

Guidance of mesoderm cell migration in the *Xenopus* gastrula requires PDGF signaling

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Summary

In vertebrates, PDGFA and its receptor, PDGFR α , are expressed in the early embryo. Impairing their function causes an array of developmental defects, but the underlying target processes that are directly controlled by these factors are not well known. We show that in the *Xenopus* gastrula, PDGFA/PDGFR α signaling is required for the directional migration of mesodermal cells on the extracellular matrix of the blastocoel roof. Blocking PDGFR α function in the mesoderm does not inhibit migration per se, but results in movement that is randomized and no longer directed towards the animal

pole. Likewise, compromising PDGFA function in the blastocoel roof substratum abolishes directionality of movement. Overexpression of wild-type PDGFA, or inhibition of PDGFA both lead to randomized migration, disorientation of polarized mesodermal cells, decreased movement towards the animal pole, and reduced head formation and axis elongation. This is consistent with an instructive role for PDGFA in the guidance of mesoderm migration.

Key words: *Xenopus*, PDGF, Gastrulation, Cell migration

Introduction

The migration of mesodermal cells away from their site of internalization is a conserved feature of vertebrate gastrulation, although the importance of this process on the mechanics of gastrulation may differ between taxonomic groups (Winklbauer, 1994). Available evidence suggests that migration is essential for gastrulation in the chicken (Harrison et al., 1993), and contributes to germ layer formation in carp (Gevers et al., 1993) and zebrafish (Yamashita et al., 2002). In the mouse, null mutations for various integrins and matrix proteins have not yet provided conclusive evidence for the functional relevance of mesoderm migration (De Arcangelis and Georges-Labouesse, 2000; Gustafsson and Fassler, 2000). In amphibians, blocking migration impedes gastrulation completely in the urodele *Pleurodeles* (Boucaut et al., 1984; Darribere et al., 1990), and less severely, though distinctly, in anurans such as *Xenopus* or *Rana* (Smith et al., 1990; Saint-Jeannet and Dawid, 1994; Winklbauer and Keller, 1996).

In *Xenopus*, migration of dorsal anterior mesoderm cells across the ectodermal blastocoel roof (BCR) relies on their interaction with a network of fibronectin (FN) containing fibrils (Winklbauer and Keller, 1996), the extracellular matrix of the BCR (Nakatsuji and Johnson, 1983a; Winklbauer, 1998). FN contributes to the adhesion of mesoderm cells to the BCR, and, most importantly, it elicits the formation of lamellipodia, the locomotory organelles of these cells (Winklbauer, 1990; Ramos and DeSimone, 1996; Winklbauer and Keller, 1996).

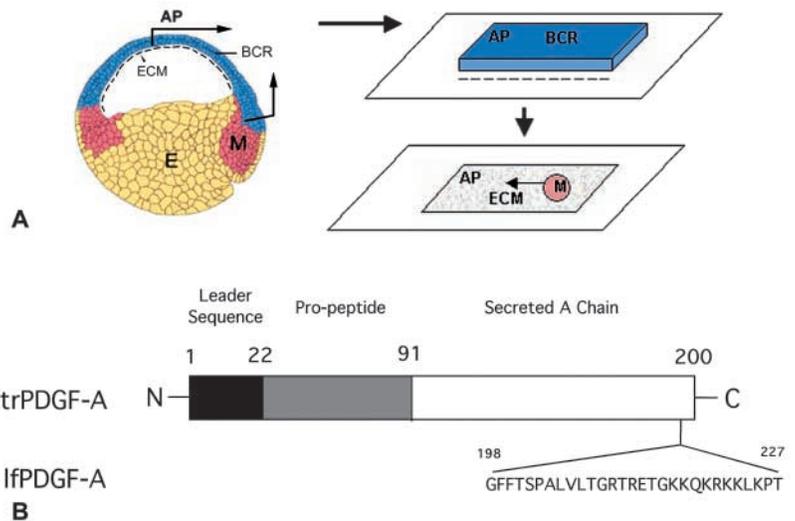
In pioneering work, Nakatsuji and Johnson (Nakatsuji and Johnson, 1983b) showed that in addition to serving as permissive substratum, the BCR extracellular matrix contains guidance cues that determine the direction of migration. When BCR is explanted

to deposit its matrix on an inert surface, dispersed mesodermal cells from several amphibian species migrate on this conditioned substratum directionally, recognizing cues that guide them to the animal pole region (Nakatsuji and Johnson, 1983b). In *Xenopus*, migration in vitro is also directional, although only when aggregates of mesodermal cells are used to probe conditioned substratum, instead of single cells (Fig. 1A). Moreover, in the *Xenopus* embryo, anterior mesoderm cells extend locomotory processes towards the animal pole, confirming that migration is directional in vivo (Winklbauer and Nagel, 1991).

To identify the nature of the guidance cues that direct mesoderm migration has been a goal since their discovery two decades ago. Previously, we have shown that signals emanating from the vegetal part of the embryo, which employ fibroblast growth factor (FGF)- and activin-like signaling pathways for propagation, polarize the BCR layer. This polarization is a prerequisite for the deposition of a correctly oriented extracellular matrix (Nagel and Winklbauer, 1999). However, the molecular basis of the matrix-borne directional cues remained elusive. We address this long-standing unresolved issue, and show that platelet-derived growth factor (PDGF) signaling plays an essential role in mesoderm guidance during *Xenopus* gastrulation.

PDGF signaling is involved in a variety of processes, including early embryonic development, formation of the nervous system, heart and lung development, and angiogenesis. It has also been implicated in several pathological conditions, such as oncogenesis, atherosclerosis, lung and kidney fibrosis, and wound healing. Major target processes of PDGF signaling are the regulation of proliferation, and the control of cell motility and migration (Ataliotis and Mercola, 1997; Heldin and Westermark, 1999; Betsholtz et al., 2001).

Fig. 1. Experimental procedures. (A) Mesoderm migration on conditioned substratum. (Left) Mesoderm cells migrate on the extracellular matrix (ECM) of the BCR towards the animal pole (AP) (arrow). Blue, prospective ectoderm; pink, mesoderm (M); yellow, endoderm (E). (Right, top) A strip of BCR (bracket in left figure) is explanted at stage 10, and cultured for 1 hour with its matrix-forming surface down. (Right, bottom) The ECM of the BCR has become deposited at the bottom of the dish. A mesodermal explant (M) placed on the conditioned substratum migrates (arrow) to the animal pole position. (B) Construction of trPDGFA. The sequence corresponding to amino acid residues 198–227, that contain the matrix binding motif, was deleted from the lfpDGFA.



The PDGF family comprises four members: PDGFA and PDGFB, which form homo- and heterodimers (Heldin and Westermark, 1999), and the CUB-domain containing PDGFC and PDGFD (Li et al., 2000; Bergsten et al., 2001). In the *Xenopus* gastrula, PDGFA is expressed in the BCR, whereas PDGFB is barely detectable, suggesting the exclusive presence of PDGFAA homodimers (Mercola et al., 1988). The A-chain occurs in two splice variants, a long form (lfpDGFA) that contains a C-terminal site involved in extracellular matrix binding, and a short form (sfPDGFA) lacking the matrix-binding motif (Raines and Ross, 1992; Andersson et al., 1994). RNAs of both variants are present in the *Xenopus* gastrula (Mercola et al., 1988). Of the possible PDGF receptor dimers (PDGFR $\alpha\alpha$, PDGFR $\alpha\beta$ and PDGFR $\beta\beta$), only PDGFR $\alpha\alpha$ recognizes PDGFAA (Ataliotis and Mercola, 1997; Heldin and Westermark, 1999), and the PDGFR α isoform is in fact expressed in the *Xenopus* mesoderm at gastrulation (Jones et al., 1993).

The complementary pattern of expression, with PDGFA ligand in the BCR and its receptor in the adjacent mesoderm (Ataliotis et al., 1995), suggests a role for paracrine PDGFA signaling in mesoderm development. Disruption of PDGF function by means of a dominant-negative PDGFR α leads to aberrant movement of the mesoderm (Ataliotis et al., 1995), and treatment of mesoderm aggregates with PDGFAA protein stimulates spreading on FN in vitro (Symes and Mercola, 1996). However, it is not known which of the several mesoderm-driven gastrulation movements in *Xenopus* is actually controlled by PDGFA.

We identify a distinct *in vivo* function for PDGFA signaling in early *Xenopus* development. We show that interfering with PDGF function randomizes mesoderm migration on conditioned substratum *in vitro*, and disrupts the orientation of migratory cells in the embryo. We propose that matrix-bound PDGFAA could be instructive in mesoderm guidance during gastrulation.

Materials and methods

Embryos and explants

Xenopus laevis embryos from induced spawnings (Winklbauer, 1990) were staged according to Nieuwkoop and Faber (Nieuwkoop and

Faber, 1967). Operation techniques and buffer (Modified Barth's Solution, MBS) have been described previously (Winklbauer, 1990).

Substratum conditioning

BCR explants were held against the bottom of Greiner tissue culture dishes (TC grade) by strips of coverslip resting on both ends on silicone grease. Before removing BCR explants by aspiration with buffer, their outlines and orientation were marked on the dish. Substrata were saturated with 50 mg/ml bovine serum albumin (BSA) and used in the migration assay (Winklbauer and Nagel, 1991) (Fig. 1A). FN-coated substratum was prepared by incubating Greiner tissue culture dishes with bovine plasma fibronectin solution (Sigma) at 200 μ g/ml for 30 minutes, followed by saturation of the substratum with 50 mg/ml BSA.

Directional migration assay

Displacement of anterior mesoderm explants (about 200 cells per explant) on conditioned substratum (one explant per substratum) was observed on an inverted microscope. Positions of explants were recorded at the start of the experiment, and 1 hour later. The positions after 1 hour are indicated by circles, with starting points all superimposed at the origin. Movement towards the position of the animal pole of the BCR was assigned positive values on the *y*-axis. Using a one-sided sign test, the null hypothesis that explants move anteriorly and vegetally with equal frequency was tested against the alternative hypothesis, that aggregates move preferentially towards the animal pole.

FN fibril and actin cytoskeleton staining

Embryos were fixed in 4% formaldehyde, BCRs were removed with needles, blocked in 5% BSA and stained with rabbit antibody to *Xenopus* FN (Winklbauer, 1998), and FITC-conjugated secondary antibody. Anterior mesoderm cells on conditioned substratum were fixed after 1 hour in 4% formaldehyde/0.1% Triton-X-100, and stained with 5 μ M TRITC-conjugated phalloidin (Sigma) for 30 minutes.

Scanning electron microscopy

Embryos were fixed in 2.5% glutaraldehyde in MBS, and BCRs were removed with needles. Specimens were postfixed in 1% OsO₄, dehydrated in a graded ethanol series, critical point dried and sputter coated with gold-palladium.

In situ hybridization

In situ hybridization was modified from the methods of Harland

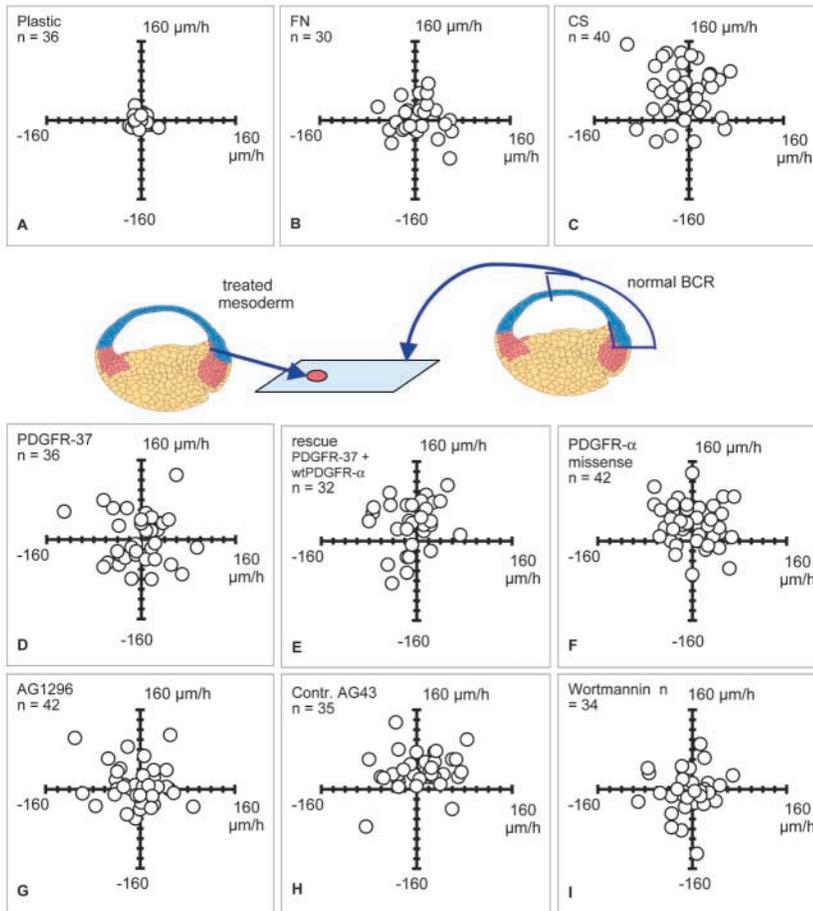


Fig. 2. Directional migration and its dependence on PDGFR α signaling. (A-C) Comparison of mesoderm migration on untreated tissue culture plastic (A), FN-coated plastic (B) and conditioned substratum (C). (D-I) Migration of PDGFR α -inhibited mesoderm on normal conditioned substratum (see explanatory scheme above). Inhibition of PDGFR α signaling by expression of dominant-negative PDGFR37 mRNA randomizes the direction of migration (D). Directed migration is restored by co-expression of wild-type PDGFR α (E). Expression of missense PDGFR α does not affect mesoderm migration (F). Mesoderm explants moving on conditioned substratum were treated during the 1 hour migration period with tyrphostin AG 1296 (G), control tyrphostin AG 43 (H) or Wortmannin (I). Both AG 1296 and wortmannin randomize the direction of migration. n, number of explants tested; results from at least three independent experiments. Explants in C,E,F,H prefer the animal pole side significantly (one-sided sign test, significance level $\alpha=0.005$ for (E) and 0.0005 for all others). In the same test, explants in A,B,D,G,I show no preference for the animal pole at any significance level.

Reagents

Human recombinant PDGFAA protein, long form (TEBU, Germany) was used at 50 ng/ml. Tyrphostin AG 1296, an inhibitor of PDGF receptor tyrosin kinases, and AG 43 control tyrphostin (Calbiochem-Novabiochem) were dissolved in DMSO (10 mg/ml) and used at a final concentration of 5 μM, Wortmannin (Sigma) at 100-200 nM. Anti-PDGFA morpholino antisense oligonucleotide (GeneTools, 5'-AGAATCCAAGCCCAGATCCTCATTG-3') and 5

bp mispaired control morpholino (exchanged bases underlined) were injected at 60 ng per embryo.

(Harland, 1991). Alkaline phosphatase substratum was BM Purple (Roche). For the antisense *Xbra* digoxigenin probe, plasmid DB30 (gift of M. Sargent, London) was linearized with *Bgl*III and used as a template for transcription with T7 RNA polymerase. *chordin* and *gsc* probes (Sasai et al., 1994; Cho et al., 1991) (gifts from H. Steinbeisser, Heidelberg) were generated by linearizing clones 59 and H7, respectively, in pBluescript SK(-) with *Eco*RI and transcription with T7 polymerase.

Constructs, mRNA synthesis and injection

Plasmids pCS2 containing wild-type *Xenopus* PDGFR α , a dominant-negative version of this receptor, PDGFR37, or a PDGFR α missense construct (Ataliotis et al., 1995), were linearized with *Nhe*I and transcribed with T7 polymerase. pGHE2 containing the wild-type long form of *Xenopus* PDGFA (Mercola et al., 1988), lfpDGFA, was linearized with *Nhe*I and transcribed with T7 polymerase. A C-terminally truncated form of lfpDGFA lacking the matrix-binding motif (amino acid residues 198-227) was generated by PCR using pGHE2-PDGFA (Mercola et al., 1988) as a template (Fig. 1B). The truncated form of PDGFA (trPDGFA) was subcloned into the *Bam*HI/*Eco*RI site of pCS2 and used for in vitro transcription with SP6 polymerase after linearization with *Xho*I. Plasmid pGHE2 harboring a processing defective mutant of mouse PDGFA that acts as a dominant-negative mutant in *Xenopus* (Mercola et al., 1990) was linearized with *Nhe*I and transcribed with T7 polymerase. Embryos were injected at the four-cell stage into all four blastomeres, marginally with 250 pg mRNA per embryo (PDGFR α , PDGFR37, missense PDGFR α), or anally with 400 pg mRNA per embryo (lfpDGFA, trPDGFA) or 200 pg mRNA per embryo (dnPDGFA).

Results

Directional migration requires PDGFR α signaling in the mesoderm

Migration of cells from the *Xenopus* gastrula is studied in vitro in simple, serum-free medium. To compare migratory behaviors of mesoderm aggregates, their translocation was observed on three different substrates: plain tissue culture plastic, an artificial substratum of purified FN, and BCR-conditioned substratum (Fig. 2A-C). In the absence of FN, explants do not move (Fig. 2A). Coating dishes with FN suffices to stimulate translocation but, as expected, the direction of movement is random relative to an arbitrary coordinate system (Fig. 2B). By contrast, aggregates move directionally towards the position of the animal pole on conditioned substratum, and migration is also more vigorous (Fig. 2C), in agreement with previous results (Winklbauer and Nagel, 1991; Nagel and Winklbauer, 1999). During substratum conditioning, FN is deposited, which promotes migration; however, additional components are apparently present that guide mesoderm explants to the animal pole.

To examine whether PDGF signaling is involved in this guidance, a dominant negative PDGFR α construct, PDGFR37 (Ataliotis et al., 1995), was expressed in mesoderm explants

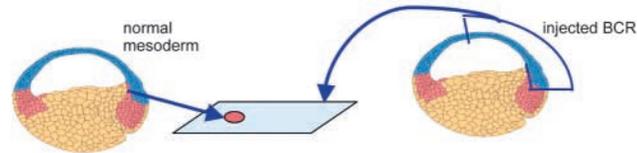
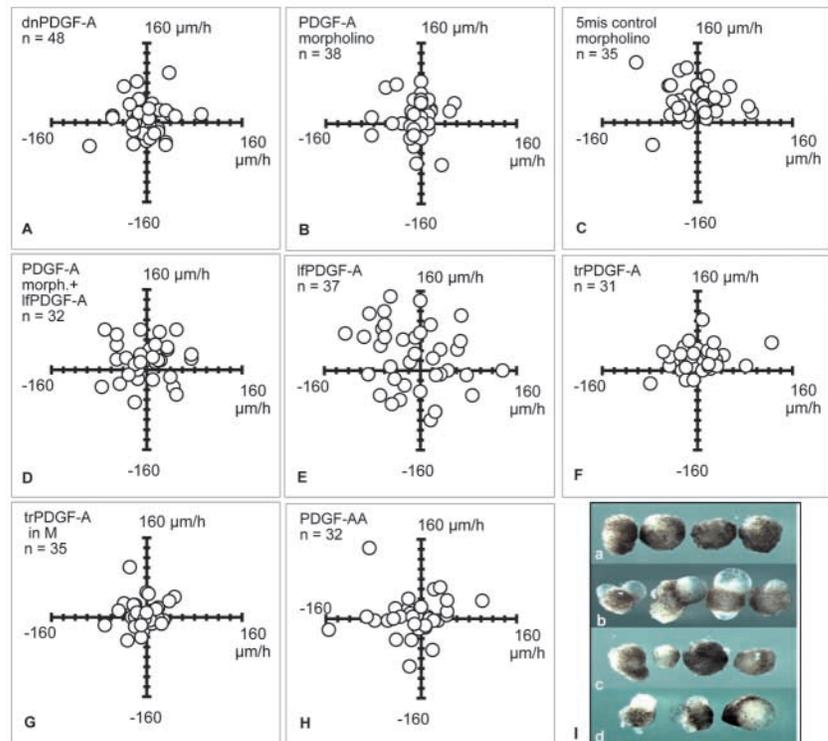


Fig. 3. Interference with PDGFA function in the BCR randomizes the migration of normal mesoderm. (A-F) Normal mesoderm on substratum conditioned with PDGFA-compromised BCR (see explanatory scheme above). Substratum was conditioned with BCR containing inhibitory dnPDGFA mRNA (A), or morpholino antisense oligonucleotides directed against PDGFA (B), 5-mismatch control morpholino (C), PDGFA morpholino together with lfpdgfa mRNA (D), lfpdgfa mRNA alone (E) or C-terminally truncated trPDGFA (F). (G,H) Mesoderm expressing trPDGFA mRNA (G) or normal mesoderm migrating in the presence of exogenous PDGFAA protein (H), on normal conditioned substratum. n, number of explants tested; results from at least three independent experiments. Explants in C,F prefer the animal pole direction significantly (significance level $\alpha=0.0005$). Explants in A,B,D,E,G,H show no preference for the animal pole [at $\alpha=0.05$ for (B,D) or any significance level for all others]. (I) Specificity of morpholino function. Animal caps were explanted at stage 9, and cultured for 3 days. (a) untreated animal caps; (b) caps from embryos co-injected with PDGFR α and lfpdgfa mRNAs; (c) caps as in b, but with additional injection of PDGFA morpholino; (d) caps as in c, but incubated in 200 ng/ml of human recombinant PDGFA protein after explantation.



migrating on conditioned substratum. Under these conditions, translocation is randomized and resembles that on plain FN substratum (Fig. 2D). Co-injection of a wild-type receptor with the dominant-negative construct rescues the directionality of migration significantly (Fig. 2E). Expression of a PDGFR α missense mutation in the migrating mesoderm does not affect directionality (Fig. 2F).

The inhibitory PDGFR37 does not impair mesoderm induction in *Xenopus* (Ataliotis et al., 1995). However, in order to ensure that its effect on migration is not due to subtle alterations of mesoderm development, PDGFR α signaling was blocked directly in migrating explants taken from normally developed mesoderm, by treatment with the cell permeable inhibitor, AG1296 (Kovalenko et al., 1997). This inhibition (Fig. 2G), but not treatment with a similar control substance, AG43 (Fig. 2H), also abolished directionality, suggesting that PDGFR signaling is required during actual migration, and not during formation or patterning of the mesoderm. As the previously reported effect of PDGFR α signaling on mesoderm motility was mediated by PI3 kinase (Symes and Mercola, 1996), aggregates on conditioned substratum were also treated with an inhibitor of phosphatidylinositol 3 (PI3) kinase, Wortmannin. In its presence, migration was non-directional (Fig. 2I), implicating this kinase in the pathway that regulates directionality of movement.

PDGFA expression in the BCR is essential for directional migration

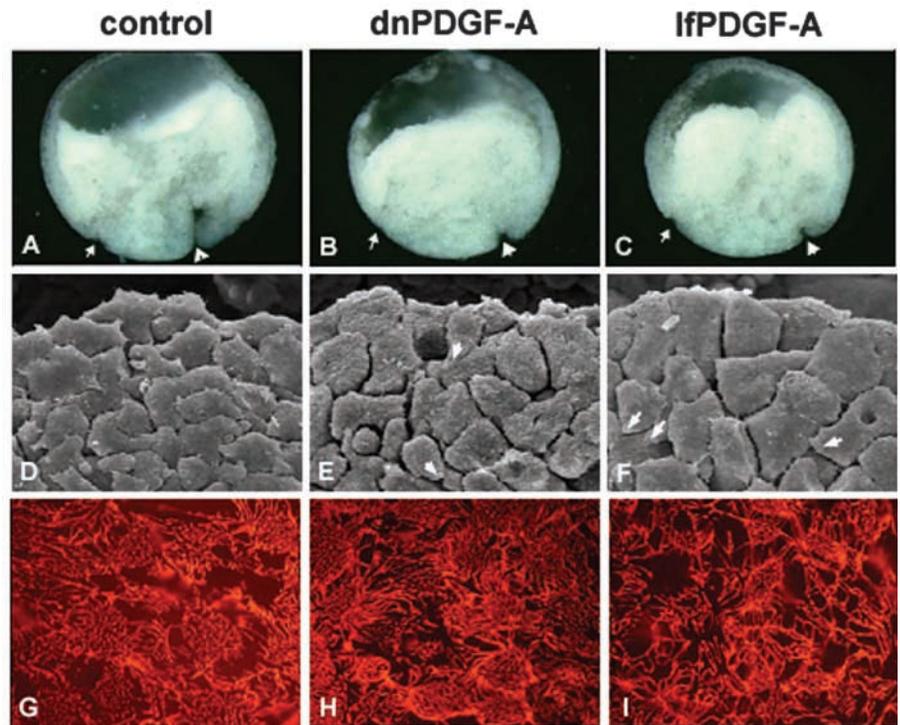
PDGF signaling was also disrupted by blocking PDGFA in the

BCR. Expression of the dominant negative dnPDGFA (Mercola et al., 1990) in the BCR leads to non-directional migration of normal mesoderm on substratum conditioned with injected BCR (Fig. 3A). Likewise, impeding translation of endogenous PDGFA mRNA in the BCR by morpholino antisense oligonucleotide randomizes translocation (Fig. 3B), whereas injection of a 5-mispaired control morpholino has no such effect (Fig. 3C). To further demonstrate specificity of the morpholino, we employed the fact that mesoderm differentiation can be induced by exogenous PDGFA in animal caps expressing PDGFR α (Ataliotis et al., 1995). In contrast to untreated caps, those co-injected with receptor and with *Xenopus* PDGFA mRNA containing the morpholino target site develop transparent bulges indicative of ventral-type mesoderm. Additional injection of anti-PDGFA morpholino completely blocks this effect, but it can be rescued by treatment of explants with PDGFA protein (Fig. 3I). This suggests that the morpholino specifically inhibits *Xenopus* PDGFA function.

Injection of mRNA coding for the matrix-binding lfpdgfa together with the anti-PDGFA morpholino does not substantially rescue directionality of migration (Fig. 3D). A rescuing effect is not to be expected, however, as overexpression of lfpdgfa alone suffices to reduce the directionality of translocation significantly (Fig. 3E). Thus, both diminishing and increasing PDGFA activity in the BCR adversely affect directionality, consistent with an instructive function for this factor in mesoderm guidance.

To guide cells on conditioned substratum, PDGFA should be

Fig. 4. Effects of compromised PDGFA signaling on gastrula. Stage 10.5 gastrulae of uninjected controls (A,D,G) are compared with sibling embryos injected with inhibitory dnPDGFA mRNA (B,E,H) and wild-type lfpPDGFA mRNA (C,F,I). (A-C) Gastrulae were fixed and fractured in the sagittal plane, dorsal is towards the right, animal towards the top; arrowheads, dorsal blastopore; small arrows, ventral blastopore; large arrows, pointed leading edges in controls, and corresponding positions in treated embryos. (D-F) In parallel, gastrulae were prepared for scanning electron microscopy. The BCR was removed to expose the surface of the leading edge mesoderm that had been in contact with the BCR substratum. Animal is towards the top. Arrow, cells with laterally or vegetally oriented protrusions. Scale bar: 50 μm . (G-I) BCRs of gastrulae were stained for the presence of a FN fibril matrix.



associated with the extracellular matrix. To see whether the matrix-binding capability of lfpPDGFA is essential for its interference with directional migration, we deleted the C-terminal matrix-interacting motif from the wild-type lfpPDGFA. When this truncated form, trPDGFA, is expressed in the BCR, directionality of migration on respective conditioned substratum is not affected (Fig. 3F). Apparently, only matrix-binding PDGFA can interfere with mesoderm guidance. This implies that it is the endogenous matrix-binding lfpPDGFA that is involved *in vivo* in determining the direction of migration on the BCR.

When trPDGFA is expressed in the mesoderm, migration on normal conditioned substratum is randomized (Fig. 3G). This shows that the trPDGFA construct is functional, and that its ectopic expression in the mesoderm interferes with the orientation of aggregates on normal substratum. In addition, exposure of mesoderm aggregates to human recombinant PDGFAA protein during migration on normal conditioned substratum abolishes directionality (Fig. 3H). This confirms that proper PDGF signaling is required during migration, and not earlier, as, for example, during patterning. Furthermore, as in the previous experiment, it suggests that the abundant, uniform presence of soluble PDGFAA interferes with the ability of migrating cells to read the cues on the conditioned substratum, arguing also in favor of an instructive role of matrix-binding lfpPDGFA in cell guidance.

PDGF signaling is required for orientation and protrusive activity of migrating cells in the embryo

By the mid-gastrula stage, vegetal rotation (Winklbauer and Schurfeld, 1999) has moved the anterior dorsal mesoderm against the BCR. The blastocoel floor is concave, and the mesoderm exhibits a pointed leading edge that is apposed to the BCR (Fig. 4A). Both the expression of inhibitory

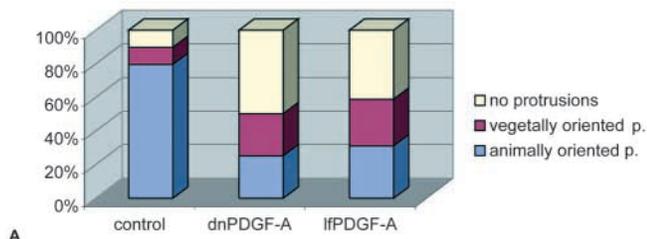
dnPDGFA (Fig. 4B) and the overexpression of wild-type lfpPDGFA (Fig. 4C) in the BCR affect gastrula morphology in a similar way: the mesoderm does not develop a pointed leading edge, and the blastocoel floor does not become concave (Fig. 4B,C). This morphology is consistent with mesoderm movement across the BCR being impeded while vegetal rotation continues to internalize vegetal cell mass.

The BCR-apposed surface of the mesoderm displays a shingle arrangement: cells underlap adjacent cells in front of them, and are underlapped by processes from cells behind, indicating an orientation of cells in the direction of the animal pole (Winklbauer and Nagel, 1991) (Fig. 4D). In dnPDGFA-expressing embryos, but also upon overexpression of lfpPDGFA, this shingle arrangement is abolished (Fig. 4E,F). Quantitative analysis shows that normally, cytoplasmic processes point almost always animally, but that protrusions in PDGF signaling-defective embryos are directed with equal frequency towards and away from the animal pole (arrows in Fig. 4E,F; Fig. 5A), indicating disruption of cell orientation.

The frequency of protrusions is also affected in dnPDGFA and lfpPDGFA expressing embryos, respectively. In both cases, it is significantly reduced to almost half its normal value (Fig. 5A). However, this does not reflect a direct control of protrusive activity by PDGF signaling. On substratum conditioned by control BCR, and by BCR injected with dnPDGFA or lfpPDGFA mRNA, respectively, the frequency of cells extending processes does not vary as strongly as in the embryo, although slightly fewer protrusions form on dnPDGFA substratum (Fig. 5B). Apparently, it is under the conditions of mesoderm movement in the embryo where proper PDGF signaling is required to increase protrusion formation.

To exclude the possibility that disorientation or absence of protrusions are due to defective FN fibril network formation

Protrusions in embryo



Protrusions of single cells on conditioned substratum

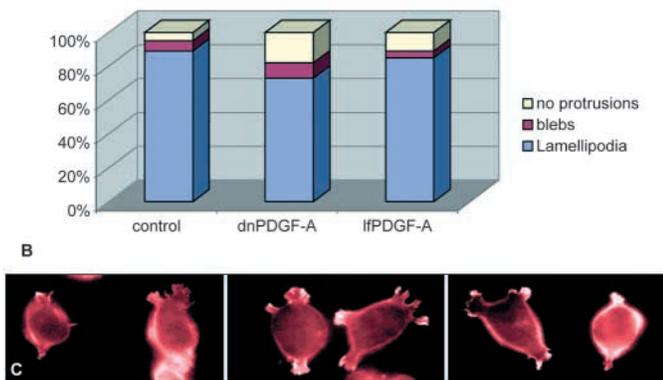


Fig. 5. PDGFA signaling and protrusive activity. (A) Protrusions in the mesoderm of normal and of PDGFA signaling-compromised gastrulae, as determined from scanning electron microscope pictures. Percentage of cells with no underlapping protrusions, with protrusions pointing animally, or vegetally, were assessed in uninjected embryos ($n=157$ cells), and in embryos injected with dnPDGFA ($n=155$) or lfpdGF-A mRNA ($n=158$), respectively. Cells were scored as animally oriented if the vector, cell center-protrusion, pointed upwards at any angle in carefully oriented SEM pictures, and vegetally oriented if otherwise. The lower percentage of protrusion-bearing cells in dnPDGFA and lfpdGF-A embryos, when compared with controls, is highly significant (significance level $\alpha=0.001$, χ^2 -test), the difference between dnPDGFA and lfpdGF-A embryos is not significant. (B,C) Protrusions of single mesodermal cells on substratum conditioned with control BCR, with BCR overexpressing lfpdGF-A or dnPDGFA. (B) The percentage of cells extending cytoplasmic protrusions (lamellipodia or pseudopodial blebs) was determined. The fraction of cells with no processes is significantly higher on dnPDGFA conditioned substratum ($n=317$ cells) when compared with control ($n=323$) or lfpdGF-A ($n=467$) substratum (significance level $\alpha=0.01$, χ^2 -test). (C) Examples of typical cell morphologies (rhodamine-phalloidin staining of the actin cytoskeleton) are shown.

(Winklbauer and Nagel, 1991; Winklbauer and Keller, 1996), the BCR was stained with FN antibody. A normal FN fibril network (Fig. 4G) is present in dnPDGF (Fig. 4H) or lfpdGF-A overexpressing embryos (Fig. 4I), or after anti-PDGFA morpholino injection (not shown). Together, these results suggest that orientation of migrating mesodermal cells and normal protrusive activity in the embryo depend on proper PDGFA signaling.

PDGF signaling controls mesoderm translocation during gastrulation

To determine what consequences disoriented migration has on

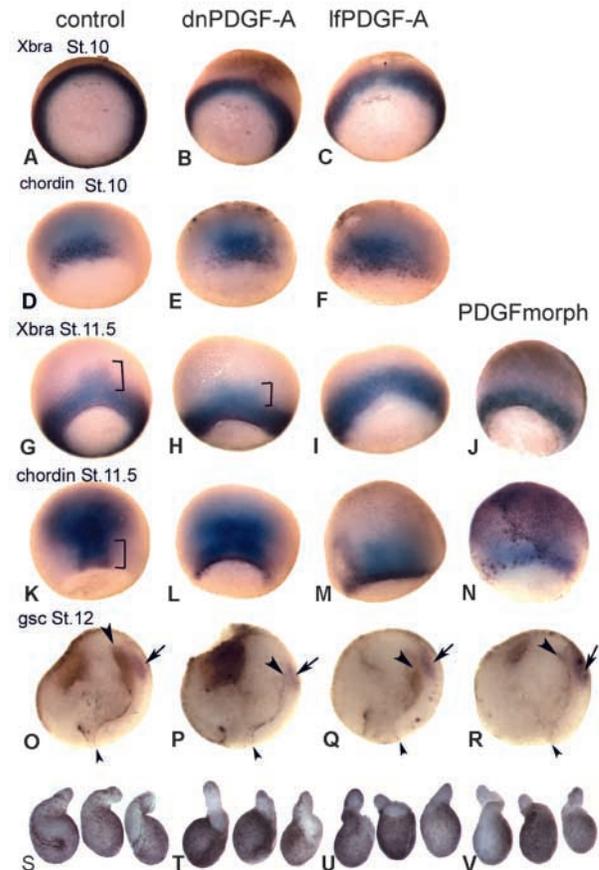


Fig. 6. Effect of compromised PDGFA signaling on mesoderm movement. (A-R) In situ hybridization. Expression of *Xbra* at stage 10 (A-C) and stage 11.5 (G-J), and of *chordin* at stage 10 (D-F) and stage 11.5 (K-N), in uninjected embryos (A,D,G,K), and in embryos injected with dnPDGFA (B,E,H,L) or lfpdGF-A mRNA (C,F,I,M) or PDGF morpholino (J,N). (A-C) Vegetal view. (D-N) Dorsovegetal view with animal towards the top. Brackets indicate extending notochord region. (O-R) Expression of *gsc* in controls (O), and in dnPDGFA (P), lfpdGF-A (Q) and PDGF morpholino (R) gastrulae fixed at stage 12 and fractured mid-sagittally. Small arrowhead, blastopore lip; large arrowhead, tip of archenteron; arrow, *gsc* expression. (S-V) Convergent extension in dorsal blastopore lips explanted at stage 10 from control (S), dnPDGFA expressing (T), lfpdGF-A overexpressing (U) and PDGF morpholino injected (V) gastrulae, fixed at stage 14 ($n=15$, three independent experiments for each treatment).

gastrulation, mesoderm translocation was inferred from the expression pattern of the mesoderm markers *Xbra* (Smith et al., 1991), *chordin* (Sasai et al., 1994) and *gooseoid* (*gsc*) (Cho et al., 1991) (Fig. 6). *Xbra* is initially expressed in a subequatorial ring (Fig. 6A), and *chordin*, a marker of head and axial mesoderm, in the dorsal lip (Fig. 6D). Expression of dnPDGFA or overexpression of lfpdGF-A in the BCR does not abolish this pattern (Fig. 6B,C,E,F). This supplements the finding that inhibition of PDGF receptor function does not affect mesoderm regionalization (Ataliotis et al., 1995).

In the mid-gastrula, *Xbra* expression labels the blastopore and the notochord region, which has begun to extend anteriorly (Fig. 6G). In dnPDGFA, lfpdGF-A and PDGF-morpholino-

injected embryos, extension of the notochord region is reduced (Fig. 6H-J). At this stage, *chordin* is normally expressed in a broad anterior area, marking the head mesoderm, and in a narrow strip behind, indicating the extending notochord region (Fig. 6K). In dnPDGFA, lfpdgfa and PDGF morpholino injected embryos, the *chordin* expression domain is compressed along the anteroposterior axis, showing that its anterior end has moved less far anteriorly (Fig. 6L-N). Likewise, compared with controls (Fig. 6O), the *gsc* domain has moved less far from the dorsal lip in injected embryos, and the archenteron is less well developed (Fig. 6P-R). Taken together, this suggests that mesoderm movement is attenuated in PDGF signaling-defective embryos. Explanted dorsal lips from control (Fig. 6S), dnPDGFA injected (Fig. 6T), lfpdgfa-overexpressing (Fig. 6U) or PDGF-morpholino-injected (Fig. 6V) gastrulae all elongate similarly, showing that convergent extension is not affected. This suggests that mesoderm migration is the primary target of PDGF signaling, and that impairment of convergent extension in the intact embryo is indirect.

Deficient mesoderm movement most probably contributes to the aberrant later development of PDGF signaling-defective embryos. As previously described (Ataliotis et al., 1995), expression of inhibitory PDGFR37 results in diminished head structures, a shortened, bent axis and split tails (Fig. 7A). A comparable phenotype was obtained after expression of dnPDGFA or wild-type lfpdgfa in the BCR (Fig. 7C,D). Phenotypes were less pronounced after injection of PDGFA morpholino (Fig. 7B), 5-mismatch control morpholino had no effect (Fig. 7E).

Discussion

PDGFA/PDGFR α signaling and mesoderm guidance in the *Xenopus* gastrula

It had been shown previously that inhibiting PDGFA signaling in the *Xenopus* embryo by expression of PDGFR-37 does not disrupt mesoderm formation and patterning, but interferes with gastrulation movements (Ataliotis et al., 1995). Presently we have demonstrated that blocking PDGFA/PDGFR α function specifically affects a guidance mechanism that orients mesoderm migration through directional cues associated with the extracellular matrix of the BCR.

We have determined that mesodermal expression of the same dominant negative PDGFR-37 used by Ataliotis et al. (Ataliotis et al., 1995) interferes with directional movement on conditioned substratum. This suggests that the previously observed gastrulation defects can at least in part be explained by an impaired guidance mechanism. Further, our results show that for directional translocation, PDGFA/PDGFR α signaling is required during actual migration, and not during mesoderm development: treatment of mesoderm explants with various agents during the 1 hour period of migration on conditioned substratum is sufficient for interference with directionality, and normal mesoderm explants move non-directionally on substratum conditioned with PDGFA-defective BCR.

In all these cases, migration is not inhibited, but randomized with respect to direction, ultimately resembling translocation on an artificial isotropic FN substratum. Coupled with the fact that blocked PDGFA/PDGFR α signaling has the same effect on directionality as a spatially homogeneous, artificial PDGF

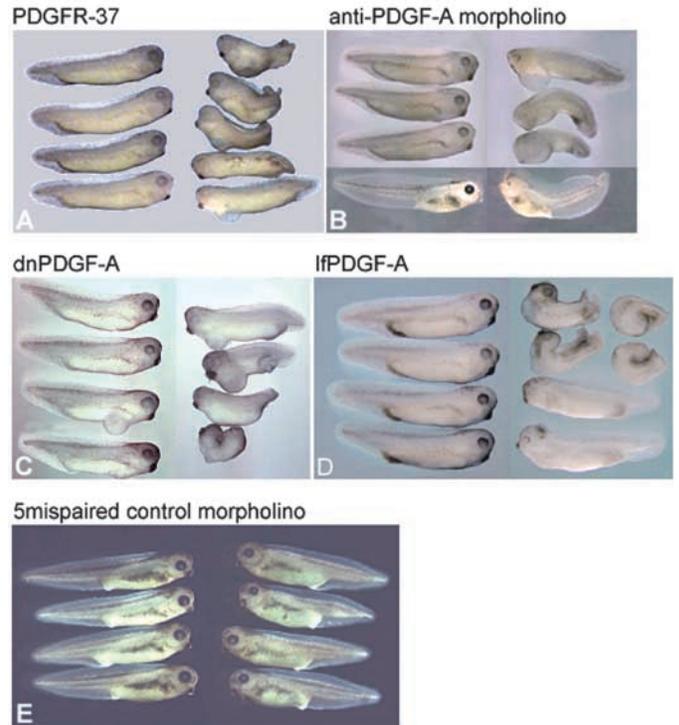


Fig. 7. Larval phenotypes after interference with PDGF signaling. Embryos injected at the four-cell stage with dominant negative PDGFR37 mRNA (A), PDGFA morpholino (B), dnPDGFA mRNA (C), wild-type lfpdgfa mRNA (D) and 5-mismatch control morpholino (E) are shown at the larval stage. Uninjected control larvae are at the left in each panel, injected ones are in the right column.

signal, this argues that PDGFA/PDGFR α activity is instructive, and directly involved in establishing the direction of migration. However, we cannot strictly rule out the possibility that PDGF signaling is permissive, but is required at exactly the normal level of activity. As expected for a matrix-dependent guidance cue, only the lfpdgfa that contains the matrix-binding motif is able to disrupt directional migration when overexpressed in the BCR.

Several possibilities exist to explain how substratum-dependent cues may guide translocation. One set of mechanisms involves the trapping of randomly migrating cells at a target site. For example, haptotaxis leads to the accumulation of cells in regions of increased substratum adhesiveness (Carter, 1965). However, BCR-conditioned substratum exhibits no spatial differences in adhesiveness (Winklbauer and Nagel, 1991). Other trapping mechanisms can be envisioned, for example, mechanisms based on the differential stimulation of motility by the substratum. However, these options are all restrained by the fact that in *Xenopus*, the mesoderm migrates as a coherent mass, where cells have limited freedom for independent random movement. On conditioned substratum, whole explants move persistently towards the animal pole, without random excursions to explore the substratum. By contrast, single mesoderm cells migrate randomly, but they are unable to accumulate in the animal pole area on conditioned substratum (Winklbauer et al., 1992).

Together, these observations argue against a trapping mechanism of mesoderm guidance.

A different type of guidance mechanism relies on orienting each migrating cell towards its target, as in eukaryote chemotaxis. The hallmark of such a mechanism, the presence of polarized cells all pointing with their locomotory processes towards the target of migration, is actually observed in the *Xenopus* gastrula, in the shingle arrangement of anterior dorsal mesoderm cells (Winklbauer and Nagel, 1991). This arrangement also forms when explants migrate directionally on conditioned substratum, but not when they move randomly on FN (Winklbauer et al., 1992). In the embryo, the shingle arrangement disappears when PDGFA function is compromised by inhibition or overexpression. In both cases, protrusions point with equal frequency towards and away from the animal pole. Apparently, a precisely controlled mode of PDGFA/PDGFR α signaling is essential for correct mesoderm cell orientation, such as when a gradient of matrix-bound PDGFA serves as a chemoattractant for the migratory cells. In situ hybridization data showing that PDGFA expression in the BCR is strongest at the animal pole, and decreases towards the marginal zone (Ataliotis et al., 1995), are consistent with this hypothesis.

Chemotaxis through PDGFR α signaling is well documented (e.g. Westermarck et al., 1990; Kundra et al., 1994; Hansen et al., 1996; Ronnstrand et al., 1999), and PDGFR α promotes a chemotactic response at least in some cells (e.g. Hosang et al., 1989; Ferns et al., 1990; Rosenkranz et al., 1999; Yu et al., 2001), although probably through a different mechanism (for reviews, see Hayashi et al., 1995; Ronnstrand and Heldin, 2001). A great deal of work on PDGF-stimulated chemotaxis relates to cultured cells in vitro. Recently, an in vivo role for the *Drosophila* PDGF/VEGF receptor in guiding border cell migration during oogenesis has been demonstrated (Duchek et al., 2001). By showing that PDGFA/PDGFR α signaling is required for directional migration of mesoderm on its endogenous matrix substratum, as well as for cell orientation in the embryo, we demonstrate such a guidance function for this receptor-ligand pair for the vertebrate embryo.

Translocation of the coherent mesodermal cell mass

Single mesoderm cells translocate vigorously in vitro. Their lamellipodia continuously extend, retract, divide and shift along the cell margin. When lamellipodia of two cells meet, they retract immediately, showing contact inhibition; however, lamellipodia are not prevented from underlapping another cell in a lamellipodia-free region (Winklbauer and Selchow, 1992; Winklbauer et al., 1992). In the embryo, the mesoderm forms a coherent mass, and random protrusive activity should lead to frequent encounters between processes and to their retraction as result of contact inhibition. This could explain the rarity of such protrusions in explants on artificial FN substratum (Winklbauer et al., 1992), but also in disoriented mesoderm on PDGFA-defective BCR. That cells extend processes more frequently in control embryos would then be an indirect consequence of their parallel orientation, which prevents collision of lamellipodia. Consistent with this, protrusion formation of single cells does not depend on PDGF signaling, and extension of cytoplasmic processes is not increased, but decreased on BCR overexpressing IfPDGFA, arguing against a direct role for PDGFA in the stimulation of protrusive activity.

The immediate function of the PDGF-mediated guidance mechanism would then be to orient cell processes into a parallel array, to ensure that protrusions can form on every cell, and that processes do not mutually neutralize their effects by pointing in random directions. In turn, to engage all cells at the interface with the substratum in active migration seems essential if the mesodermal mass is to move as a whole.

Facilitating the shear movement between mesoderm and BCR is crucial, as is evident from the phenotypes of PDGF signaling-compromised embryos. That phenotypes are similar, regardless of whether PDGFA function is diminished or increased, argues that they are due to a common primary defect. Moreover, similar phenotypes are obtained when migration is blocked by FN antibodies (Ramos and DeSimone, 1996), or when the shear between BCR and mesoderm is mechanically alleviated by the fusion of the two layers (Winklbauer et al., 2001). This suggests that the common denominator is the attenuation of mesoderm advance across the BCR, as betrayed by marker gene expression patterns. Because in all cases, convergent extension (Keller, 2002) is not deficient in blastopore lip explants, shortened axes and the lack of blastopore closure must be secondary effects, caused by indirect inhibition of convergent extension in the embryo through arrested anterior mesoderm movement. PDGFA and its receptor are also expressed in a complementary pattern at later stages, e.g. in the cranial neural crest (Ho et al., 1994), and interfering with its late functions may contribute to the larval phenotype observed, for example, to head reduction.

PDGFA/PDGFR α signaling and mesoderm migration in other vertebrates

As in the frog, PDGFA and PDGFR α show complementary expression in the mouse gastrula, with the ligand in the epiblast and visceral endoderm, and the receptor in the mesoderm (Palmieri et al., 1992; Orr-Urtreger and Lonai, 1992). The role of PDGFA and PDGFR α in mouse mesoderm migration is not clear, however. The mutant *patch* includes the deletion of the PDGFR α (Smith et al., 1991; Stephenson et al., 1991). Homozygous *patch* embryos are defective in mesoderm development, but the primary cause for this has not been revealed (Orr-Urtreger et al., 1992). Embryos carrying a targeted null mutation for PDGFR α accumulate various defects during early development, but again, data bearing directly on mesoderm migration are lacking (Soriano, 1997). The same is true for embryos homozygous for targeted null alleles of PDGFA (Bostrom et al., 1996).

In the zebrafish, PDGFA and PDGFR α are co-expressed in all cells of the gastrula (Liu et al., 2002a; Liu et al., 2002b). Despite this striking difference in expression, which suggests autocrine signaling, PDGFA is involved in the control of mesoderm migration, as in *Xenopus*. However, the role of PDGFA differs. In the fish, PDGFA signaling through PI3-kinase and PKB is needed for cell polarization and protrusion formation; inhibition of this pathway decreases protrusive activity and the velocity of migration, but cells are still oriented towards the animal pole (Montero et al., 2003). By contrast, *Xenopus* anterior mesoderm cells are intrinsically polarized (Winklbauer and Selchow, 1992; Wacker et al., 1998), and the frequency of protrusion formation is only indirectly affected in PDGFA misexpressing embryos. In *Xenopus*, PDGF signaling

is required for the orientation of cells towards the animal pole, and for directional migration.

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