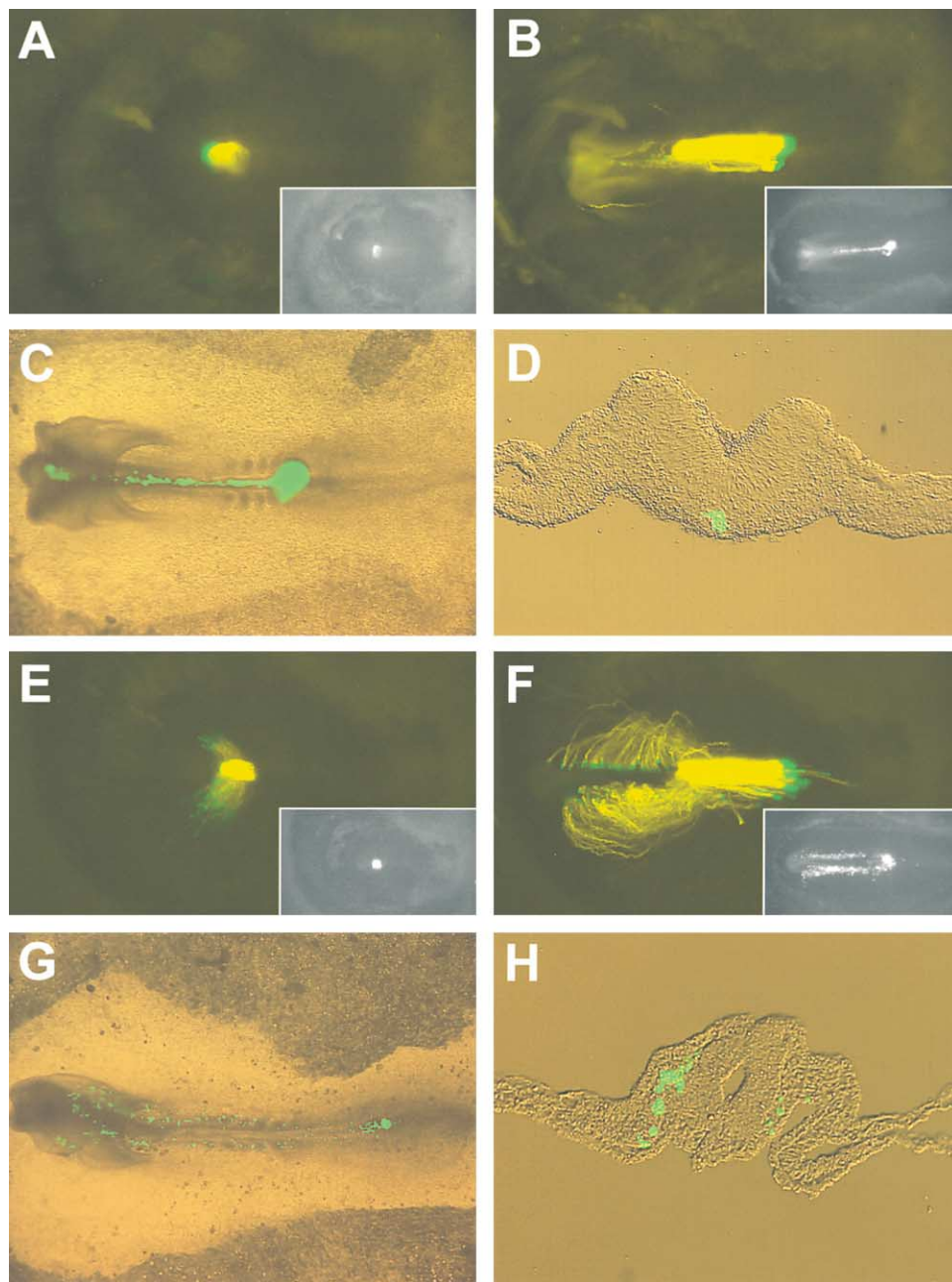


Figure 1. Cell Movement Trajectories and Fate of Cells from the Node and Anterior Streak

Embryos were transfected with an EGFP expression vector at HH stage 3, and, after 4–5 hr incubation, either the node (A–D) or the anterior streak (E–H) from a transfected donor embryo was transplanted into a nontransfected host embryo. Images were taken over the next 15 hr at 3 min intervals.

(A) Movement pattern of EGFP-expressing node cells during the first 50 images, (150) minutes after transplantation. The lines represent the movement tracks of individual cells, and they have been processed to show the direction of movement. The initial part of the track is yellow, and the movement during the last 20 images is shown in green. The track shows that, during the first 50 images, the streak still extended toward the anterior of the embryo (on the left in all panels). The inset in the lower right hand corner shows a still image taken at the start of the experiment.

(B) Cell movement patterns over 15 hr of development show that some cells have moved forward (thin lines going to the left); the thick track shows the regression of the node during the course of the experiment. The inset image in the right hand corner shows a still fluorescence image taken at the end of the experiment. It can be seen that, during regression, many labeled cells stay in the node, while others are left behind to form the notochord (C and D). The inset in the lower right hand corner shows a still image taken at the end of the experiment.

(C) Fluorescent and phase contrast merged images of a similar embryo to that shown in (A) and (B) after 15 hr of development.

(D) Transverse section through the embryo shown in (C). GFP-expressing cells are found mainly in the notochord.

(E and F) Movement patterns of EGFP-expressing cells emerging from the anterior streak.

(E) Movement during the first 150 minutes.

(F) Movement of the cells during 15 hr of the experiment. It is clearly shown that the cells move out laterally from the streak, and they move

Here we perform a detailed analysis of cell movement patterns of cells emerging at different anterior-posterior positions from the fully extended primitive streak stage, HH stage 4 (Hamburger and Hamilton, 1951), in the chick embryo. We have established a novel approach where we graft GFP-expressing primitive streak cells into unlabeled host embryos cultured in New culture (New, 1955), allowing us to follow the cell movement of individual cells *in vivo* for 12–20 hr of development. We find that cells in different positions along the streak show different movement patterns and that their movement is controlled by signals from the surrounding cells. Streak cells are attracted by the head process and factors produced in the border between the area opaca and the area pellucida. We also show that streak cells are attracted to FGF4 and repelled by FGF8b and propose that these factors control the movement of the cells in the embryo.

Results

Movement Patterns of Cells Emerging from the Streak in an HH3 Stage Embryo

To investigate the movement patterns of individual cells emerging from various anterior-posterior positions of the streak of an HH stage 4 embryo, we grafted pieces of EGFP- or EYFP-expressing streak tissue into an unlabeled host embryo as described in Experimental Procedures. Grafts were either placed in the same position (homotypic) or in a different position (heterotypic), and the movement of the cells was analyzed by taking images every 3 min during the next 12–15 hr of development. The resulting images were processed as described in Experimental Procedures to visualize the movement tracks of individual cells. The movement tracks of individual cells (thin lines) or groups of cells, such as the graft (thick lines), are shown in yellow from the beginning of the experiment, while the movement over the last hour of the experiment is shown in green (see Supplemental Data at <http://www.developmentalcell.com/cgi/content/full/3/3/425/DC1> for examples of the movies obtained for every experiment described in this paper). Using this technique we have examined the behavior of cells in the node, the streak just posterior to the node, the middle primitive streak, and the most posterior part of the primitive streak.

Movement of Cells in the Node

When we grafted GFP-labeled node tissue into the equivalent position of an unlabeled host, we found that, during the very last phase of streak extension, a few cells moved out from the node and forward, where they form part of the head process. During node regression fluorescent cells were left behind in a narrow continuous line in the midline of the embryos (Figures 1A, 1B, and 1C). Sections of several of these embryos showed that

these cells formed mostly notochord, in agreement with fate-mapping experiments (Figure 1D; Psychoyos and Stern, 1996). During regression many fluorescent cells stayed in the node, suggesting that these cells may be part of a stem cell population that forms the notochord during regression.

Movement of Cells in the Anterior and Middle Streak

Analysis of movement of the cells, which originate from just behind the node, showed a very different type of behavior. These cells initially moved out laterally, away from the streak, and, once the node had regressed past these cells, they moved back in again toward the midline of the embryo (Figures 1E, 1F, and 1G), effectively resulting in a movement “around” the node. Serial sections of these embryos showed that most of the cells formed medial somites (Figure 1H). The outward lateral movement was even more pronounced in the cells that emerged from the middle primitive streak (Figures 2A and 2B). Initially these cells moved rapidly outward to the periphery of the embryo, and then their movement slowed down. In many instances, they seemed to pause until the node had regressed past them, and then the cells started to move inward again. These cells gave rise mainly to lateral plate mesoderm (Figures 2C and 2D). Quantitative analysis of the cell movement speed with a specialized optical flow detection algorithm (Siegert et al., 1994) showed that the cells moved at 2–3 $\mu\text{m}/\text{min}$ during the outward movement from the streak, then slowed down to $<1 \mu\text{m}/\text{min}$ until the node had regressed past them, and then speeded up again to 2–3 $\mu\text{m}/\text{min}$ during the inward movement.

Movement of Cells in the Caudal Streak

The cells that originated from the caudal primitive streak moved out in lateral and caudal directions, giving rise to a wide fan-shaped pattern of cell movement (Figures 2E and 2F). These cells move very directionally and at relatively high speed and give rise mainly to extraembryonic structures (Figure 2G). These experiments demonstrated that cells in the anterior streak move along very directed trajectories with very little random movement. Furthermore, cells in anterior regions of the streak made two distinctly different types of movement: first, cells moved outward, away from the streak, and this was followed by a phase of movement directed inward, back toward the central midline of the embryo. When we labeled cells on only one side of the streak, we found that, in addition to the movement patterns described above, a proportion of cells crossed the midline and emerged on the other side of the embryo (Figure 2H). In general these cells displayed similar movement behavior to the cells that had not crossed over, and they moved outward, away from the streak.

back in toward the midline and forward after the node has regressed passed them. This is even more apparent in the video images, which can be viewed in Supplemental Data at <http://www.developmentalcell.com/cgi/content/full/3/3/425/DC1>.

(G) Image of a similar embryo at the end of the experiment showing that the cells from the grafted anterior streak are mainly found in somitic tissue.

(H) Transverse section through the embryo shown in (G) showing that, at the end of the experiment, the GFP-expressing cells (green) are located in somites.

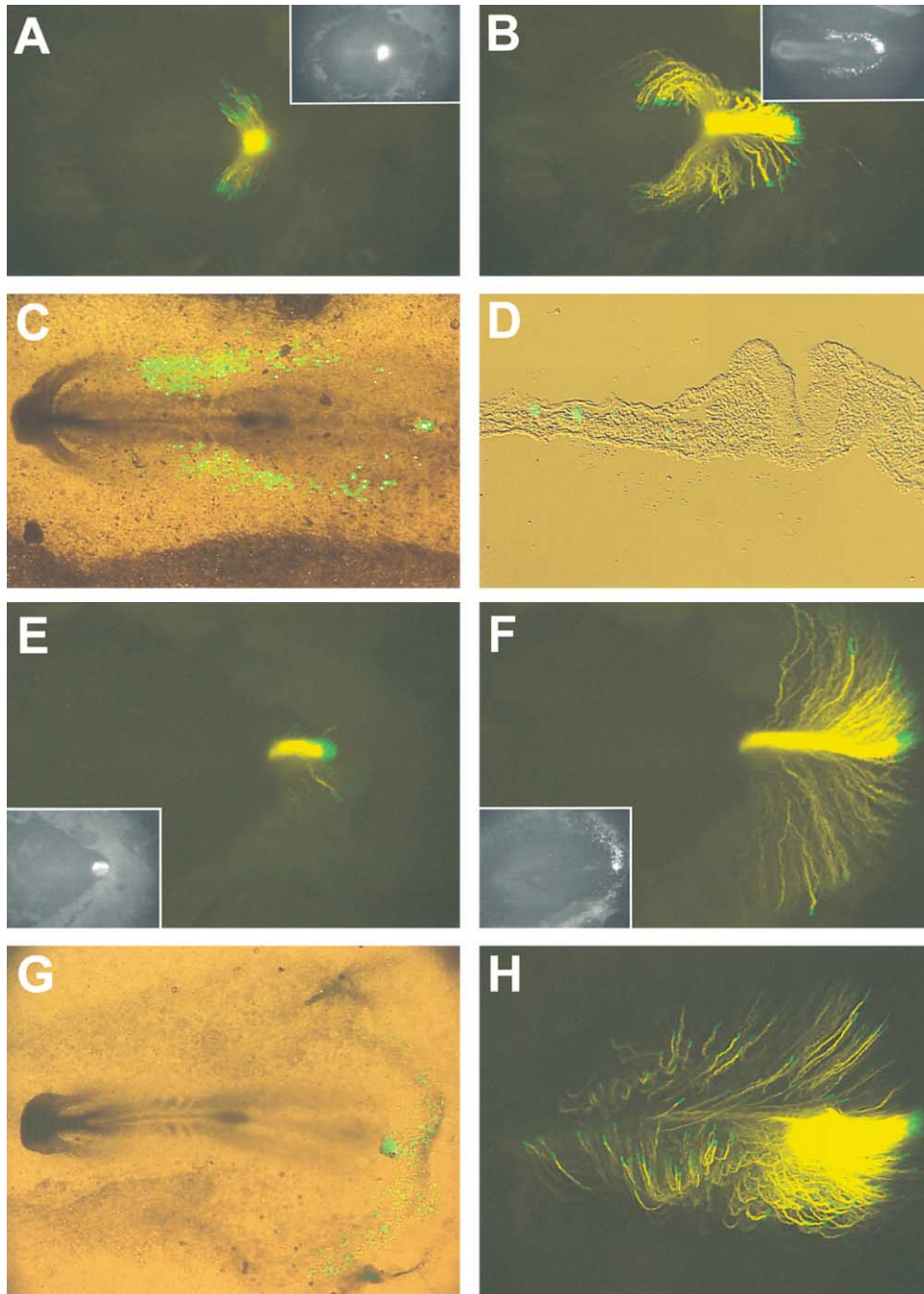


Figure 2. Movement Trajectories and Fate of Cells from the Middle and Posterior Primitive Streak

Conditions of the experiment are as described for Figure 1.

(A and B) Movement of cells from the middle primitive streak during the first 150 min after transplantation (A) and tracks of cells over the 15 hr from the time of transplantation (B). It can be seen that the cells move out from the streak laterally and then most move toward the midline to form lateral plate mesoderm (C and D).

(C) Whole-mount picture of an embryo similar to the one shown in (A) and (B).

(D) Transverse section through the embryo showing that the labeled cells end up mainly in the lateral mesoderm.

(E–G) Movement trajectories of cells transplanted to the posterior streak during the first 150 min (E) and over 15 hr of the experiment (F). The cells move out in a fan-like pattern and form mostly extraembryonic tissue (G). All other conditions are as described for Figure 1.

(H) Movement of GFP-expressing cells in an embryo, in which only the cells on the left side (bottom half) of the streak have been labeled. It shows that the cells in the anterior half of the streak move out and in again, while the cells in the caudal part of the streak move laterally and caudally. It can also be seen that some cells cross the midline and exit the streak at the opposite side of the embryo.

The insets in (A) and (E) and in (B) and (F) show images taken at the beginning and end of the experiments, respectively.

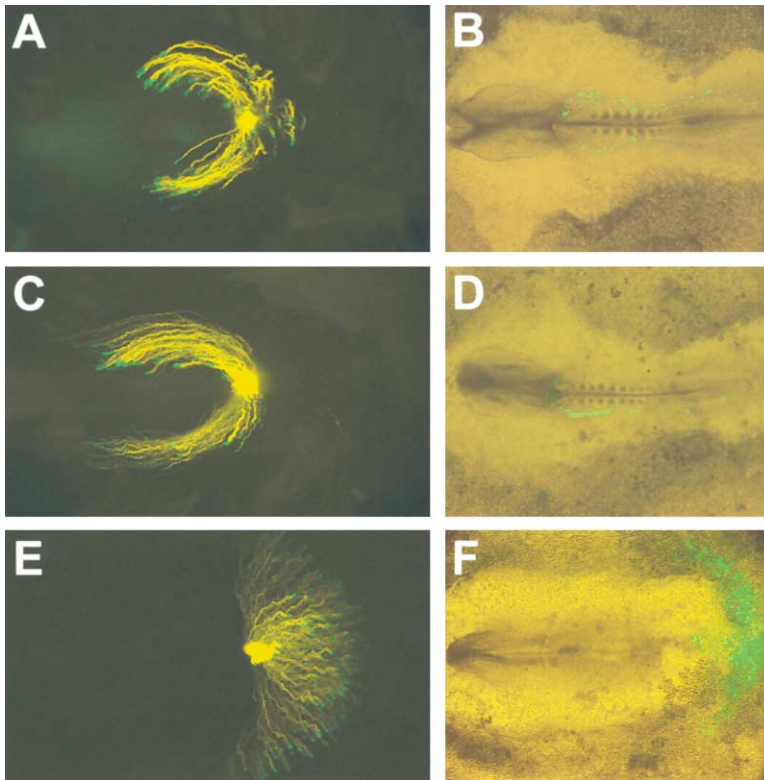


Figure 3. Cell Movement Is Determined by the Surrounding Tissue

Cells transplanted from the anterior streak to the middle primitive streak behaved as middle primitive streak cells. Their movement trajectories (A) were similar to movement trajectories of middle primitive streak cells (C), and the cells formed lateral plate mesoderm (B), as did cells from the middle primitive streak (D). Cells from the anterior streak transplanted to the posterior streak moved out laterally (E), as did posterior streak cells, and formed extraembryonic tissues (F), as did posterior streak cells. These experiments show that the cell's movement pattern is dependent on its position in the streak and is not a cell-autonomous property. The movement trajectories in these figures are shown relative to the GFP-expressing grafted streaks, whose position is fixed in the middle of these images to make comparison of the tracks easier.

Cell Movement Patterns of Cells Emerging from the Streak Are Controlled by Signals from the Surrounding Tissues

In order to investigate whether the movement patterns observed were cell autonomous or mostly determined by the surrounding tissues, we grafted GFP-expressing cells from the region just behind the node to a position either in the middle primitive streak or the caudal primitive streak and analyzed their movement trajectories. As demonstrated above these cells would normally move outwards and then move back toward the midline to form somites (Figures 1F, 1G, and 1H). If transplanted to the middle streak, however, the anterior streak cells behaved like middle streak cells, i.e., they moved out significantly laterally before they moved back to give rise mainly to lateral plate mesoderm (Figures 3A and 3B), as control middle cells would do (Figures 3C and 3D). Similarly, if anterior streak cells were transplanted to the posterior streak, they behaved like posterior streak cells, i.e., they moved out, fan-like, in lateral and caudal directions (Figure 3E) and formed mostly extraembryonic structures (Figure 3F). In the latter scenario the cells never showed any inward movement toward the midline, typical of anterior streak cells. These experiments clearly show that signals from the surrounding tissues determine cell movement behavior.

Streak Cells Are Attracted by FGF4 and Repelled by FGF8

On the basis of the evidence from *Drosophila*, *Xenopus*, and mice that FGF's are involved in controlling gastrulation movements and the fact that FGFs 2, 4, and 8 and

their receptors FGFR1, FGFR2, and FGFR3 are expressed in the early chick embryo (Lawson et al., 2001a; Riese et al., 1995; Shamim and Mason, 1999; Walshe and Mason, 2000), we decided to test the possibility that cells leaving the primitive streak might be responsive to FGFs. We developed an assay in which GFP-labeled explants of primitive streak were grafted to the area opaca. This allowed us to challenge the cells with different FGF signals, which were supplied on beads placed in positions nearby. We found that, when streak cells were transplanted from the embryo to the area opaca, they would behave very similarly to the way they behave in the primitive streak and move radially outward from the streak explant (data not shown).

When heparin beads soaked in FGFs were placed some distance (2–4 mm) away from a GFP-labeled primitive streak explant (derived from an HH stage 4 embryo), we found a strong attraction of cells toward the FGF4-releasing bead (n = 8/10), while, in contrast, an FGF4 bead placed next to a bead soaked in the FGF receptor inhibitor SU5402 (Mohammadi et al., 1997) was clearly less attractive (12/14; Figure 4A). Furthermore, the outward movement of the cells was completely abolished by incubating explants for 10 min in a 20 μM solution of SU5402 before grafting (8/8; data not shown). This result implies that streak cells respond directly to FGF4, rather than to another molecule induced by FGF4. Control beads soaked in PBS did not influence the movement behavior of cells (data not shown). The attraction was highly specific to FGF4. We tested other FGFs; FGF2 did not show a detectable, or showed only a very weak, chemotactic response, while FGF8b acted clearly as a repellent (Figure 4B). When cells were challenged

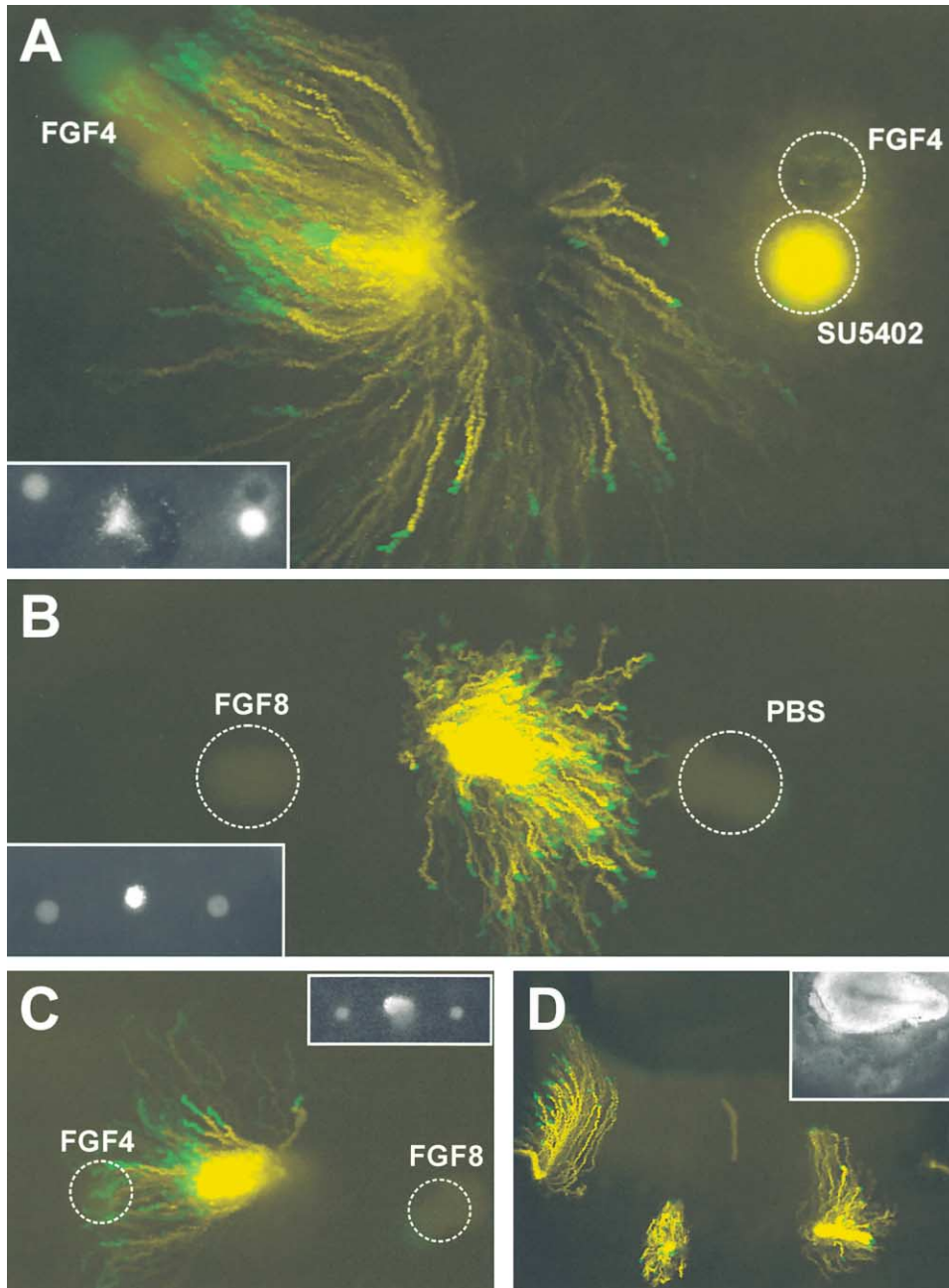


Figure 4. Middle Streak Cells Move Chemotactically toward FGF4 and Are Repelled by FGF8b

(A) Transplantation of a piece of EGFP-expressing middle primitive streak into the area opaca. Movement tracks (8 hr) show that the cells emerging from the graft are strongly attracted by a heparin bead soaked in FGF4 (50 $\mu\text{g}/\text{ml}$), while they are much less attracted to a bead soaked in FGF4 (50 $\mu\text{g}/\text{ml}$) if it is placed next to a bead soaked in the FGFR1 inhibitor SU5402.

(B) If middle primitive streak cells are placed in between a bead soaked in FGF8b (50 $\mu\text{g}/\text{ml}$) and a bead soaked in PBS, they show an active repulsion by the FGF8b bead. Insets show pictures taken at the start of the calculation of the tracks.

(C) Middle primitive streak cells are placed in between beads soaked in FGF4 and FGF8 (50 $\mu\text{g}/\text{ml}$). They move strongly in the direction of the FGF4 bead and are repelled from the FGF8 bead.

(D) Middle primitive streak cells are strongly attracted toward the border between the area opaca and the area pellucida. Pieces of GFP-expressing middle primitive streak were grafted into various positions along the anterior-posterior axis of the embryo in the area opaca. The embryo is oriented with the head fold to the left. Strong attraction can be seen toward the head (left) and the caudal (right) part of the embryo. The cells emerging from the graft placed in the area opaca opposite the middle of the embryo (middle patch) showed no clear directional motion of the cells, presumably because it was placed too far away from the boundary of the embryo. From these experiments we conclude that this region is a source of an as yet unidentified chemoattractant for streak cells. The cells were followed over a period of 12.5 hr, and the tracks were color coded to indicate the direction of migration, as described in Fig 1.

with a PBS- and an FGF8b-soaked bead, they actively avoided the FGF8b bead, while their movement behavior was not influenced by the PBS bead (20/27; Figure 4B). If cells were given a choice between an FGF4 and an FGF8b bead, they moved toward the FGF4 bead, while they actively avoided the FGF8b-releasing bead (Figure 4C; 5/5). There exist eight splice variants of the first exon of FGF8 in mice and four in human that show differences in their transforming potential (MacArthur et al., 1995a, 1995b). In chick only two FGF8s have so far been identified, FGF8a and FGF8b (Sato et al., 2001). When we tested the commercially available human FGF8c isoform, we did not detect any effect in our movement assay, even when tested at concentrations as high as 1 mg/ml (12/12).

During the course of these explant experiments, we noted that middle primitive streak cells were often strongly attracted toward the embryo proper, more specifically, toward the border between the area opaca and the area pellucida, suggesting that this area might release a potent chemoattractant of an as yet unidentified nature. To investigate this in greater detail, we placed GFP-expressing middle streak cells in various anterior and posterior locations around the embryo. We found that the middle streak cells move strongly to the boundary between the area opaca and the area pellucida (6/8). An example of such an experiment is shown in Figure 4D, where the streak cells are strongly attracted to the anterior and posterior end of the embryo. Once the cells reach the border between the area pellucida and the area opaca, they seem to disperse along this boundary, suggesting that they can either not cross this boundary or that this is the source of the chemoattractant. We are in the process of mapping the precise spatiotemporal regulation of this attractive activity and establishing its nature.

We also tested whether FGF4 and FGF8b beads could act as chemoattractants and chemorepellents in the context of the embryo. We grafted labeled middle primitive streak cells into an unlabeled embryo and placed an FGF4 bead in the periphery of the embryo. The results clearly showed that cells were strongly attracted to the bead and that this attraction prevented them from moving inward toward the midline compared with cells in the control half of the embryo (21/23; Figures 5A and 5B). We also compared the action of FGF4 with that of FGF8b and found that, also in the context of the embryo proper, the cells would avoid the FGF8b bead, i.e., the cells turn inward away from the bead (6/6; Figures 5C and 5D). Because of the massive cell movement in the embryo, the bead is moved around, as is evident from the track of the bead over time (Figure 5D).

We observed that, after anterior and middle streak cells moved out, they moved back in toward the midline as soon as the node started to regress and the notochord started to form, suggesting that the notochord may be the source of a chemoattractant guiding the cells back in toward the midline. To test this directly we grafted middle streak cells in the area opaca next to a piece of newly formed notochord from an HH stage 5 embryo. These experiments (22/32) showed that notochord attracts middle streak cells in this situation (Figure 5E), just as would FGF4 beads (Figure 4A). Furthermore, when we placed a piece of notochord lateral to the

middle primitive streak, we found that it could attract middle streak cells (5/7) (Figure 5F), just as did implants of FGF4 beads (Figures 5A and 5B).

Expression of a Dominant-Negative FGFR1c Receptor Results in Altered Cell Movement Patterns

It has been described that FGF4 is expressed in the extending primitive streak, the node, and the forming head process (Lawson et al., 2001a; Shamim and Mason, 1999; Streit, 1999), while FGF8 is expressed in most of the primitive streak (Boettger et al., 1999; Lawson et al., 2001a). On the basis of the expression patterns described and our findings that FGF4 acts as a chemoattractant and that FGF8 acts as a chemorepellent, we hypothesize that cells are moving away from the streak because of the chemorepellent action of FGF8 but then are attracted back toward the midline by a signal coming from the forming head process once the node has started to regress. In order to test this hypothesis, we decided to interfere with FGF detection by the cells by expressing a dominant-negative FGFR1c receptor. This receptor has been shown to be an effective means of interfering with gastrulation movements in *Xenopus* (Amaya et al., 1991; Kroll and Amaya, 1996). We constructed an expression construct, based on the chick FGFR1c receptor, in which the receptor tyrosine kinase domain was replaced by GFP. This allowed us to visualize and follow the fate of the cells expressing the dominant-negative receptor protein. We found that the dnFGFR1c receptor GFP construct was expressed readily in cells in the epiblast and that the fusion protein localized predominantly at the plasma membrane (data not shown). Expression of the construct in the embryo at HH stage 3 resulted, in most cases, in very severe malformations. In many cases the forming streak seemed to dissolve upon expression of the dnFGFR1 construct, resulting in a block of further development (22/35 embryos transfected), while, in other cases, there was a very noticeable expansion of the head at the expense of somite formation and tail structures (Figure 6A) (10/35 embryos). Control embryos transfected with GFP only showed mostly normal development (Figure 6B). This is very reminiscent of the results obtained in *Xenopus*, where expression of the same construct resulted in similar severe malformations of the embryo, i.e., the embryos were defective in the extension of the dorsal body axis (Amaya et al., 1991; Kroll and Amaya, 1996). These observations suggested that the dominant-negative receptor blocked FGF signaling.

To investigate the effect of expression of dnFGFR1c on FGF4 chemotaxis, we repeated the experiment in which we transplanted dnFGFR1c-expressing middle streak cells to the area opaca and challenged them with an FGF4 signal supplied by a heparin-coated FGF4 bead. The results invariably showed only a few cells expressing dnFGFR1c receptor moving away from the graft; however, the cells that did move showed no attraction by the FGF4 bead (Figure 6C). Further experiments showed that expression of the dominant-negative receptor could also block the movement of dnFGFR1-expressing cells toward the FGF4 bead in vivo (data not shown). Also, here, only very few dnFGFR1-positive cells were able to move out of the streak. The cells that moved

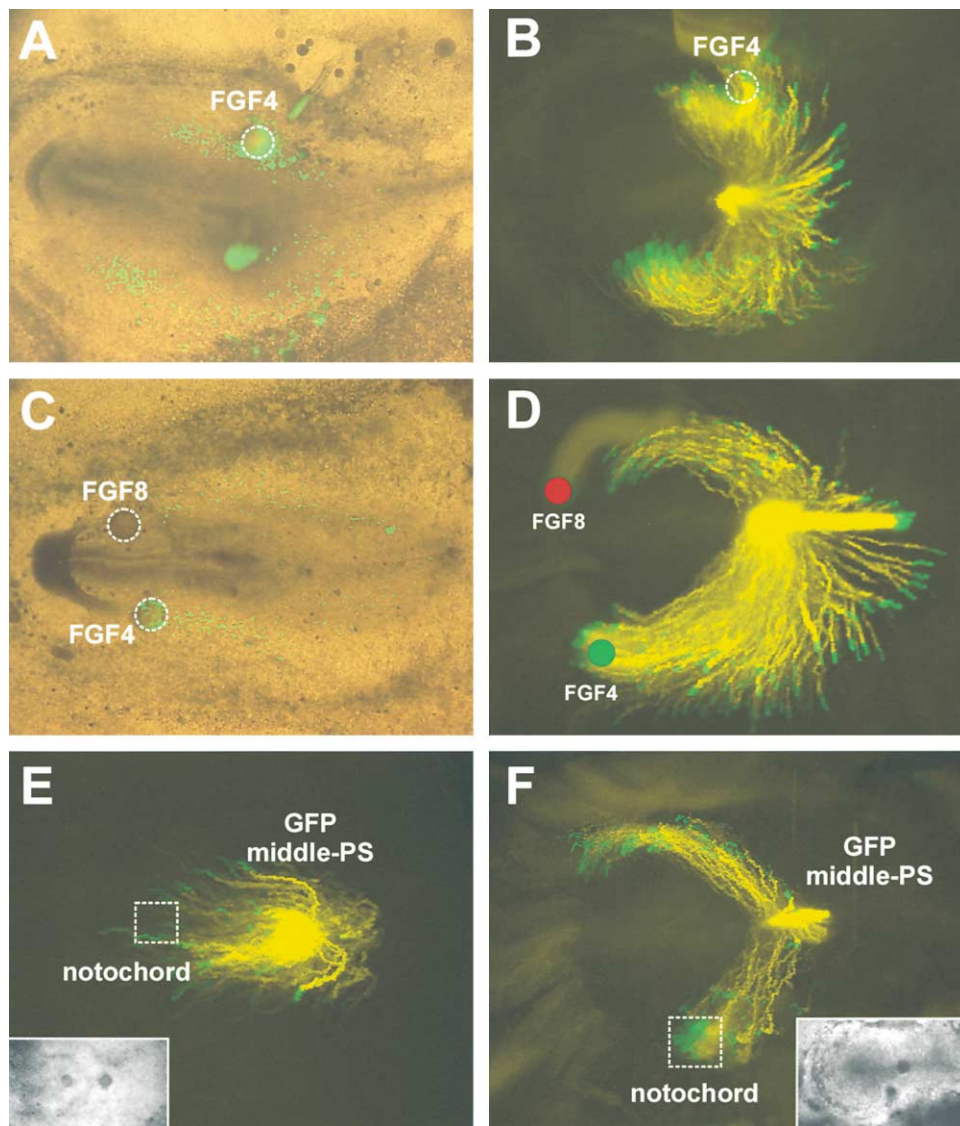


Figure 5. Cells Are Attracted toward FGF4 Beads and Repelled by FGF8 Beads In Vivo

(A) Middle primitive streak cells were attracted by an FGF4 bead in the periphery of the embryo. The image taken at the end of the experiment shows fluorescent cells that have accumulated on the FGF4 bead, while cells have started to move toward the midline in the untreated half of the embryo.

(B) Movement tracks of the GFP-expressing cells emerging from the middle primitive streak during 15 hr experiment. The cells moved outward and toward the FGF4 bead (indicated by a stippled circle in the top half of the panel); they did not move toward the midline of the embryo, as did the cells in the control half of the embryo. Conditions are as described for Figures 1 and 2.

(C) A still image taken at the end of a 15 hr experiment, in which an FGF8 bead (top) and FGF4 bead (bottom) were implanted into the side of an embryo. This experiment showed clearly that the cells have accumulated on the FGF4 bead, while they have avoided the FGF8 bead.

(D) Tracks of GFP-expressing cells emerging from the middle primitive streak. Many cells have moved onto the FGF4 bead, while cells never contacted the FGF8 bead. The tracks also show that the FGF8 bead was displaced by the cells moving forward (the movement trajectory of the bead can be seen as a pale green track).

(E) Attraction of GFP expressing middle primitive streak cells by a small piece of notochord isolated from an HH stage 5 embryo and grafted into the area opaca. The inset shows a brightfield image at the start of the experiment.

(F) Attraction of middle primitive streak cells by a piece of notochord grafted into the epiblast, laterally to the streak. The cells moved toward the piece of notochord and did not move back in toward the midline, as did cells in the upper control half of the embryo. This result is similar to that obtained with an FGF4 bead (compare panels [B] and [F]). The inset shows a brightfield image at the start of the experiment.

out did not travel the same distance as control cells during the experiment, since they moved at a lower average speed (0.5–1.5 $\mu\text{m}/\text{min}$). We often noted that, in contrast to GFP expression alone, which was stable for long periods of time, dnFGFR1 expression rapidly diminished over the course of the experiment, typically

resulting in few labeled cells at the end of the experiment after 15 hr. We frequently observed cells that contained fluorescent material in intracellular vesicular structures in the later stages of the experiments, showing that the cells are able to downregulate the receptor from the membrane. We have not yet been able to determine

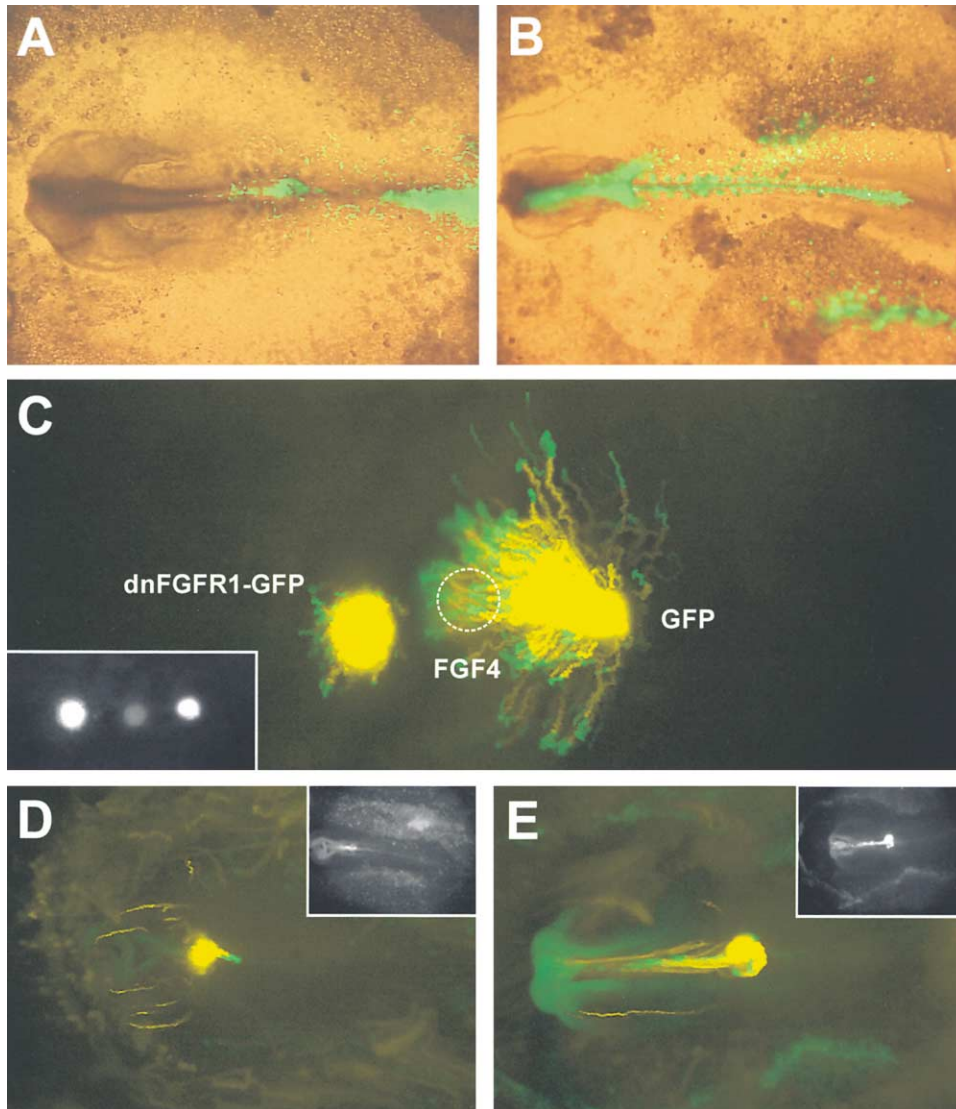


Figure 6. Expression of a Dominant-Negative FGFR1c Receptor Results in Big Heads and Inhibits Chemotaxis toward Beads Soaked in FGF4. Expression of an EYFP-tagged dominant-negative FGFR1c-EYFP receptor construct in embryos resulted in the complete disappearance of the streak (data not shown) or the formation of a large head (A) at the expense of the formation of axial mesoderm compared with control embryos expressing only EYFP (B). Expression of the dnFGFR1c construct in middle primitive streak cells resulted in few cells moving out from the graft compared with cells expressing just GFP. The cells expressing the dnFGFR1c construct that did move out from the graft were not attracted by the FGF4 bead, while middle streak cells expressing only GFP were strongly attracted (C). Expression of the dnFGFR1c construct in the node allowed the initial extension of the streak to occur, but inhibited the regression (D) compared with a control node expressing GFP (E). This often resulted in split brains (see inset).

whether these cells die or survive. The experiments with the dominant-negative receptor were phenocopied by experiments in which we soaked GFP-expressing middle primitive streak cells for 5–10 min in a 20 μ M solution of the FGFR1 receptor inhibitor SU5402 before grafting to the host. This completely blocked the outward movement of the cells (data not shown).

When we expressed dnFGFR1c in the node, it showed strongly reduced regression movement, typically resulting in embryos with abnormal head structures and few somites (Figures 6D and 6E). In contrast, regression took place normally when cells in the anterior, middle, and posterior streak were replaced by cells expressing the dominant-negative FGFR1 construct (data not shown),

in agreement with the data obtained with the FGFR inhibitor SU5402.

Discussion

The Dynamic Movement of Cells Emerging from the Primitive Streak Follow Highly Specific Trajectories, which Depend on the Position of the Cells within the Streak

Analysis of the movement of GFP-expressing cells from HH stage 3b to the 5–6-somite stage of development has revealed characteristic trajectories that cells located in the different areas of the primitive streak follow during this period. There exists a strong correlation between

the anterior-posterior position of the cells in the streak and the extent of their lateral movement. Some of the cells in the node are left behind as a thin line of cells that will form the notochord during regression, in agreement with earlier fate map studies (Catala et al., 1996; Psychoyos and Stern, 1996; Selleck and Stern, 1991), while others stay resident in the node and form an apparent stem cell population. The cells in the anterior half of the streak moved back in toward the midline when the node had regressed. The cells in the posterior half of the streak moved out and stayed out to become extraembryonic tissue. Overall, the movement patterns are entirely consistent with results obtained from vital dye and quail chick chimera experiments, which found that the anterior-posterior position of cells within the streak determined their eventual mediolateral position within the embryo (Garcia-Martinez and Schoenwolf, 1992; Hatada and Stern, 1994; Psychoyos and Stern, 1996; Schoenwolf et al., 1992; Selleck and Stern, 1991). Heterotopic grafting experiments confirmed and extended previous observations by these workers and showed that the movement of the cells depends on the position of the cells within the streak and is not a cell autonomous property but is determined predominantly by the surrounding tissue (Garcia-Martinez and Schoenwolf, 1992). Specifically, anterior streak cells behaved appropriately for the position they found themselves in, i.e., they behaved like middle or caudal streak cells in this experiment (Figure 3). This suggests that cells along the anterior-posterior position of the streak are guided by different signals and that anterior streak cells are competent to respond to the whole spectrum of anterior, middle, and posterior signals. We noted that, during the movement of the middle primitive streak cells, the cells moved out laterally to the periphery of the embryo, where they seemed to pause for a while, and then started to move toward the midline again, once the node had regressed past them. This strongly suggested that the outward and inward movements are regulated by different signals. We have shown that the border between the area pellucida and the area opaca and the notochord can both attract middle streak cells (Figures 4D, 5E, and 5F). Therefore, it seems most likely that the signal that attracts the anterior streak cells back in toward the midline originates from the forming notochord, in agreement with data that show that the notochord can influence the formation of somites (Nicolet, 1971a, 1971b).

FGF4 and FGF8 Positively and Negatively Direct Cell Movement

Our experiments show that FGF4 is a strong chemoattractant for cells emerging from a piece of primitive streak tissue, both in explants grafted to the area opaca and in the embryo proper, while FGF8b does not attract cells but seems to act as a repellent. It is well established that both FGF4 and FGF8 are expressed in the streak and show partially overlapping expression patterns. FGF4 is expressed initially by all cells in the streak and in the node, as well as by cells in the head process, in the forming notochord, as shown schematically in Figure 7 (Shamim and Mason, 1999). FGF8 is expressed initially in the anterior streak and the forming node but, during the later stages of streak-extension expression, is excluded from the node and the anterior part of the streak

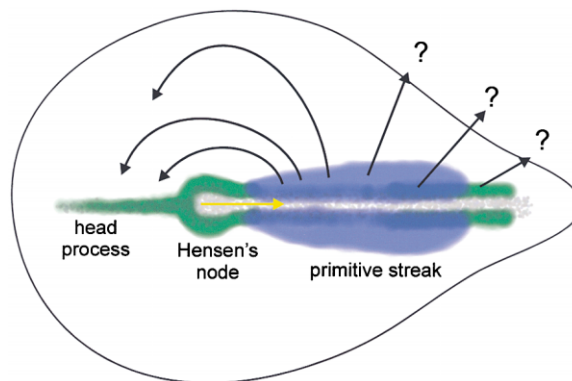


Figure 7. Model Suggesting that Cell Movement Is Directed by Repulsion from the Streak Mediated by FGF8 and Attraction toward the Midline in Response to FGF4

The FGF4 expression pattern is shown schematically in green, and the FGF8 expression pattern is shown in blue. The area in the middle primitive streak, which expresses both FGF4 and FGF8, appears dark blue in the overlay. The black arrows show the trajectories of cell movement observed in this study in relation to the FGF4 and FGF8 expression patterns. The yellow arrow indicates the direction of node regression. We propose that the movement away from the streak is the result of negative chemotaxis in response to FGF8 produced by cells in the middle primitive streak, while the inward movement of the cells emerging from the anterior streak is the result of attraction by FGF4 secreted by cells in the head process and notochord. The cells in the caudal part of the embryo are initially repelled by FGF8 and subsequently attracted by a signal of unknown identity, secreted in the border region between the area opaca and the area pellucida (question marks).

(Lawson et al., 2001a). On the basis of the expression patterns of FGF4 and FGF8 and our demonstration of cell movement trajectories in real time, we propose the following model for the control of the observed cell movement patterns (Figure 7). In the region where the cells move out from the streak, the cells are exposed to high levels of FGF8, and we suggest that FGF8 acts as a chemorepellent and sends the cells away from the streak. This suggestion is consistent with data obtained in homozygous FGF8 knockout mice, which show an accumulation of mesoderm cells in the streak of the embryo (Sun et al., 1999). The behavior of the cells after they have moved out depends on their position. The cells just behind the node come under the influence of the FGF4 signal secreted by the forming notochord after the node starts to regress. These cells are attracted back in toward the midline to form somites. The cells in the middle streak show a similar movement pattern but experience a longer period of repulsion by FGF8, resulting in their extended lateral movement. The cells in the caudal streak respond to the repulsive action of the FGF8 produced by the cells in the middle streak and move away from this signal in a caudal direction. These cells end up in the extra embryonic region, and, in this position, these cells never sense the attractive influence of the forming notochord and somites. It is likely that, besides the repulsive action of FGF8 produced by cells in the middle streak, these cells are attracted by another factor produced at or near the border between the area opaca and the area pellucida (Figure 4D).

Interference with FGF Signaling Severely Affects Cell Movement Behavior

We performed two types of experiments designed to interfere with FGF signaling. We locally applied the FGF receptor inhibitor SU5402 and investigated the effects on movement. Furthermore, we expressed a dominant-negative form of the FGFR1c receptor in various pieces of streak and investigated the effect on cell movement. The most prominent effect of both the inhibitor and the expression of dnFGFR1c was that the ability of the affected cells to move out from the streak was severely compromised. For cells to move from the streak, they have to downregulate E-cadherin, resulting in the epithelial to mesenchymal transition (EMT; Burdsal et al., 1993). Mice lacking the FGFR1 receptor die postimplantation (Yamaguchi et al., 1994), but chimeric analysis of FGFR1^{-/-} and wild-type mice has shown that FGFR1 signaling is required for EMT to occur in mouse embryos (Ciruna et al., 1997; Yamaguchi et al., 1994). Failure to downregulate E-cadherin results in the affected cells being trapped in the streak (Ciruna and Rossant, 2001; Ciruna et al., 1997). The requirement for FGF signaling in the downregulation of adhesion molecules makes the experiments with dominant-negative FGF receptors less straight forward, since only few cells succeed in moving out. However, it appears that the few cells that manage to escape from the streak do not show any strong directed motion toward FGF4 beads either in the area opaca (Figure 6C) or in the embryo (data not shown). We also observed that the cells that moved out moved considerably slower (short tracks) than did control cells, suggesting that the dnFGFR1-expressing cells may also be less sensitive to the FGF8b signal, which sends them away from the streak (graft). The FGFR1c receptor has been shown to be an effective transducer for FGF4 signals in a cell proliferation assay; however, this receptor is significantly less efficient for transduction of FGF8b mitogenic signals (Ornitz et al., 1996). At this stage we do not know whether the receptor specificity measured in the mitogenic response assay is relevant to the signaling pathway involved in chemotactic movement response nor do we know whether the dnFGFR1c construct used can interfere with signaling through other FGF receptors that are more likely to transduce the FGF8 signal.

The observation that regression of the streak can be blocked by expression of the dominant-negative FGFR1 receptor in the cells in the node, but not by a graft of similar size from anywhere else in the streak, is in agreement with the finding made recently in which it was shown that, when cells in the node are electroporated in ovo with a dnFGFR1 construct, the streak does not elongate properly (Mathis et al., 2001). It was suggested that FGF signaling is required to maintain a cell population of neural progenitor cells in the node and that this zone of stem cells is somehow required for the regression process. Our results were obtained at a slightly earlier stage of development but appear to support the observation that FGF signaling is required for node regression. It remains to be determined which FGF is involved in the regression process; however, on the basis of its expression pattern, FGF4 is a likely candidate.

In *Xenopus* embryos the injection of a dominant-negative FGFR1c into early blastomeres results in defects in

both mesoderm induction and gastrulation movements. The striking phenotype is that the embryos expressing a dominant-negative FGFR1 receptor show defects in the formation of the somites and notochord and have a severely shortened back (Amaya et al., 1991; Kroll and Amaya, 1996). This phenotype has been attributed to a failure of convergent extension, resulting in defects of the formation of notochord, somites, and neural plate. We observed that expression of dnFGFR1c in chick resulted in the formation of embryos with disproportional big heads (Figures 6A and 6D), which could result from a failure of node regression resulting in a hyperneural induction (Diez-del Corral et al., 2002; Streit et al., 2000).

It is now becoming increasingly clear that chemotaxis is not only a very important principle in the development of simple organisms, such as the cellular slime mold *Dictyostelium*, *C. elegans*, and *Drosophila* (Branda and Stern, 1998, 2000; Dormann et al., 2000; Dossenbach et al., 2001; Duchek and Rorth, 2001; Duchek et al., 2001; Montell, 1999), but also that, in the development of vertebrates, chemotaxis to growth factors may be very important. Both positive and negative chemotaxis have been shown to be important principles in axonal guidance (Brose and Tessier-Lavigne, 2000; Kramer et al., 2001). There is increasing evidence that movement in response to FGFs is important in various developmental contexts. FGF4 and FGF8 have been shown to function as potent chemoattractants during limb bud development and migration of mesencephalic neural crest cells in the mouse (Kubota and Ito, 2000; Li and Muneoka, 1999), and we propose that, on the basis of our experiments shown here, FGFs control movement of mesoderm cells by acting as chemoattractants and repellents in conjunction with other as yet unidentified factors (Figure 7).

Experimental Procedures

White Leghorn chick embryos (Needle Farm, Sussex) were incubated for 15 hr at 37°C until they reached stage 3–4. New cultures were prepared (Chapman et al., 2001; New, 1955). A total of 0.5 μ l DNA (0.5 μ g/ μ l) of the GFP expression plasmid (pEGFP-C3, Clontech) was microinjected in the space between the vitelline membrane and the epiblast. Cells were transfected by electroporation with two successive square pulses of 25 V and 50 ms and platinum wire electrodes 1.5 mm apart with a home made “electroporator” (Momose et al., 1999; Yasuda et al., 2000). After 3–5 hr many cells displayed strong GFP fluorescence and had moved to the streak. Grafts were made, in which pieces of GFP-expressing streak cells, roughly 200 \times 200 μ m, were excised and grafted into different positions of unlabeled host embryos. The embryos were transferred to a custom-made air-heated microscope incubation chamber, which was kept at 38°C and 90% humidity, and the imaging was started. In general embryos developed well up to HH stage 9 on the microscope in New culture (New, 1955). For every experiment the operations were performed on 4–8 embryos, and one embryo was selected for time-lapse analysis, while the development of the other embryos was recorded by photographing the embryos at different stages. All types of experiments were performed on at least 4 different days, resulting in at least four different 12–15 hr time-lapse movies of each type.

Brightfield and fluorescence images were taken every 2 or 3 min with a Hamamatsu cooled CCD camera as described previously (Dormann et al., 2001). Tracks were generated by successive logical addition of images with software written with the Optimas VI imaging library.

The heparin beads (Sigma; H5263) were incubated in FGF4, FGF8b, and FGF8c (R&D systems) at a concentration of 50 μ g/ml for at least 1 hr at room temperature. Before they were used, the

beads were washed once in PBS. The inhibitor SU5402 was dissolved in DMSO to a concentration of 1 mg/ml. Biorex AG1-X2 (Bio-Rad 140-1241) beads were soaked in inhibitor for 1 hr at room temperature and washed once in PBS before transplantation. The pieces of tissue were incubated for 10 min in 20 μ M SU5402 in PBS, washed once in PBS, and then transplanted.

The dominant-negative receptor FGFR1c was constructed by amplifying the extracellular domain and transmembrane region of the receptor with PCR and pFR3 (Amaya et al., 1991) as a template. We used the following primers (forward, GGTGAATCCAGTCGCTGAG CAGT; reverse, CTGGAATCCTTACCTGTCTGCGCA) to introduce EcoRI sites, to enable us to clone the fragment into the Clontech pEYFP-N1 expression vector.

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