

PDGF signalling controls the migration of mesoderm cells during chick gastrulation by regulating N-cadherin expression

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In the early chick embryo, *Pdgfra* is expressed in the epiblast, outlining the migration route that mesoderm cells expressing the receptor, *Pdgfra*, follow to form somites. Both expression of a dominant-negative PDGFR α and depletion of endogenous PDGFR α ligands through injection of PDGFR α -Fc fragments, inhibit the migration of mesoderm cells after their ingress through the primitive streak. siRNA-mediated downregulation of *Pdgfra* expression in the epiblast on one side of the streak strongly blocks the migration of mesoderm cells into that side. Beads soaked in PDGFA elicit a directional attractive movement response in mesoderm cells, showing that PDGFA can provide directional information. Surprisingly, however, PDGF signalling is also required for directional movement towards other attractants, such as FGF4. PDGF signalling controls N-cadherin expression on mesoderm cells, which is required for efficient migration. PDGF signalling activates the PI3 kinase signalling pathway in vivo and activation of this pathway is required for proper N-cadherin expression.

KEY WORDS: Gastrulation, Cell movement, N-cadherin, PDGF signalling, PI3 kinase signalling

INTRODUCTION

During the early gastrulation stages of the chick embryo, cells in the epiblast move towards the primitive streak where they undergo an epithelial-to-mesenchymal transition (EMT) during invagination, after which these mesenchymal cells migrate outward between the epiblast and the hypoblast. Depending on their anterior-posterior location in the streak, these cells are fated to form different mesodermal structures. Anterior streak cells give rise to somites, whereas middle streak cells predominantly form intermediate and lateral plate mesoderm, and posterior streak cells form extra-embryonic mesoderm and cells that give rise to the haematopoietic system (Imura et al., 2007; Psychoyos and Stern, 1996). The signals that guide the migration of the epiblast cells towards the primitive streak followed by their ingress and migration away from the primitive streak are not yet understood in detail, nor are the mechanisms by which these cells move. FGF signalling plays a major role in mesodermal cell migration. We have provided evidence that migration away from the primitive streak involves FGF8 signalling, and that movement back in towards the midline involves chemotaxis towards factors produced by the forming headprocess and notochord, notably FGF4 (Yang et al., 2002). It is, however, to be expected that a wider array of guidance molecules is required to guide the different mesodermal cell populations to their correct targets. Indeed, experiments in other organisms, especially frogs, fish and mice, have implicated PDGF signalling in particular in the control of mesodermal cell movement during early development.

In *Xenopus* it has been shown that PDGFA is expressed in the ectoderm, whereas the PDGF receptor (PDGFR) is expressed in the deeper mesodermal cells migrating anterior along the blastocoel

roof. Expression of a dominant-negative PDGFR, with a single-point mutation that prevents activation of the receptor, results in severe gastrulation defects (Ataliotis et al., 1995). The embryos showed a loss of anterior structures and failure of proper closure of the neural tube resulting in spina bifida, suggesting a defect in the anterior migration of mesodermal cells. Explanted prechordal plate mesoderm cells are able to follow a directional signal, in a conditioned extracellular matrix deposited on a plastic surface by animal cap cells, in a direction that corresponds to anterior (Nagel and Winklbauer, 1999). This directional migration is blocked by expression of dominant-negative PDGFR or by morpholino knockdown of *Pdgfr* in the migrating prechordal plate cells (Nagel et al., 2004). PDGF signalling is also required for the correct attachment of the prechordal plate cells to the matrix lining the blastocoel roof and for anterior migration in vivo (Van Stry et al., 2004). Furthermore, it has been shown that isolated zebrafish mesodermal cells orient their protrusive activity towards an ectopic PDGF source, which involves localised PI3 kinase signalling in the leading edge of the migrating cells. Inhibition of PI3 kinase signalling in vivo results in the markedly slower, but still directed, movement of mesoderm cells (Montero et al., 2003).

In higher vertebrates there are two PDGF receptors, PDGFR α and PDGFR β , that form both homo- and heterodimers, and there are at least four PDGF genes, *Pdgfa*, *Pdgfb*, *Pdgfc* and *Pdgfd*, that give rise to PDGFAA, PDGFAB, PDGFBB, PDGFCC and PDGFDD ligands. PDGFAA, PDGFAB, PDGFCC and PDGFBB are ligands for the PDGFR α homodimer, whereas PDGFAB, PDGFCC, PDGFBB and PDGFDD are ligands for the PDGFR α -PDGFR β dimer, and PDGFBB and PDGFDD for the PDGFR β homodimer (Heldin and Westermark, 1999; Tallquist and Kazlauskas, 2004). Owing to this complexity, the effects of PDGF signalling on early development, especially gastrulation, are not yet well characterised. In mice, PDGFA is expressed in the epiblast and PDGFR α in the mesoderm (Mercola et al., 1990). Knockouts exist for both *Pdgfa* and *Pdgfra*. Knockout of *Pdgfra* results in developmental defects including incomplete cephalic closure and increased apoptosis on the pathways followed by migrating neural

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crest cells. Furthermore, alterations in the formation of the sternum and ribs appear to result from deficiencies in the formation of the myotome (Hoch and Soriano, 2003; Soriano, 1997). Knockout of *Pdgfa* leads to the death of half of the embryos before E10; the other half die shortly after birth owing to a failure of alveolar septation and lung emphysema (Bostrom et al., 1996). The variable phenotype has been attributed to differences in genetic background, compensation by expression of *Pdgfb*, maternal factors, placental transfer of PDGF and/or embryonic position in the uterus. The cause of the early developmental lethality at E10 has not been analysed in detail.

Based on all this evidence indicating an important role for PDGF signalling in the control of mesodermal cell migration in vertebrates, we have investigated its role in the chick embryo, in which it is possible to study the migration of mesoderm cells in situ (Sweetman et al., 2008; Yang et al., 2002; Yue et al., 2008). In this study, we characterise the expression of *Pdgfa*, *Pdgfb* and *Pdgfc* and of their receptor *Pdgfra* and show that signalling through PDGFA and PDGFR α is required for the migration of mesoderm cells in vivo, and that the effects are mediated through the control of N-cadherin expression and PI3 kinase signalling.

MATERIALS AND METHODS

Embryos and imaging

All experiments were performed with Brown Leghorn chick embryos (Henry Stewart, Louth, UK). Embryos were incubated at 38°C until they reached HH2-3 (Hamburger and Hamilton, 1951), then early chick (EC) cultures prepared (Chapman et al., 2001) followed by electroporation-based transfection as described (Leslie et al., 2007). Imaging of cell movement during early gastrulation was performed as described (Yang et al., 2002). Brightfield and fluorescence images were taken every 3 minutes for periods of up to 25 hours. A computer-controlled motorised stage allowed us to record the development of six embryos simultaneously. Typically, we recorded three experimental embryos and three controls; the remaining surviving embryos were photographed at the beginning and end of the experiment. Each experiment was repeated at least three to four times.

Molecular biology

cDNA probes for in situ hybridisation were obtained from the Chick EST Consortium (Boardman et al., 2002). We used the following cDNAs: *Pdgfa* (ChEST460a12, linearised with *SacII*), *Pdgfb* (ChEST115n16, linearised with *BglII*), *Pdgfc* (ChEST270m4, linearised with *NheI*), *Pdgfd* (ChEST74b4, linearised with *SacII*), *Pdgfra* (ChEST404I3, linearised with *HpaI*). cDNAs were transcribed with T3 RNA polymerase. In situ hybridisation was performed according to standard procedures (Wilkinson and Nieto, 1993).

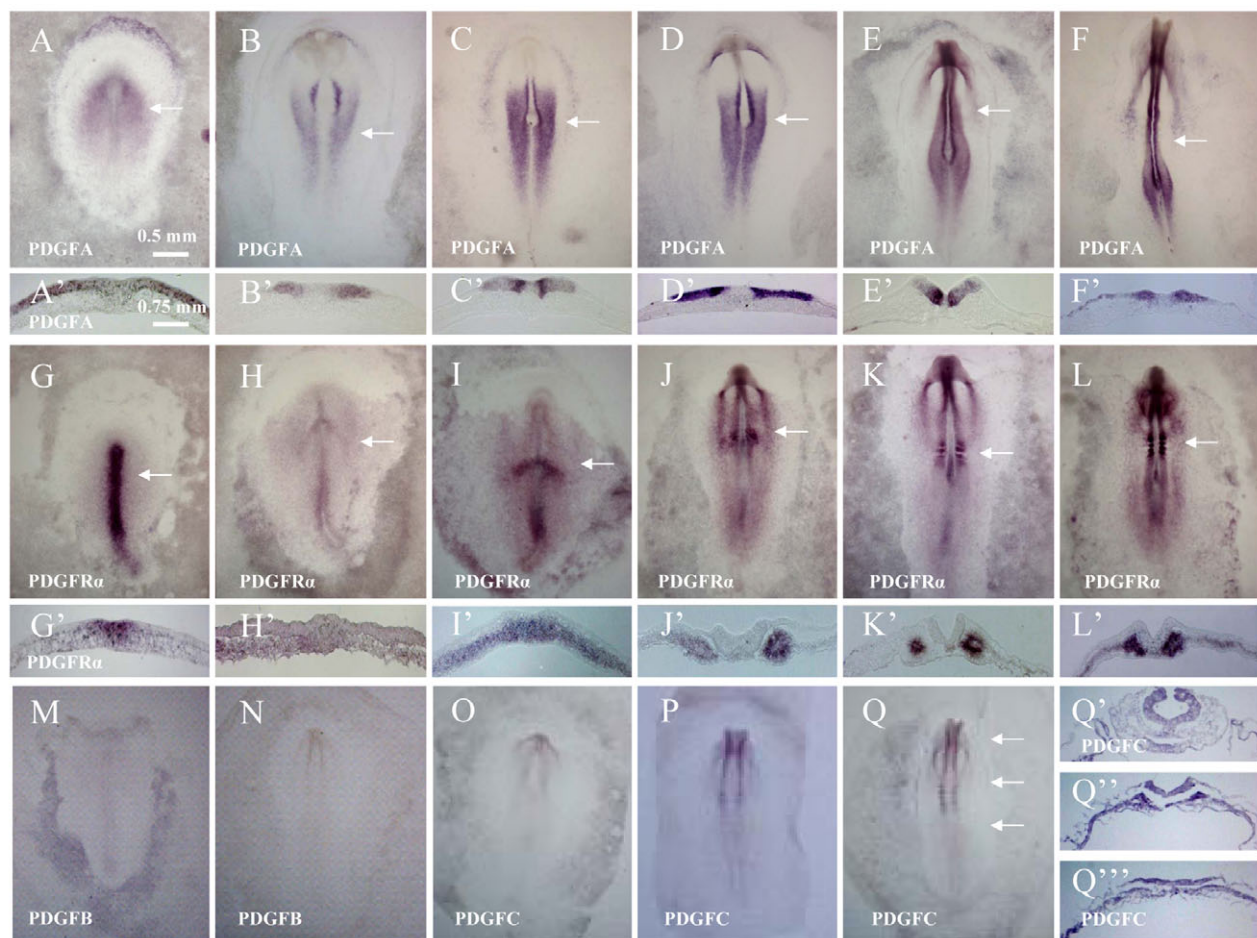


Fig. 1. Expression *Pdgfa*, *Pdgfra*, *Pdgfb* and *Pdgfc* during early chick development. (A-F) *Pdgfa* expression in HH3-8 chick embryos. (A'-F') Sections through the embryos shown in A-F at the level of the arrows. (G-L) *Pdgfra* expression in HH3-8 embryos. (G'-L') Sections through the embryos shown in G-L at the level of the arrows. (M,N) Expression of *Pdgfb* is almost undetectable in HH3-8 embryos. (O-Q) Expression of *Pdgfc*. (Q'-Q''') In later embryos (HH6-8), *Pdgfc* is expressed in the brain and forming neural tube and in the forming somites. Probes used in these and all further figures are indicated in the bottom left-hand corner of relevant panels.

The pCA β -PDGFR37-IRES-GFP expression construct was made by amplifying the coding sequence from the original vector, containing *Xenopus* PDGFR37 in PCS2, with gene-specific primers: PDGFR37 forward, 5'-GAATATATTAGCGGCCGCATGATGCCTGCCATGAGG-3'; PDGFR37 reverse, 5'-GAATATATTAGAATTCTCACAGGAACT-GTCTC-3'. The fragment was inserted into the pCA β /GFP expression vector, which has an internal ribosome entry site (IRES) directing the expression of GFP (Yue et al., 2008). The dominant-negative N-cadherin receptor was constructed by cloning the extracellular and transmembrane domains of chick N-cadherin in pEGFP-N1 (Invitrogen) as a *Bgl*II-*Sal*I fragment, amplified using primers 5'-TTGATATAGATCTCGGGCC-ATGTGCC-3' and 5'-TTATCACGGCGCTCGACCATACTACGAACATCA-3' from a full-length N-cadherin cDNA. To label cells with GFP, they were transfected with pEGFP-N1. *Pdgfa* knockdown was achieved by electroporation of a chick *Pdgfa* siRNA Smartpool (10 μ g/ml) custom designed by Dharmacon using previously described procedures (Leslie et al., 2007). The full-length N-cadherin expression construct was made by PCR amplification of N-cadherin with the primers 5'-TTGATATAGATCTCGGGCCATGTGCC-3' and 5'-CGACCGCGTGGCGGCCGCTCTAGAACT-3' and cloning as a *Bgl*II-*Not*I fragment into pCA β /GFP. Proper expression of these constructs was tested by transfection into COS7 cells followed by western analysis of cell lysates.

Antibodies and immunocytochemistry

Immunocytochemistry was performed as described (Leslie et al., 2007). Antibodies used were phosphospecific (S473) AKT (PKB) (Cell Signaling Technologies), β -catenin (15B8, Sigma), chick N-cadherin (6B3), chick integrin α 6 (P2C6C4) and chick integrin β 1 (CSAT), chick laminin (310/31-2) and chick fibronectin (B3/D6, Developmental Studies Hybridoma Bank). Secondary antibodies were HRP-conjugated (Promega) and detection was performed using the Tyramide Signal Amplification System (Molecular Probes). For neutralising PDGFR α ligands, 0.5 μ l 100 μ g/ml recombinant mouse PDGFR α -Fc chimera

(R&D Systems, Cat. 1062-PR-050) was microinjected into each embryo. For PDGF stimulation experiments, heparin beads were soaked in recombinant rat PDGFAA short form (R&D Systems, Cat. 1055-AA-050) or human PDGFAA (R&D Systems, Cat. 221-A) long form at 20 μ g/ml at room temperature for 3 hours, followed by several washes with PBS before implantation into the embryo. FGF4 beads were prepared as described (Yang et al., 2002).

RESULTS

Expression of *Pdgfr*, *Pdgfa* and *Pdgfc* in the chick embryo

We performed in situ hybridisation to investigate the expression of *Pdgfr α* and its ligands *Pdgfa*, *Pdgfb* and *Pdgfc*. *Pdgfa* expression was detected in the epiblast from HH stage 3 onwards, on both sides of the primitive streak. The intensity of expression increased from posterior to anterior, as far as a region just anterior of the node where expression stopped abruptly at the anterior border of the future neural plate (Fig. 1A-F'). Expression of *Pdgfr α* was detected from HH3 onwards in cells in the primitive streak that were about to ingress, and in migrating mesoderm cells (Fig. 1G-L). Expression appeared to be upregulated in the forming somites, especially in the sclerotome, from HH7-8 onwards (Fig. 1G'-L'). The *Pdgfr α* message in the forming somites was highly localised in the side lining the somatic cavity (Fig. 1K'). Expression of *Pdgfb* was very weak up to HH8 (Fig. 1M,N), making it unlikely that PDGFAB or PDGFBB are major signalling molecules during these early stages of development. Expression of *Pdgfd* was undetectable (not shown). *Pdgfc*, however, showed weak expression in the early streak stages (Fig. 1O-Q), predominantly in the forming brain, neural tube and in the forming somites (Fig. 1Q'-Q''').

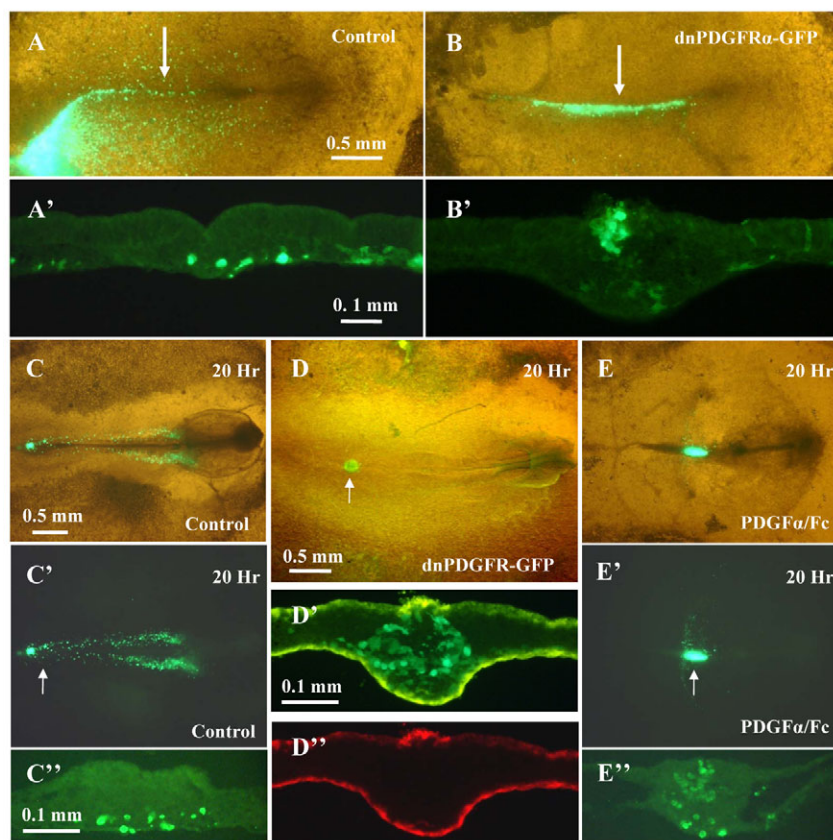


Fig. 2. Inhibition of PDGF signalling inhibits the migration of mesoderm cells away from the streak.

(A) Chick embryo transfected on one side of the streak with a GFP expression construct (pCA β /GFP). (A') Fluorescent image of section through the embryo at the level of the arrow in A. (B) Embryo transfected with a dominant-negative (dn) *Pdgfr α* construct. (B') Section through the embryo at the level of the arrow in B. (C-C') GFP-expressing streak cells grafted in a control embryo of similar age migrate away from the site of transplantation in the anterior primitive streak to form somites and lateral plate mesoderm. (C') Fluorescent image of embryo shown in C. (C'') Section taken at the arrow in C'. (D-D') Streak cells expressing a dn-PDGFR do not migrate away from the site of transplantation in the streak. (D') Section of embryo taken at the level of the arrow in D. (D'') β -catenin staining of the section shown in D'. (E-E') Embryo 20 hours after injection with 0.5 μ l 100 μ g/ml mouse dn-PDGFR α -Fc and transplantation of GFP-expressing middle streak cells. Depletion of PDGFR α ligands results in failure of most GFP-expressing cells to migrate away from the site of implantation. (E') Fluorescent image of embryo shown in E. (E'') Section taken at the arrow in E'.

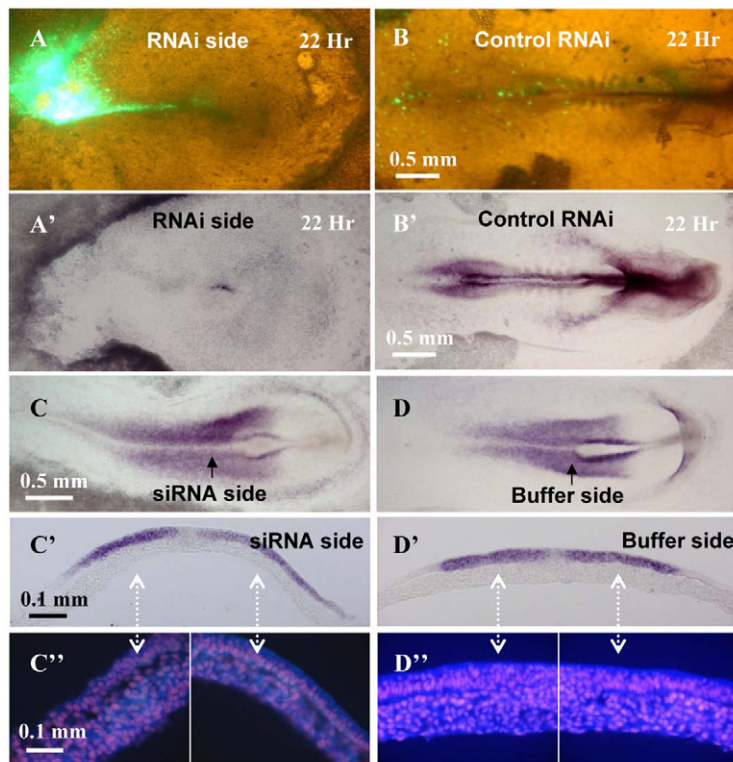


Fig. 3. Downregulation of endogenous *Pdgfra* expression inhibits movement of cells out of the streak. (A) Chick embryo 22 hours after transfection with a mixture of GFP and Smartpool *Pdgfra* siRNA. Labelled cells migrate to the streak, but do not move out of the streak; development is arrested at the extended streak stage. (A') *Pdgfra* in situ hybridisation of the embryo shown in A. (B) Embryo transfected with GFP and a control siRNA (cyclophilin). GFP-expressing cells migrate away to form paraxial mesodermal structures. (B') *Pdgfra* in situ hybridisation of embryo shown in B. (C) *Pdgfra* in situ hybridisation of an embryo 22 hours after half-sided *Pdgfra* siRNA knockdown. (C') Section taken at the position of the arrow in C. (C'') High-magnification image of section shown in C' after staining of nuclei with propidium iodide. (D) *Pdgfra* in situ hybridisation of an embryo 22 hours after half-sided transfection with siRNA-dilution buffer. (D') Section taken at the position of the arrow in D. (D'') High-magnification image of section in D' after staining of nuclei with propidium iodide.

Blocking signalling through PDGFR α blocks migration of mesoderm cells away from the primitive streak

The expression pattern of *Pdgfra* in the mesoderm and the strong expression of *Pdgfra* in the ectoderm suggested that they might be the major signalling partners involved in the control of migration of mesoderm cells. We therefore investigated the effects of interfering with PDGFR α and PDGFA signalling on mesodermal cell migration. Expression of a dominant-negative (dn) PDGFR α from a vector with an internal ribosome entry site driving the simultaneous expression of GFP allowed us to track the movements of transfected GFP-expressing cells during gastrulation. In experiments in which a control construct expressing GFP was electroporated in one half of the epiblast of a HH3 embryo, we observed that the transfected epiblast cells migrated towards the primitive streak, ingressed, then moved out on both sides of the streak to form paraxial mesoderm (5/5 embryos) (Fig. 2A). Expression of dn-PDGFR in one half of the epiblast resulted in the migration of transfected cells towards the primitive streak, but the ability of these cells to migrate away from the streak was severely compromised (8/8 embryos) (Fig. 2B). To investigate whether this effect might be due to a failure of the transfected cells to undergo EMT, we sectioned some of the embryos. This revealed that most of the cells expressing higher levels of the dn-*Pdgfra* construct accumulated in the upper layers of the streak (Fig. 2B'). Some cells expressing lower levels of the dn-*Pdgfra* construct managed to ingress and migrate a little distance away from the streak. In control embryos, most cells ingressed through the streak and then migrated away from the streak (Fig. 2A').

The inhibitory effect of dn-PDGFR on cell migration is cell-autonomous, as cells expressing the dn-*Pdgfra* construct grafted in the streak of an untransfected embryo also showed an almost complete inhibition of migration away from the streak (Fig. 2D), whereas control cells expressing only GFP underwent EMT and migrated out to form somites and lateral plate mesoderm (Fig. 2C-

C''). Sectioning confirmed that the dn-*Pdgfra*-expressing cells were found, as before, in the deeper layers of the embryo, but the majority of the cells failed to migrate away (Fig. 2D'). Staining for β -catenin, a protein enriched in epithelial adherens junctions, confirmed that many of the cells expressing dn-*Pdgfra* had undergone EMT, as they did not express the high levels of β -catenin associated with the apical adherens junctions seen typically in epiblast cells (Fig. 2D').

Depletion of PDGFR α ligands blocks the migration of mesoderm cells

To investigate signalling through PDGFR α in more detail, we depleted PDGFR α ligands by injection of recombinant PDGFR α -Fc protein, while monitoring the migration of GFP-labelled streak cells (Fig. 2E). GFP-expressing streak cells derived from a GFP-transfected donor embryo were grafted into a homotypic position in an age-matched unlabelled host embryo, after which the host embryo was injected with recombinant PDGFR α -Fc protein. In these experiments, very little migration of the GFP-expressing cells out of the streak was observed (Fig. 2E', E''). Injection of the carrier protein BSA, used to stabilise the PDGFR α -Fc protein, had no effect on the migration of the mesoderm cells (data not shown). Embryos injected with PDGFR α -Fc protein consistently developed much less well than the control embryos. Embryos injected with BSA developed a recognisable head and somites (5/6), whereas those injected with PDGFR α -Fc did not form somites and showed only limited head development (6/6) (Fig. 2E).

Knockdown of *Pdgfra* impairs cell migration away from the primitive streak

PDGFR α can bind several PDGF ligands and therefore the observed phenotypes could be caused by depletion of any one or a combination of ligands. Since *Pdgfra* is the most prominent PDGF ligand expressed during the early stages of streak development, we knocked down *Pdgfra* expression by electroporation of *Pdgfra* siRNA

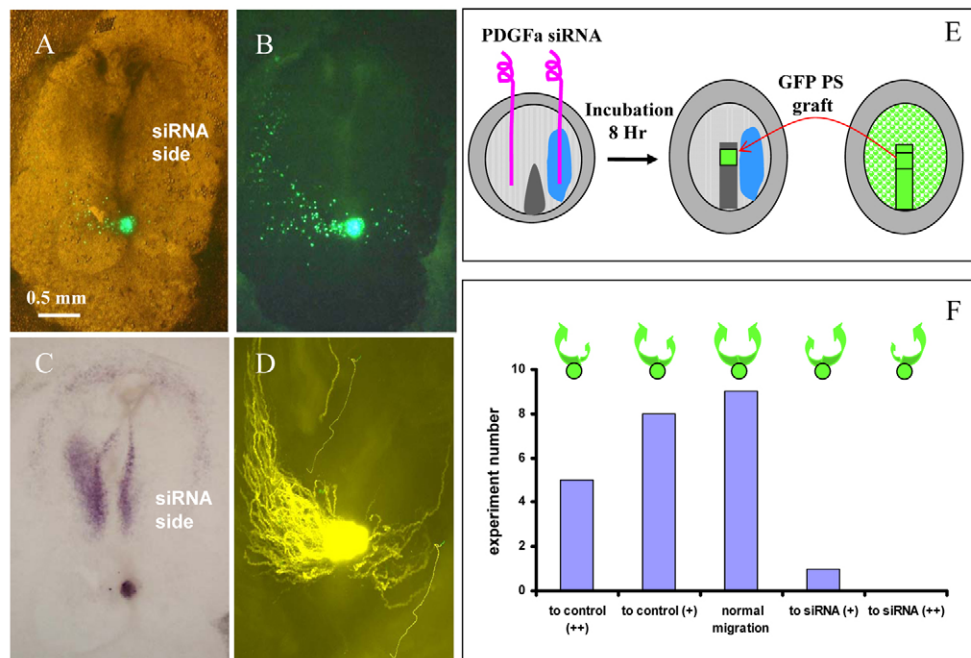


Fig. 4. Half-sided knockdown of *Pdgfa* results in reduced migration in the side of reduced *Pdgfa* expression. (A) Merged brightfield and fluorescence image of a chick embryo 20 hours after transfection with *Pdgfa* siRNA on one side of the streak. (E) GFP-expressing middle streak cells from a control embryo were transplanted in a homotypic position in the streak of the siRNA-transfected embryo. (B) Fluorescent image of the embryo shown in A. (C) In situ hybridisation of the embryo shown in A. (D) Tracks of the fluorescently labelled cells followed over the 20 hours of the experiment. (F) The accumulated results from 23 embryos show that in 13 out of the 23 experiments, the cells move preferentially into areas of normal *Pdgfa* expression, as shown in B,D; in nine experiments, there was no clear effect and in one experiment the cells migrated towards low PDGF (see Movie 1 in the supplementary material). To score the migration, we binned cases in which 80-100% of the tracks went to one side of the streak as strongly positive, cases in which 60-80% of the cells went to one side as positive, and cases where 40-60% went to one side as normal.

to study the effects on cell migration away from the streak. (Fig. 3A,A'). Our previous experiments established that siRNA is transfected effectively in all cells of the transfected region. Although GFP expression from transfected plasmid is more mosaic (Leslie et al., 2007), simultaneous transfection with GFP does allow us to track the movement of at least some of the cells. Embryos transfected with GFP alone, or with a mixture of GFP and a control siRNA (cyclophilin siRNA), served as controls (Fig. 3B,B'). Embryos transfected with control siRNA showed normal development and extensive migration of GFP-expressing cells towards the streak, followed by ingress and migration away from the streak to form paraxial mesodermal structures (Fig. 3B,B'). The embryos developed well and after 22 hours had reached HH8 or 9 (26.2% and 25%, respectively; $n=24$ embryos) and showed normal *Pdgfa* expression (Fig. 3B'). Embryos transfected with *Pdgfa* siRNA did not develop well (Fig. 3B,B'). Development was arrested at the extended streak stage at HH4, 5 and 6 (17.9%, 41% and 20.5%, respectively; $n=39$ embryos) and the GFP-expressing cells were generally unable to migrate out of the streak, similar to what was observed after expression of the dn-*Pdgfra* construct (compare Fig. 2B with Fig. 3A). In situ hybridisation showed an almost complete absence of *Pdgfa* expression in these embryos (Fig. 3A'). These experiments show that PDGFA signalling is crucial in the control of mesodermal cell movement away from the streak.

Using conditions that resulted in a partial inhibition of *Pdgfa* expression (Fig. 3C), sections revealed significantly less mesoderm on the side in which *Pdgfa* expression was downregulated in the ectoderm, as compared with the non-transfected control side (Fig. 3C',C'') and with mock-transfected control embryos (Fig. 3D',D'').

Quantitative analysis of embryos from three independent experiments showed that the number of mesoderm cells in the *Pdgfa* siRNA-transfected side was reduced to $76.6 \pm 6.5\%$ ($n=5$ embryos, five consecutive sections each) of that of the non-transfected control side of the same embryo (probability of distributions being the same at $<10^{-8}$, one-tailed Student's *t*-test). In control experiments in which one side of the embryo was transfected with buffer, there was no significant difference in the number of mesoderm cells on each side ($100.3 \pm 4.9\%$, $n=5$ embryos).

To investigate whether the lack of mesodermal cells on the side of the streak where *Pdgfa* expression was reduced resulted from defective migration, or from a possible indirect effect on their differentiation, we assayed the effect of half-sided *Pdgfa* knockdown on the migration of GFP-labelled mesoderm cells derived from a normal donor embryo (Fig. 4E). In these experiments, streak cells are provided with a choice, either to migrate to the side of the embryo where *Pdgfa* expression is normal or to that where *Pdgfa* expression has been knocked down (Fig. 4C). In the majority of cases, the cells preferentially migrated to the side where *Pdgfa* expression was not affected, avoiding the side where *Pdgfa* expression had been knocked down (Fig. 4B,D,F).

PDGFA can act as an instructive ligand for the migration of mesoderm cells

The data in frogs and fish suggest that PDGFA could have an instructive role in guiding mesoderm cells to more-anterior positions after their migration out of the streak, acting as an attractive guidance cue. We tested whether ectopically applied

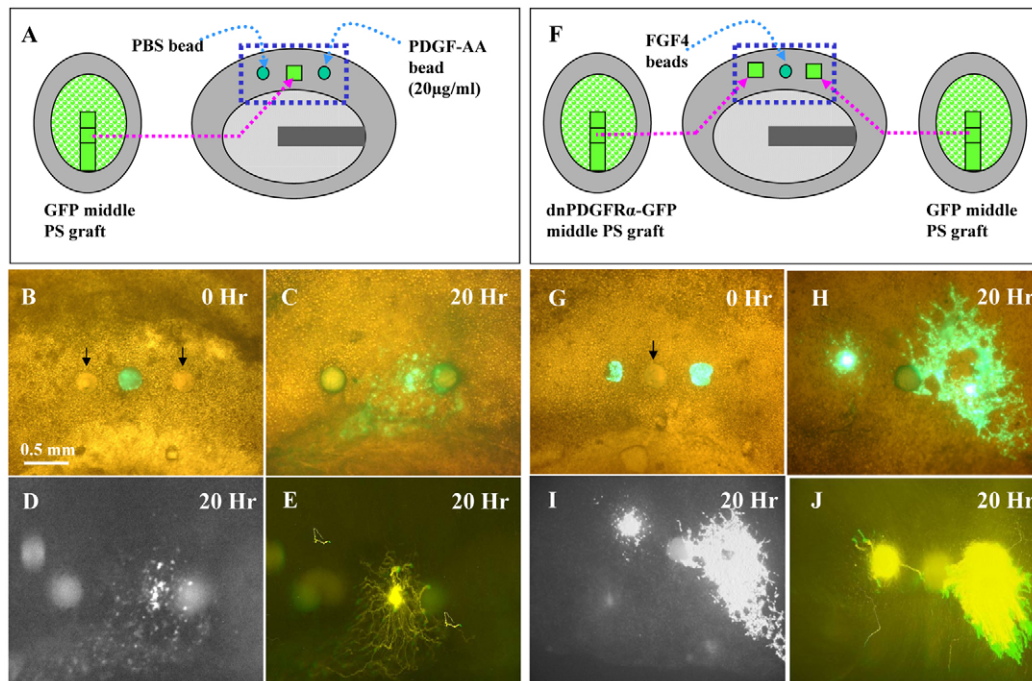


Fig. 5. Signalling through the PDGFA receptor enhances, and is required for, cell migration. (A) GFP-expressing streak cells are grafted in the area opaca between a bead soaked in PBS and a bead soaked in rat PDGFAA short form (20 µg/ml). (B) Merged fluorescence and brightfield image at the start of the experiment. Arrows indicate the position of the PBS- and PDGFA-soaked beads as shown in A. (C) Image of the same region after 20 hours incubation. (D) Fluorescence image after 20 hours development. (E) Cell tracks followed over the duration of the experiment. For further details, see Movie 2 in the supplementary material. A positive response was scored when many cells were seen to migrate towards the beads and to accumulate on their surface. Owing to movements of the embryos during many of the experiments and despite computational fixation of the position of the beads, the resulting tracks are often distorted. (F) Middle streak cells expressing either GFP alone or together with *dn-Pdgfra* are grafted into the area opaca on the right and left sides, respectively, of a bead soaked in FGF4 (100 µg/ml). (G) Merged fluorescence-brightfield image taken at the start of the experiment. Arrow indicates the position of the FGF4-soaked bead as shown in F. (H) Image of the same area after 20 hours of the experiment. (I) Fluorescence image after 20 hours. (J) Cell tracks over the course of the experiment. For further details, see Movie 3 in the supplementary material.

recombinant PDGFAA could act as a chemoattractant in an *in vivo* chemotaxis assay (Yang et al., 2002). We grafted GFP-expressing anterior or middle streak cells into the area opaca of an unlabelled host embryo, in between two heparin-coated beads soaked in recombinant rat PDGFAA (short form) or carrier protein, respectively (Fig. 5A). In a significant number of cases, there was a preferential migration in the direction of the beads soaked in recombinant rat PDGFAA (Fig. 5B-E) (7 positive responses in 12 experiments). Although many cells moved in the direction of the PDGFAA-coated bead, we note that the migration of the cells was not as strong or as directional as we had described previously for the movement of these cells towards FGF4 in the same assays (Yang et al., 2002). This could indicate that the short form of PDGFAA used in the current experiment diffused rapidly and did not form a stable gradient. However, similar experiments performed with the long form of PDGFAA, which contains an N-terminal matrix-binding motif and is assumed to be less diffusible, did not show significantly different results (data not shown). The finding that localised PDGF signalling elicits only a weak directional movement response, but that blocking PDGF signalling results in a strong inhibition of migration of mesoderm cells, strongly suggests that the role of PDGF signalling is not confined to providing directional information, but that it might also be required for another aspect of mesodermal cell movement. We therefore investigated whether inhibition of PDGF signalling affects movement to ectopic sources of other attractants, in this case

FGF4 (Fig. 5F). Experiments in which GFP and *dn-Pdgfra*-expressing cells were challenged with FGF4 confirmed that streak cells expressing GFP show a strong chemotactic response to FGF4 (Fig. 5G-J). Cells expressing the *dn-Pdgfra* construct, however, were severely impeded in their ability to migrate towards the FGF4 source (7/8 experiments).

PDGF signalling controls N-cadherin expression

We explored several possibilities regarding the mechanisms by which PDGF signalling modulates movement towards FGF4. We found no evidence for an effect of PDGF signalling on FGF receptor expression, nor for an effect of *Pdgfra* knockdown on either FGF8 or FGF4 expression (data not shown). Therefore, we directed our attention towards the possibility that PDGF signalling might control some other, more basic aspect of cell movement. We found that PDGF signalling modulates N-cadherin expression on mesoderm cells. Half-sided knockdown of *Pdgfra* resulted in defective development of the transfected side, accompanied by strongly reduced N-cadherin expression as detected by antibody staining (Fig. 6A). Sections showed that in the side where *Pdgfra* expression is knocked down, there are significantly fewer mesoderm cells, resulting in small and malformed somites (Fig. 6A1). However, there were not only fewer mesoderm cells, but also the cells present expressed less N-cadherin on a per-cell basis than those on the control side (Fig. 6A1'A2'). Detailed examination of the cellular N-cadherin

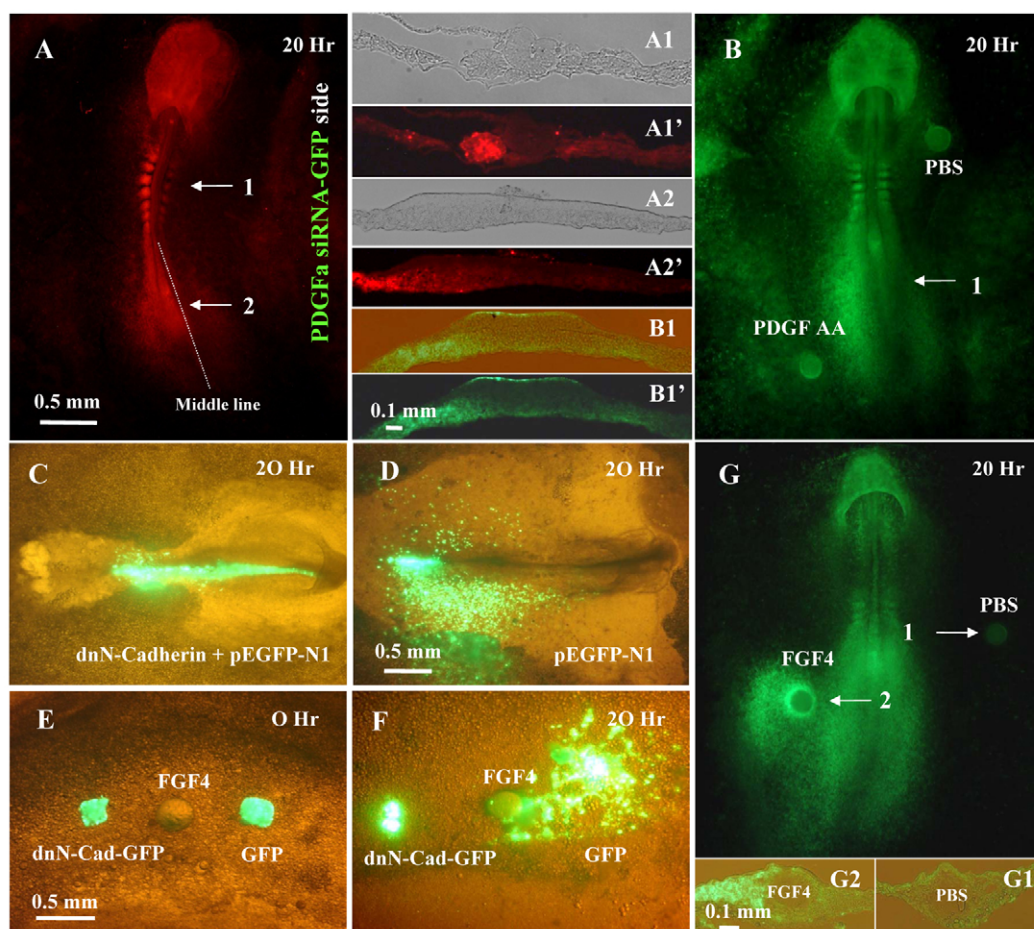


Fig. 6. Effect of PDGF signalling on N-cadherin expression. (A) Reduced N-cadherin expression as detected by immunofluorescence in a chick embryo in which *Pdgfa* has been knocked down in one half of the embryo. The embryo was transfected at stage HH3+ and allowed to develop for 20 hours in early chick (EC) culture. Brightfield image (A1) and N-cadherin immunofluorescence (A1') of section of the embryo in A at level 1. Brightfield image (A2) and N-cadherin immunofluorescence (A2') of section of the embryo in A at level 2. (B) Implantation of a heparin bead soaked in PDGFA results in increased N-cadherin expression at the side of implantation of the bead, which is confirmed in N-cadherin immunofluorescence (B1') and merged brightfield-immunofluorescence (B1) images of section taken at position 1 in B. (C,D) Expression of a dn-N-cadherin construct results in cells migrating to the streak but failure to migrate out, in contrast to control cells expressing just GFP. (E,F) Expression of dn-N-cadherin inhibits chemotaxis of middle streak cells to a bead soaked in FGF4. (E) Merged brightfield and fluorescence images taken at the start of the experiment. (F) The same embryo after 20 hours of development. (G) Local application of an FGF4 bead in a developing embryo results in the attraction of many N-cadherin-expressing cells to the FGF4 bead, which is not the case with a PBS-soaked control bead. Sections through the PBS control and FGF4 beads show that the FGF4 bead has attracted many N-cadherin-expressing cells (G2), whereas the PBS bead has not (G1).

distribution showed that most of the protein is expressed on the cell surface, with a strong enrichment in cellular protrusions, features that were reduced in mesoderm cells on the *Pdgfa* siRNA-treated side of the embryo (see Fig. S1 in the supplementary material). Implantation of a bead soaked in PDGFA short form next to the streak in a HH3+ embryo resulted in strong upregulation of N-cadherin expression on the side of the bead after 20 hours of development (Fig. 6B,B1'). Interestingly, increased N-cadherin expression was not observed in cells immediately surrounding the bead, but was clearly evident in the presomitic mesoderm cells on the side of the streak where the PDGFA bead was implanted, suggesting that PDGF signalling does not control the induction but the stability of N-cadherin. In agreement with this, we found no evidence for a direct effect of PDGF signalling on N-cadherin RNA expression, suggesting that the effect is most likely mediated through a post-transcriptional mechanism (see Fig. S2 in the supplementary material).

Transfection of epiblast cells with a dn-N-cadherin construct resulted in the migration of transfected cells towards the primitive streak, where they underwent EMT but failed to migrate away from the streak (Fig. 6C). By contrast, cells expressing GFP migrated out on both sides of the streak (Fig. 6D). This behaviour phenocopies that observed after expression of dn-PDGFR α (compare Fig. 2B with Fig. 6C). Furthermore, expression of the dn-N-cadherin construct also abolished the ability of mesoderm cells to migrate towards a localised FGF4 signal in the area opaca (Fig. 6E,F), again mimicking the effect of the expression of dn-PDGFR α (compare Fig. 5I with Fig. 6F). These results show that N-cadherin function is required for the migration of mesoderm cells and that PDGF signalling plays a major role in the control of N-cadherin expression on mesoderm cells. We noted that an ectopic FGF4 source strongly attracts N-cadherin-expressing cells (Fig. 6G,G1,G2). We found little direct effect of FGF4 signalling on N-cadherin RNA expression (data not shown). These experiments show that

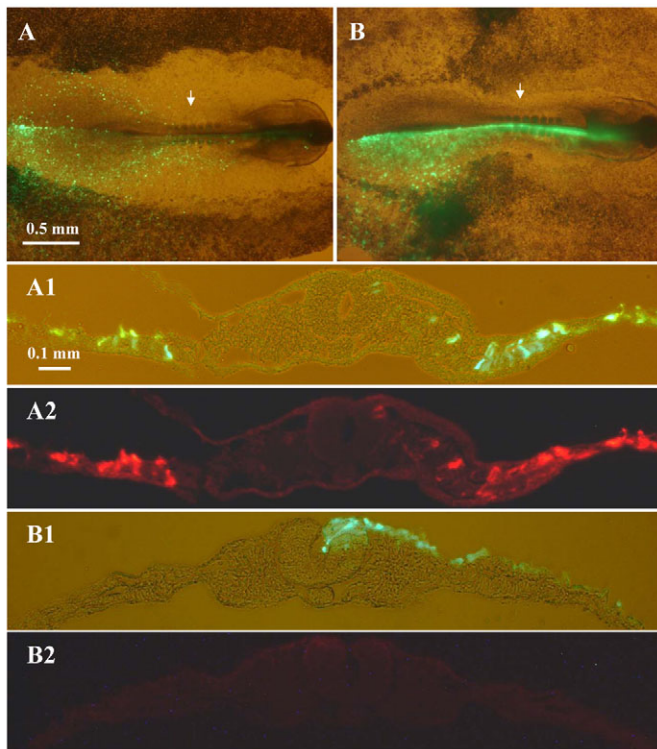


Fig. 7. Expression of N-cadherin rescues mesodermal cell migration after inhibition of PDGF signalling. (A) Cells co-transfected with a dn-PDGFR expression vector (dn-NPDGFR-pCA β /GFP) and full-length N-cadherin expression vector (N-Cad-pCA β /GFP) 20 hours after transfection. Arrow, level of section in A1,A2. (A1) GFP-expressing cells migrate into the mesoderm on both sides of the streak. (A2) N-cadherin expression as detected by 6B3 antibody. (B) Cells co-transfected with dn-NPDGFR-pCA β /GFP and pCA β /GFP 20 hours after transfection. Arrow, level of section in B1,B2. (B1) GFP-expressing cells fail to migrate into the mesoderm on either side of the streak. (B2) N-cadherin expression as detected by 6B3 antibody. B2 was taken at the same exposure as A2, showing that N-cadherin overexpression levels are high compared with endogenous expression levels, which are almost undetectable at these settings.

mesoderm cells that respond to an FGF signal express N-cadherin and confirm that FGF4 appears to be a much more potent attractant than PDGFA (Fig. 6 compare B with G). An important question is whether the inhibitory effects of decreased PDGF signalling on cell migration can be attributed to reduced N-cadherin expression? To investigate this, we co-transfected the dn-PDGFR with a full-length N-cadherin expression construct. Cells co-expressing dn-PDGFR and full-length N-cadherin migrated out of the streak on both sides of the embryo (Fig. 7A,A1,A2), whereas cells that were co-transfected with dn-PDGFR and a GFP expression construct remained confined to the streak and ectoderm/neural tube (Fig. 7B,B1,B2). Expression of dn-PDGFR resulted in severe downregulation of endogenous N-cadherin expression in the few cells that managed to ingress into the mesoderm in a cell-autonomous manner (see Fig. S3 in the supplementary material). We found no evidence for strong effects of PDGF signalling on the regulation of integrins or extracellular matrix formation: we did not detect any effects of siRNA-mediated knockdown of *Pdgfa* on integrin α 6, integrin β 1, laminin or fibronectin expression (see Fig. S4 in the supplementary material). Taken together, these

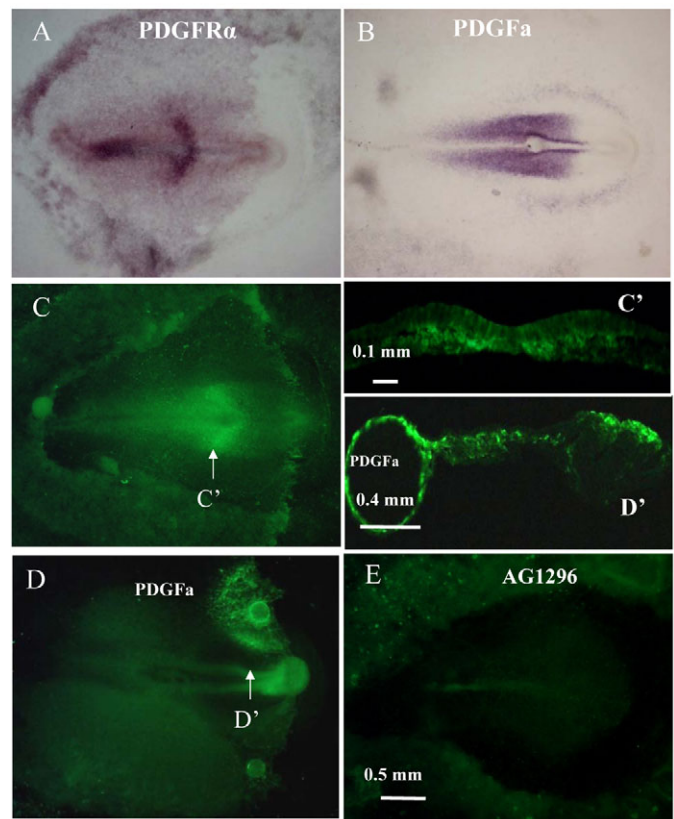


Fig. 8. Coincidence of PDGF and PI3 kinase signalling domains. (A,B) Expression of *Pdgfra* (A) and PDGFA (B). (C) AKT (PKB) activation, measured as phospho-AKT (S473), shows a remarkably similar pattern to that of *Pdgfra* expression (A). (C') Section through the embryo at the arrow in C, showing that the mesoderm cells have activated the PI3 kinase pathway. (D) Application of an ectopic PDGFA bead results in localised activation of the PI3 kinase pathway, measured as AKT phosphorylation (S473) around the bead. (D') Section through the embryo in D at the site of the bead (arrow) shows that the cells that activate the PI3 kinase pathway are mostly mesodermal. (E) AKT staining of an embryo incubated for 10 hours on a substrate containing the PDGFR inhibitor AG1296 (50 μ M).

experiments show that the major defects caused by reduced PDGF signalling can be accounted for by its effects on N-cadherin expression.

PDGFR α signals through PI3 kinase to AKT activation

Experiments in mice have suggested that one of the major signalling pathways downstream of PDGFR α is activation of PI3 kinase (Klinghoffer et al., 2002; Pickett et al., 2008). In previous experiments, we showed that PIP3 signalling is necessary for the directionality of the movement response of mesoderm cells during gastrulation in the chick embryo (Leslie et al., 2007). In the present study, we observed that the expression pattern of PDGFR α and the phosphorylation pattern of AKT (PKB) on serine 473 are very similar during the early stages of gastrulation (Fig. 8, compare A with C). Sectioning showed that AKT phosphorylation was essentially confined to mesoderm cells, the same cells that also express PDGFR α (compare Fig. 8C' with Fig. 11'). High-magnification observations showed that activated AKT was, to a large extent, localised at the plasma membrane of mesoderm cells (data not shown). Inhibition of PDGF signalling

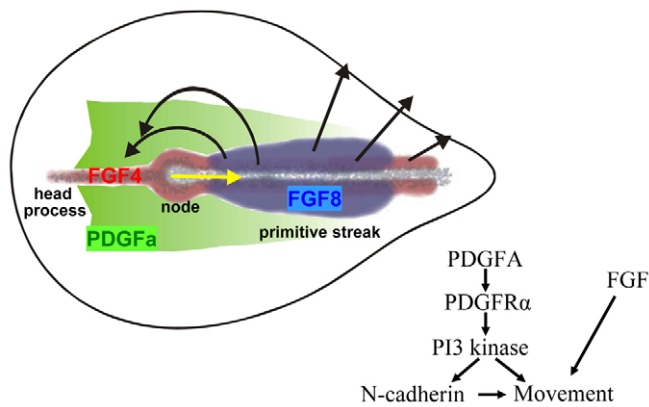


Fig. 9. PDGF and FGF signalling control mesodermal cell migration. PDGFA (green) is expressed in the epiblast on both sides of the streak in a posterior-to-anterior-directed gradient that controls the migration of PDGFR-expressing mesoderm cells. PDGF signalling is most likely to have a dual role in mesodermal cell migration. Through a PI3 kinase-dependent signalling pathway, it controls the expression of N-cadherin on mesoderm cells, which allows them to gain the traction necessary to migrate. Secondly, in cooperation with FGF signalling, it provides instructive information for the anterolateral migration of the mesoderm cells towards their final destination (Yang et al., 2002). The black arrows indicate the patterns of movement of the mesoderm cells after their ingression through the streak. The yellow arrow indicates the direction of regression of Hensen's node.

through application of the PDGFR inhibitor AG1296 resulted in severe depression of AKT phosphorylation in the embryo (Fig. 8E), suggesting that the PDGF signalling pathway is one of the major activators of PI3 kinase signalling in vivo. Application of this inhibitor also resulted in a significant block to development, similar to that observed after downregulation of *Pdgfa*. Local application of the short form of PDGFAA on beads resulted in the activation of the PI3 kinase pathway, as measured by AKT phosphorylation some distance away from the beads (Fig. 8D,D'), whereas application of the long form resulted in activation of AKT phosphorylation in the immediate vicinity of the beads (data not shown). These experiments show that PDGF signalling is a major activator of the AKT pathway in vivo, and through this it may not only control the directionality of cell movement but also modulate N-cadherin expression. To investigate the latter directly, we measured N-cadherin protein expression after incubation of embryos for 6 hours in the presence of the PI3 kinase inhibitor LY294002. The results clearly showed that inhibition of PDGF signalling through the PDGFR inhibitor AG1269, as well as the PI3 kinase inhibitor LY294002, almost completely inhibited N-cadherin expression as measured with N-cadherin antibody (see Fig. S5 in the supplementary material), while there was no effect on N-cadherin RNA expression over this period of time (data not shown). Together, these data make it highly likely that PDGF signalling controls N-cadherin expression through activation of the PI3 kinase pathway.

DISCUSSION

The experiments performed in this study show that PDGFA and PDGFRα are the main ligand and receptor expressed during early chick development. PDGFA signalling through PDGFRα is needed for the migration of cells away from the primitive streak, as migration is blocked effectively by transfection with dn-PDGFRα or by depletion of its ligands. The results of this study show that

PDGF signalling is required for N-cadherin protein expression. N-cadherin expression is known to be widespread on mesodermal cells in the chick embryo, especially in presomitic and somitic mesoderm (Hatta et al., 1987). Our experiments show that N-cadherin function is required for efficient mesodermal cell migration, even in the presence of strong guidance cues such as an ectopic source of FGF4. N-cadherin expression has recently been shown to be important in mesodermal cell migration in zebrafish (Warga and Kane, 2007), and disruption of N-cadherin in mice results in embryonic lethality E10 and in severe adhesion defects in the primitive heart, as well as malformation and fragmentation of somites (Horikawa et al., 1999; Radice et al., 1997), suggesting defective migration of mesodermal precursor cells during the earlier stages of gastrulation, in agreement with our present findings in chick.

It is likely that N-cadherin expression is required for the mesoderm cells to gain traction from their neighbouring cells, as suggested by the strong enrichment in cellular protrusions contacting other cells. Although cells also need to gain traction from the extracellular matrix, and PDGF signalling has been implicated in regulating this in other systems, our experiments have not uncovered a role for PDGF signalling in the expression of integrins or extracellular matrix components. However, it is likely that PDGF signalling, possibly through N-cadherin expression, modulates integrin function rather than expression and, thereby, cell-matrix interactions (Chen and Gumbiner, 2006; Czirok et al., 2004; Nagel et al., 2004; Symes and Mercola, 1996).

We do not yet know exactly how PDGF signalling controls N-cadherin expression, but it most likely involves a post-translational mechanism as we do not detect a direct effect of PDGF signalling on N-cadherin RNA expression (see Fig. S2 in the supplementary material). Although there are many possibilities for post-translational control of N-cadherin expression, it has been shown that the extracellular domain is proteolytically cleaved by metalloproteases, notably ADAM10 (Maretzky et al., 2005; Reiss et al., 2005; Uemura et al., 2006), and that after further processing the resulting intracellular domain of N-cadherin may have a nuclear signalling function in early development (Shoval et al., 2007).

In our experiments, PDGFA acted as a weak attractant and therefore we cannot rule out the possibility that it has an instructional role in mesodermal cell migration. The RNA expression pattern would suggest that gradients of PDGFA exist that could direct migration of mesoderm cells in an anterior direction, as proposed in frogs and fish (Ataliotis et al., 1995; Montero et al., 2003; Nagel et al., 2004). Ectopic sources of both the long and short form of PDGFA can attract mesoderm cells (Fig. 5), and the experiments in which *Pdgfa* expression in the epiblast was reduced in one half of the embryo tended to show less anterior migration in the half with reduced PDGFA expression (Fig. 4B,D), compatible with the hypothesis that PDGFA acts as an instructive signal.

The effects of manipulating PDGF signalling in the chick embryo appear to be stronger than those described in the mouse embryo after deletion of *Pdgfra* or *Pdgfa* (Bostrom et al., 1996; Soriano, 1997). The strong effects observed with dn-PDGFRα in the chick embryo could be due to its ability to form dimers with PDGFRβ and thus to effectively inhibit all PDGF signalling by inactivating all possible receptor complexes. Likewise, injection of PDGFRα-Fc fragments will bind all ligands for this receptor (the PDGFAA, PDGFAB and PDGFCC dimers) and thus potentially block signalling by multiple ligands (Heldin and Westermark, 1999; Tallquist and Kazlauskas, 2004). However, the very strong inhibitory effects of *Pdgfa* knockdown on the migration of mesoderm cells in the chick embryo

suggest that this is a major *in vivo* signal, in agreement with the low expression levels of *Pdgfb* and *Pdgfc* observed in the early chick embryo.

The expression pattern of PDGFA in the chick shows considerable similarity to the pattern of AKT S437 phosphorylation. PDGF stimulation induces AKT phosphorylation, whereas application of the PDGFR inhibitor AG1296 reduces *in vivo* AKT phosphorylation on S473 almost to zero, as well as blocking development. These findings strongly suggest that signalling through PDGFR is a major *in vivo* signal activating the PI3 kinase pathway during gastrulation and that this pathway is crucial for normal development. Our data strongly suggest that the PDGF signalling-dependent regulation of N-cadherin expression is mediated by the PI3 kinase signalling pathway, which therefore not only controls the directionality but also the speed of cell migration. These data are in good agreement with findings in zebrafish in which application of AG1295 resulted in slow and defective anterior migration of prechordal plate mesoderm cells (Montero et al., 2003).

In conclusion, it is evident that PDGF signalling is required for the proper migration of mesoderm cells during the early stages of gastrulation in the chick embryo. PDGFA is the major form of PDGF expressed during the early stages of chick embryo development. We propose that the PDGFA signalling domain outlines a route along which paraxial mesoderm cells move efficiently, a process that is likely to involve control of the expression of N-cadherin on mesodermal cells as well as the provision of guidance information for the cells to move forward. This guidance system has to cooperate with other signalling systems, such as the FGF8-mediated repulsion that is required to send cells away from the streak and the FGF4-mediated attractive movement towards the anterior and back towards the midline (Fig. 9) (Yang et al., 2002).

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/21/3521/DC1>

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