PDGFR controls contact inhibition of locomotion by regulating N-cadherin during neural crest migration

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ABSTRACT

A fundamental property of neural crest (NC) migration is contact inhibition of locomotion (CIL), a process by which cells change their direction of migration upon cell contact. CIL has been proven to be essential for NC migration in amphibians and zebrafish by controlling cell polarity in a cell contact-dependent manner. Cell contact during CIL requires the participation of the cell adhesion molecule N-cadherin, which starts to be expressed by NC cells as a consequence of the switch between E- and N-cadherins during epithelial-to-mesenchymal transition (EMT). However, the mechanism that controls the upregulation of N-cadherin remains unknown. Here, we show that platelet-derived growth factor receptor alpha (PDGFRα) and its ligand platelet-derived growth factor A (PDGF-A) are co-expressed in migrating cranial NC. Inhibition of PDGFR-α/PDGFRα blocks NC migration by inhibiting N-cadherin and, consequently, impairing CIL. Moreover, we identify phosphatidylinositol-3-kinase (PI3K)/AKT as a downstream effector of the PDGFRα cellular response during CIL. Our results lead us to propose PDGFR-A/PDGFRα as a switch from E- to N-cadherin during migration of cranial NC cells (Carmona-Fontaine et al., 2008b; Davis et al., 2012; Kay et al., 2012; Stramer and Mayor, 2016). EMT in Xenopus and zebrafish cranial NC is defined by an acquisition of CIL, which has been linked to a switch from E- to N-cadherin (also known as cadherins 1 and 2, respectively) (Scarpa et al., 2015). This N-cadherin upregulation has been shown to be essential for CIL-dependent polarity in NC collective migration (Mayor and Etienne-Manneville, 2016; Theveneau et al., 2010, 2013). However, the mechanism of N-cadherin upregulation during NC migration remains unknown. The platelet-derived growth factor (PDGF) receptor tyrosine kinase pathway has been implicated in EMT during cancer invasion (Eckert et al., 2011; Jechlinger et al., 2006; Thiery and Sleeman, 2006), and it is essential for the correct development of several NC derivatives (Morrison-Graham et al., 1992; Soriano, 1997; Tallquist and Soriano, 2003). Furthermore, evidence suggests that the involvement of the PDGF pathway in the formation of NC derivatives is related to the control of NC cell migration and proliferation (Eberhart et al., 2008; He and Soriano, 2013; Smith and Tallquist, 2010). However, the specific mechanism by which PDGF controls the formation of NC-derived tissues has not been completely elucidated. The PDGF signalling pathway is activated by five soluble, disulphide-linked, homo- or heteromeric ligands (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, PDGF-DD) that bind to three receptor tyrosine kinases (PDGFRα/β, PDGFRβ/Rβ, PDGFRα/Rβ), leading to the subsequent activation of downstream signalling cascades (Hoch and Soriano, 2001). These can affect a wide range of cellular events, such as proliferation, migration, survival and EMT. Functional in vivo interaction studies in mice demonstrated that platelet-derived growth factor A (PDGF-A) and PDGF-C activate platelet-derived growth factor receptor alpha (PDGFRα) signalling (Boström et al., 1996; Ding et al., 2004; Soriano, 1997). PDGFRα is expressed in cranial NC cells in Xenopus, zebrafish and mouse embryos (Ho et al., 1994; Liu et al., 2002b; Orr-Urtreger et al., 1992; Takakura et al., 1997; Fantuzzoz and Soriano, 2016). PDGFRα signalling, together with its ligand PDGF-A, has been suggested to work as a chemoattractive cue for NC cells (Eberhart et al., 2008; He and Soriano, 2013; Kawakami et al., 2011). Perturbations of PDGFRα signalling in mouse and zebrafish lead to severe defects.
in cranial NC cell-derived tissues, suggesting a role for PDGFRα signalling in the development of the NC towards its craniofacial targets (Eberhart et al., 2008; He and Soriano, 2013; Morrison-Graham et al., 1992; Soriano, 1997; Tallquist and Soriano, 2003). By contrast, PDGFRβ signalling does not seem to be required for NC cell development (Levéen et al., 1994; McCarthy et al., 2016; Tallquist et al., 2000). However, a recent publication showed that PDGFRα and PDGFRβ can form a functional heterodimer, and that double knockdown mutants exhibit a more severe craniofacial phenotype than those with either mutation alone (Fantauzzo and Soriano, 2016). Analysis of the downstream signalling binding sites of PDGFRα during mouse craniofacial development revealed the phosphatidylinositol-3-kinase (PI3K)/AKT signalling pathway as the primary signalling effector (Klinghoffer et al., 2001; McCarthy et al., 2013; Vasudevan et al., 2015). However, very little is known about early roles of PDGFRα signalling in cranial NC migration.

Here, we use *Xenopus* cranial NC cells to investigate the role of PDGF signalling in NC migration. We show that PDGF-A and its receptor PDGFRα are specifically co-expressed in pre-migratory and migratory NC cells. We find that PDGF-A works as a chemotactic signal for migratory, but not pre-migratory, NC cells. Analysis of this pre-migratory phenotype shows that inhibition of PDGFR-A/PDGFRα blocks cell dispersion by downregulation of N-cadherin, which is required for CIL acquisition during EMT. Furthermore, we find that this novel role of PDGF signalling in the NC requires downstream activity of the PI3K/AKT signalling pathway.

**RESULTS**

**PDGF-A and PDGFRα are co-expressed in the NC and are required for NC migration**

We first analysed the expression of PDGFRα and PDGF-A by *in situ* hybridization and RT-PCR. We found that PDGFRα is expressed in pre-migratory (stage 18) and migrating (stage 24) cranial NC cells, as shown by comparison with the specific NC markers *slug* and *twist* (Fig. 1A-F).

Expression of *pdgfa* was found in pre-migratory NC (Fig. 1G) and also in tissues surrounding the migrating NC (Fig. 1H-I), as previously described (Ho et al., 1994). To confirm this finding, we performed RT-PCR in NC dissected from stage 18 embryos (pre-migratory), and observed strong expression of *pdgfa* in the dissected tissue (Fig. 1J). To test for non-NC tissue contamination, we also performed RT-PCR for a neural plate marker (*Sox2*), an ectoderm marker (*e-Keratin*) and a mesoderm marker (*brachyury*), and for an NC marker (*Sox9*) as a positive control. We did not detect any of the non-NC tissue markers in our NC samples, which were positive for the NC marker (Fig. 1J).

The expression of PDGFRα in the NC was further confirmed by immunostaining (Fig. 1K) and western blotting (Fig. 1L-M). These data strongly support the notion that PDGF-A and PDGFRα are co-expressed in the migrating NC.

In order to analyse the role of PDGF-A/PDGFRα in NC migration, we developed an anti-sense morpholino (PDGFRα MO), which reduced PDGFRα protein levels with high efficiency (Fig. 1L,M). In addition, we used previously published tools, such as a morpholino against the receptor ligand (PDG-F-A MO) (Nagel et al., 2004) and a dominant-negative form of PDGFRα (PDGFRα37 mRNA) (Ataliotis et al., 1995). Depletion of PDGFRα, or its ligand PDGF-A, led to the significant inhibition of NC cell migration *in vivo* (Fig. 2A,B), without affecting NC specification (Fig. 2C,D), suggesting that it affected a specific mechanism during migration without any effect on NC cell induction. To verify the specificities of the receptor and ligand morpholinos, we co-injected them with mouse mRNA, which does not hybridize with the *Xenopus laevis* target sequence in the morpholinos (see Materials and Methods), and analysed the effect on NC migration. For both morpholinos (PDGF-A MO and PDGFRα MO), co-injection with their respective mRNAs rescued NC migration back to wild-type levels (Fig. 2E-H).
To investigate possible changes in NC cell motility resulting from PDGF signalling depletion, NC cells were dissociated and single-cell migration was monitored using time-lapse microscopy. Analysis of cell motility did not reveal any difference in cell velocity between PDGFαRα MO-injected and control cells (Fig. 2I,J), suggesting that inhibition of PDGFαRα does not affect the motility of single cells. Taken together, these data indicate that inhibition of PDGF-A/PDGFRα signalling impairs NC migration in vivo and that this phenotype is not caused by an effect on cell motility per se.

**Migratory but not pre-migratory NC cells chemotax towards PDGF-A**

Inhibition of migration by depletion of PDGF signalling could be due to decreased chemotaxis, as PDGF-A has been suggested to work as a chemoattractant for NC cells in zebrafish and mouse (Eberhart et al., 2008; Kawakami et al., 2011). To test whether impaired NC migration after PDGFαRα inhibition is caused by inhibition of chemotaxis towards PDGF-A, we used a previously published in vitro chemotaxis bead assay (Théveneau and Mayor, 2011; Théveneau et al., 2010). Control and PDGFαRα MO-injected explants were plated in close proximity to PDGF-A protein-coated beads, and their migratory behaviour was analysed by time-lapse microscopy. Indeed, control explants showed strong chemotaxis towards the PDGF-A source (Fig. 3A,B; Movie 1). Furthermore, depletion of PDGFαRα inhibited chemotaxis towards the PDGF-A beads (Fig. 3A,B; Movie 1), indicating that PDGF-A might work as a chemoattractant. To test whether the inhibition of chemotaxis in PDGFαRα MO-injected NC is caused by loss of transmission of the chemotaxis signal and not by loss of migratory behaviour, we investigated whether PDGFαRα-depleted explants are still able to migrate towards the known NC chemoattractant stromal cell-derived factor 1 (SDF-1) (Belmadani, 2005; Olesnicky Killian et al., 2009; Théveneau et al., 2010). We observed no difference in migration towards the SDF-1 source between the PDGFαRα-depleted and control NC explants (Fig. 3A,B; Movie 1), indicating that the effect of PDGF-A/PDGFRα chemotaxis is independent of the role of SDF-1 in NC cell migration. Taken together, these results suggest that PDGF-A might work as a chemoattractant in Xenopus NC cells.

As PDGFαRα is expressed at pre-migratory and migratory stages, we performed a temporal analysis of the chemotaxis response. We found that although migratory NC cells (stage 22) exhibited a strong chemotaxis response (Fig. 3C, mig.; Movie 2), pre-migratory NC cells (stage 18) did not migrate towards PDGF-A (Fig. 3C, premig.; Movie 2). To verify that this lack of chemotaxis in pre-migratory NC cells is...
not due to a general inability of pre-migratory NC cells to react to a chemotactic cue, we performed a pre-migratory chemotaxis assay with SDF-1. No change in chemotaxis behaviour towards the SDF-1 protein source in either pre-migratory or migratory NC explants was observed (Fig. 3C,D; Movie 2). Taken together, these data indicate that chemotaxis of NC towards PDGF-A is present only in migrating NC cells, and its absence in pre-migratory cells suggests a role of PDGF-A/PDGFRα at these early stages that does not involve chemotaxis. As a role of PDGF on NC chemotaxis has already been described (Eberhart et al., 2008; He and Soriano, 2013; Kawakami et al., 2011), we decided to focus our investigation on the early nonchemotactic role of PDGF.

**PDGF-A/PDGFRα controls dispersion via N-cadherin regulation**

Initial migration of NC requires EMT; therefore, we tested the possibility that impaired NC migration could be caused by defects in EMT. To assess the potential influence of PDGF signalling on EMT, NC cell dispersion was analysed in the pre-migratory NC (stage 18). Nuclear fluorescence-labelled NC cell clusters were monitored by time-lapse microscopy, and cell dispersion was quantified by measuring the distance between the nucleus of an NC cell and that of its nearest neighbour using Delaunay triangulation (Carmona-Fontaine et al., 2008b). Inhibition of PDGF-A (Fig. 4E,G) drastically reduced NC cell dispersion with SDF-1 protein source in either pre-migratory or migratory NC explants (Fig. 4A,G; Movie 3). Additionally, we observed a similar decrease in N-cadherin staining in PDGF-A-depleted cells (Fig. 4H,I; Movie S1C,D). These data suggest that PDGF-A/PDGFRα signalling controls NC EMT and cell dispersion at pre-migratory stages by regulating N-cadherin levels.

**N-cadherin-dependent CIL is regulated by PDGF-A/PDGFRα signalling**

Our results show that inhibition of PDGF-A/PDGFRα reduces the levels of N-cadherin protein at cell-cell contacts and, at the same time, reduces cell dispersion. How can this decrease in N-cadherin be related to a reduction in cell dispersion? It is known that blocking N-cadherin leads to a loss of CIL behaviour in NC (Carmona-Fontaine et al., 2008b; Moore et al., 2013; Scarpa et al., 2015). Therefore, we investigated the impact of PDGF-A/PDGFRα signalling on N-cadherin levels by western blot analysis of pre-migratory NC extracts. We observed a reduction of N-cadherin protein levels in PDGF-A MO-injected NC cells (Fig. 4J,K), but no change in E-cadherin (Fig. S1A,B). The specific decrease in N-cadherin, but not E-cadherin, was further confirmed by immunofluorescence in PDGF-A-depleted NC cells (Fig. 4H,I; Movie S1C,D). Additionally, we showed that increased cell dispersion of wild-type NC cells (Fig. 4B,G; Movie 3) or cells depleted of PDGFα (Fig. 4D), but it was unable to promote dispersion in cells lacking PDGFα (Fig. 4F,G; Movie 3), showing again the specificity of the inhibition by PDGF-A MO and PDGFα MO. This suggests that PDGF-A/PDGFRα signalling regulates NC dispersion at early pre-migratory stages.

One of the outcomes of EMT is that it promotes cell dispersion by reducing cell-cell adhesion or increasing cell motility. As showed that depletion of PDGF-A/PDGFRα inhibits cell dispersion without affecting cell motility, we decided to analyse cell-cell adhesion. Members of the cadherin protein family are key cell-cell adhesion molecules, and a switch from E- to N-cadherin is essential for NC migration (Scarpa et al., 2015; Rogers et al., 2013). Therefore, we investigated the impact of PDGF-A/PDGFRα signalling on N- and E-cadherin levels by western blot analysis of pre-migratory NC. Our results show that inhibition of PDGF-A/PDGFRα signalling controls NC EMT and cell dispersion at pre-migratory stages by regulating N-cadherin levels.

**CIL**

We have previously observed that both PDGFα and PDGF-A are co-expressed by NC cells and are functionally active in these cells (Movie 3). Moreover, addition of the ligand, PDGF-A, further increased cell dispersion of wild-type NC cells (Fig. 4B,G; Movie 3) or cells depleted of PDGFα (Fig. 4D), but it was unable to promote dispersion in cells lacking PDGFα (Fig. 4F,G; Movie 3), showing again the specificity of the inhibition by PDGF-A MO and PDGFα MO. This suggests that PDGF-A/PDGFRα signalling regulates NC dispersion at early pre-migratory stages.

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**CIL**

Inhibition of PDGF-A (Fig. 4E,G) and PDGFα (Fig. 4E,G) drastically reduced NC cell dispersion compared with control explants (Fig. 4A,G), confirming our previous observation that both PDGFα and PDGF-A are co-expressed by NC cells and are functionally active in these cells (Movie 3). Moreover, addition of the ligand, PDGF-A, further increased cell dispersion of wild-type NC cells (Fig. 4B,G; Movie 3) or cells depleted of PDGFα (Fig. 4D), but it was unable to promote dispersion in cells lacking PDGFα (Fig. 4F,G; Movie 3), showing again the specificity of the inhibition by PDGF-A MO and PDGFα MO.

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performed using pre-migratory NC cells (stage 18). First, when two cells undergoing CIL collide they remain briefly in contact and then they move away from each other (Fig. 5A); however, if CIL is impaired the two colliding cells remain in contact for longer (Stramer and Mayor, 2016). We measured the time that pairs of colliding cells remain together as an outcome of CIL. Our results show that cells injected with PDGFRα MO remain in contact significantly longer compared with control cells (Fig. 5B), with some PDGFRα MO-injected cells never separating even after 10 h of culture, indicating an impairment in the CIL response. Second, when two cell explants that exhibit CIL are confronted they do not overlap; therefore, an overlap between adjacent explants is an indication of CIL impairment (Stramer and Mayor, 2016). Two NC explants fluorescently labelled with distinct colours, Fluorescein-Dextran and Rhodamine-Dextran, were cultured at a short distance and the overlapping area between them was analysed. Although control explants did not overlap, a clear overlap was observed in explants in which the PDGFRα was inhibited, indicating a clear reduction in CIL response (Fig. 5C,D). Third, a direct consequence of CIL is the acquisition of cell polarity, where cells extend a larger protrusion away from the contact and become elongated, which has been linked to N-cadherin-dependent cell adhesion (Scarpa et al., 2015; Theveneau et al., 2010). To assess whether inhibition of PDGFRα changes polarity, we measured the protrusion area away from the cell contact and cell circularity. We found a significantly lower protrusion area and higher circularity in PDGFRα MO-injected cells compared with control cells (Fig. 5E-H), suggesting a change in polarity. Overall, these data support the idea that PDGFRα is controlling CIL via N-cadherin regulation.

PDGF-A/PDGFRα controls NC migration via the PI3K/AKT signalling pathway

It is known that PDGFR can activate several signalling pathways, such as the PI3K, MAPK, PKC, JAK-STAT and Src pathways (Demoulin and Essaghir, 2014). Therefore, we were interested in identifying the pathways involved in controlling CIL/N-cadherin in pre-migratory NC cells. Studies in mouse and zebrafish during...
Fig. 5. PDGF signalling controls CIL. (A,B) Single cell collision assay of the time between first contact and separation. (A) Representative diagram of CIL. (B) PDGFRα MO explants have impaired CIL, as indicated by the duration of control-control (n=58) and PDGFRα MO-PDGFRα MO CIL events (n=63); data are mean±s.d. from three independent experiments. (C) CIL assay, in which the overlap between two differentially labeled explants is analyzed. Thresholded images of explant invasion assay. Scale bar: 100 µm. (D) Overlap percentage between two NC explants of control (n=52) and PDGFRα MO (n=59) explants from three independent experiments. Scatter plots show median and interquartile range. (E) Protrusion formed away from the cell contact (labelled in white). Scale bar: 30 µm. (F) Protrusion area (µm²) of control (n=51) and PDGFRα MO (n=41) cells. Scatter plots show median and interquartile range. (G) Circularity index; representative examples of circularity (indicated by yellow dashed line) of control and PDGFRα MO-injected NC cells. (H) Circularity of control (n=79) and PDGFRα MO (n=73)-injected NC cells. ns, not significant; *P<0.05; ***P<0.001.

Craniofacial NC migration suggested P13K signalling as the main downstream effector of PDGF signalling (He and Soriano, 2013; Klinghoffer et al., 2001; Vasudevan et al., 2015). Therefore, we asked whether the same pathways were activated in pre-migratory NC cells during CIL.

To investigate the role of P13K as a downstream component of PDGFRα signalling, we expressed a biosensor (ph-AKT-GFP) of PI3K activity, consisting of AKT pleckstrin homology (ph) domain fused to GFP (Montero et al., 2003), in NC cells and analysed PI3K/AKT activity by high time resolution microscopy. Activation of PI3K results in the addition of a phosphate molecule to phosphoinositides, generating phosphatidylinositol 3,4,5-trisphosphate (PIP3), ph-AKT-GFP has a high and specific affinity for PIP3 and therefore translocates to the plasma membrane upon binding PIP3 (Hoch and Soriano, 2003). Thus, a change in GFP intensity from the cytosolic to the membrane-bound form can be used as a read-out of PI3K pathway activation. The treatment of ph-AKT-GFP-expressing cells with PDGFA protein induced a clear increase in membrane GFP intensity compared with control cells not treated with PDGFA protein (Fig. 6A,B,D-F; Movie 4). As a positive control, we co-injected the cells with P13K-CAAX mRNA, a dominant active form of P13K, which resulted in a strong membrane GFP response without the addition of PDGFA protein (Fig. 6C,D).

To further examine the role of P13K/AKT signalling, we tested for endogenous pathway activation by western blot analysis of phosphorylated AKT (pAKT). Treatment with PDGFA protein led to an increase in AKT phosphorylation in NC cells (Fig. 6G,H), suggesting an involvement of P13K/AKT signalling downstream of PDGFA/PDGFRα. We observed no decrease in AKT phosphorylation in PDGFRα MO-injected cells, probably because the basal levels of AKT are already too low in control cells.

To investigate the role and specificity of P13K/AKT signalling in NC migration, we used pharmacological inhibitors (Fig. 7A) of PDGF (AG1296), P13K (LY294002), AKT (MK2206) and MEK (UO126). In vivo treatment with PDGFR, P13K and AKT inhibitors at the pre-migratory stage led to inhibition of NC migration (Fig. 7B-G), supporting our earlier findings. No significant effect of MEK inhibition on NC migration was detected (Fig. 7F,G). Next, we investigated the effect of P13K/AKT on pre-migratory NC cell dispersion in vitro, which is considered as a CIL assay (Stramer and Mayor, 2016). Treatment with P13K (Fig. 7I,P, black bars) and AKT (Fig. 7J,P, black bars) inhibitors led to inhibition of NC cell dispersion compared with control NC cells (Fig. 7H,P, black bars). Remarkably, treatment with the MEK inhibitor also resulted in a reduction in NC cell dispersion (Fig. 7K,P, black bars), but had no effect on NC cell migration in vivo (Fig. 7F,G). As some of these inhibitors could also have nonspecific or off-target effects on other pathways present in the NC, different to the one activated by PDGFA, we proceeded to use the inhibitors after treating the cell clusters with PDGFA protein. As previously shown, we observed that treatment of NC cells with PDGFA led to cell dispersion (Fig. 7L,P, grey bars). However, this dispersion was dramatically impaired when PDGFA-treated cells were co-incubated with inhibitors of P13K (Fig. 7M,P, grey bars) and AKT (Fig. 7N,P, grey bars), but not with the MEK inhibitor (Fig. 7O,P, grey bars). These results show that PDGFA promotes cell dispersion in a P13K/AKT-dependent manner, but independently of MEK, which is consistent with the effect of the inhibitors of these pathways on NC migration in vivo. The results using UO126, a MEK inhibitor, suggest some off-target effects in vitro, but not in vivo, possibly because higher levels of the inhibitor are reached in cells directly exposed to the culture medium in vitro but not in vivo. Immunoblotting for pAKT confirmed a decrease after PDGF inhibitor treatment (Fig. 7Q,T), similar to treatment with the inhibitors of P13K (Fig. 7R,U) and AKT (Fig. 7S,V). Taken together, these data show that PDGFA/PDGFRα controls NC migration and dispersion via the P13K/AKT signalling pathway.

N-cadherin-dependent CIL is regulated via P13K/AKT signalling downstream of PDGFA/PDGFRα signalling

It is well-established that a cell cluster undergoing CIL will disperse (Davis et al., 2015; Scarpa et al., 2015; Stramer and Mayor, 2016). Treatment with PI3K (Fig. 7I,P, black bars) and AKT (Fig. 7J,P, black bars) inhibitors led to inhibition of NC cell dispersion compared with control NC cells (Fig. 7H,P, black bars). Remarkably, treatment with the MEK inhibitor also resulted in a reduction in NC cell dispersion (Fig. 7K,P, black bars), but had no effect on NC cell migration in vivo (Fig. 7F,G). As some of these inhibitors could also have nonspecific or off-target effects on other pathways present in the NC, different to the one activated by PDGFA, we proceeded to use the inhibitors after treating the cell clusters with PDGFA protein. As previously shown, we observed that treatment of NC cells with PDGFA led to cell dispersion (Fig. 7L,P, grey bars). However, this dispersion was dramatically impaired when PDGFA-treated cells were co-incubated with inhibitors of P13K (Fig. 7M,P, grey bars) and AKT (Fig. 7N,P, grey bars), but not with the MEK inhibitor (Fig. 7O,P, grey bars). These results show that PDGFA promotes cell dispersion in a P13K/AKT-dependent manner, but independently of MEK, which is consistent with the effect of the inhibitors of these pathways on NC migration in vivo. The results using UO126, a MEK inhibitor, suggest some off-target effects in vitro, but not in vivo, possibly because higher levels of the inhibitor are reached in cells directly exposed to the culture medium in vitro but not in vivo. Immunoblotting for pAKT confirmed a decrease after PDGF inhibitor treatment (Fig. 7Q,T), similar to treatment with the inhibitors of P13K (Fig. 7R,U) and AKT (Fig. 7S,V). Taken together, these data show that PDGFA/PDGFRα controls NC migration and dispersion via the P13K/AKT signalling pathway.
Migrating (Fig. 8K), the NC senses PDGF-A from the surrounding tissues, which induces chemotaxis and promotes directional NC migration as described previously in other animal models (Eberhart et al., 2008; He and Soriano, 2013; Kawakami et al., 2011).

Consistent with our data, expression of PDGFRα has been reported to be NC cell specific in mouse, zebrafish and Xenopus (Ho et al., 1994; Liu et al., 2002b; Orr-Urtreger and Lonai, 1992). However, in contrast to our finding that PDGF-A is expressed in Xenopus cranial NC, the expression of PDGF-A in other animal models has so far been attributed only to the NC cells surrounding tissues (Ho et al., 1994; Liu et al., 2002a; Orr-Urtreger and Lonai, 1992). In support of PDGF-A being produced by the NC, we show that depletion of PDGF-A in the NC by a morpholino inhibited NC dispersion in vitro, in a condition in which the only possible source of PDGF is the NC cells. So far, analysis of PDGF-A expression in Xenopus migratory NC has only been performed using radioactive in situ hybridization, in which expression can be confused with the background signal (Ho et al., 1994). We confirmed our results on PDGF-A expression in NC by using RT-PCR, which is a much more sensitive technique than in situ hybridization. Our data suggest that revisiting studies on PDGF-A expression during early cephalic NC migration in other model organisms with more recent and sensitive approaches would be worthwhile.

Various interactions between different ligands (PDGF-A, PDGF-BB, PDGF-CC, PDGF-DD) and receptors (PDGFRα/Rα, PDGFRβ/Rβ) have been described in vitro, but only the depletion of PDGF-A and PDGF-C, upstream of PDGFRα signalling, have been shown to be functionally important during mouse embryonic development (Boström et al., 1996; Ding et al., 2004; Soriano, 1997). We cannot rule out a potential role of PDGF-C in Xenopus NC migration; nonetheless, a PDGF-C ligand has so
Fig. 7. Small molecule inhibition of PI3K/AKT signalling. (A) Schematic of the PDGF-PI3K-AKT signalling axis. Signalling is activated upon PDGF ligand (PDGF-AA) binding to the PDGF receptor (PDGFRα), inducing receptor dimerization and subsequent autophosphorylation. PI3K is activated, leading to the phosphorylation of phosphoinositol (PI) residues in the plasma membrane, converting PIP2 to PIP3. Downstream effector kinase AKT binds [with its pleckstrin homology (ph) domain] to PIP3 residues and is activated. (B-F) In situ hybridization for twist in stage 24 embryos treated with the indicated inhibitors from stage 14: (B) DMSO control, (C) AG1296 (20 μM), (D) LY294002 (40 μM), (E) MK-2206 (100 μM), (F) UO126 (100 μM). Scale bar: 100 μm. Stars indicate the eye and arrows indicate the neural crest stream. (G) NC migration normalized to the control average of each experiment in DMSO control (n=256), AG1296 (n=101), LY294002 (n=40), MK-2206 (n=106) and UO126 (n=68) embryos. Data are mean±s.e.m. from three independent experiments. Scheme showing how the migration was quantified is shown underneath the graph. (H-O) Dispersion analysis, using Delaunay triangulation, of NC cultured in vitro for 720 min with the indicated treatments. (P) Analysis of NC dispersion, showing average Delaunay triangulation area normalized to the control of each experiment. Control (n=92), LY294002 (5 μM, n=21), MK-2206 (5 μM, n=16), UO126 (25 μM, n=14), control and PDGF-A (n=55), LY294002 (5 μM) and PDGF-A (n=35), UO126 (25 μM) and PDGF-A (n=19) explants; for all PDGF-A conditions, a PDGF-A concentration of 50 ng/ml was used. Data are mean±s.d. (Q-S) Western blots against pAKT using lysates of whole embryos treated with small molecule inhibitors as indicated. tAKT was used as a loading control. (T-V) Quantification of the western blots shown in Q-S, respectively; intensity of pAKT normalized to tAKT control. Data are mean±s.d. of three independent experiments. AU, arbitrary units. ns, not significant; **P<0.01; ***P<0.001.
far not been described for *Xenopus laevis*. Although PDGFRβ expression has yet to be determined during *Xenopus laevis* development, PDGFRα and PDGFRβ can form a heterodimer that has been shown to be functionally active during NC migration (Fantauzzo and Soriano, 2016; Klinghoffer et al., 2002; Richarte et al., 2007), and future investigations should look at the potential role of PDGFRβ during NC migration in the *Xenopus*.

We showed that loss of function of both PDGF-A and PDGFRα inhibits NC cell migration in vivo using morpholinos against PDGF-A and PDGFRα, a dominant-negative form of PDGFRα and pharmacological inhibition of PDGFR phosphorylation with AG1296. The morpholinos and dominant-negative form of PDGFRα did not affect NC specification, excluding that the inhibition of NC migration is caused by a defect in NC specification. In line with our data, mouse and zebrafish PDGFRα knockdown studies have shown defects in cranial and cardiac NC-derived tissues (Eberhart et al., 2008; Soriano, 1997; Tallquist and Soriano, 2003). In zebrafish and mouse cranial NC, PDGF-A has been suggested as a chemokine during migration (Eberhart et al., 2008; Kawakami et al., 2011). Here, we show that PDGF-A can work as a chemoattractant, at least in vitro, for migratory, but not pre-migratory, NC. Why pre-migratory NC cannot undergo chemotaxis towards PDGF remains to be investigated.

**PDGF signalling and CIL**

Analysis of the cellular behaviour controlled by PDGFRα revealed that NC dispersion is inhibited by PDGFRα depletion in pre-migratory stages. Consistent with our observation, conditional PDGFRα knockout mice have shown defects in explant outgrowth (He and Soriano, 2013). NC cells are known to undergo EMT-like dispersion in vitro (Kuriyama et al., 2014) due to a switch in the cell-cell adhesion molecules E-cadherin to N-cadherin (Scarpa et al.,...
In line with these data, we demonstrate that PDGFα signalling controls NC cell-cell adhesion by regulating N-cadherin levels. Importantly, overexpression of N-cadherin mRNA resulted in rescue of inhibition of dispersion, strongly suggesting that PDGFα signalling works upstream of N-cadherin levels.

N-cadherin has been shown to be required for CIL, and a cell-cell adhesion complex formed by N-cadherin, p120, α-catenin and β-catenin is transiently assembled upon cell-cell interactions in cranial NC (Kuriyama et al., 2014; Théveneau et al., 2010). More recently, the acquisition of CIL behaviour has been linked to EMT and a switch to N-cadherin in *Xenopus* cranial NC cell migration (Scarpa et al., 2015). In context with these findings, we demonstrate that PDGFα MO-injected NC cells were not able to undergo efficient CIL (Fig. 5), supporting the hypothesis that PDGFα is regulating CIL. N-cadherin expression promotes polarization of RAC1 activity towards the leading edge during CIL, and N-cadherin-depleted cells display a reduction in protrusion size (Théveneau et al., 2010). As expected, owing to the reduction in N-cadherin, PDGFα MO-injected NC cell explants displayed a decrease in protrusion area, indicating a loss of polarity. This suggests that PDGFα signalling controls EMT in a CIL-dependent manner by regulating N-cadherin levels. NC cell EMT and migration do have many similar characteristics with malignant cancer invasion. In line with this and our data, PDGFα signalling has been implicated in EMT during cancer invasion (Eckert et al., 2011; Jechlinger et al., 2006; Thierry and Sleeman, 2006). Furthermore, mouse studies reported that conditional *pdgfra* knockout in NC, using a Wnt1-Cre1 driver, resulted in placogenosis defects, linked to delayed migration of NC in the frontonasal prominence (He and Soriano, 2013; Tallquist and Soriano, 2003). This delayed migration in mouse could be consistent with the observed phenotype of CIL reduction in PDGFα-depleted NC.

**Signalling downstream of PDGF in NC**

Using a biosensor and pharmacological inhibition, we were able to link PDGFα signalling to the PI3K/AKT downstream pathway. PI3K signalling downstream of PDGFα appears to be a conserved mechanism in development. In mouse, PDGFα depletion of PI3K activation results in abnormalities in craniofacial development (Klinghoffer et al., 2002). Also, an increase in PI3K activity is able to rescue craniofacial development in zebrafish from a PDGFα knockdown background (McCarty et al., 2013). Although these studies focus on the later, frontonasal migration of cranial NC cells, PI3K/AKT cytoplasmic signalling appears to be conserved for NC development.

Spatial PI3K activation in the leading edge of migrating cells has been shown to be a crucial intracellular guidance cue in cell culture assays and *Dictyostelium* (Cain and Ridley, 2009; Merlot and Firtel, 2003; Yamaguchi et al., 2015). Contrary to this, we did not detect high levels of ph-AKT-GFP localization without PDGF-A protein addition at the free edge. This is most likely due to low levels of PDGF-A protein, and a more sensitive sensor might reveal intracellular spatial differences in PI3K localization.

We were able to control the CIL-dependent dispersion process in NC cells by modulating PDGF-A/PDGFRα signalling. Depletion of PDGF-A/PDGFRα signalling inhibited dispersion. Most importantly, the inhibition of dispersion by PDGFα depletion could be rescued by co-injection with N-cadherin, thus proving N-cadherin as the downstream target of the PDGFα cellular response. Further analysis by immunoblotting showed that pharmacological inhibition of the PDGFR/P13K/AKT axis does indeed lead to a downregulation of N-cadherin. This demonstrates N-cadherin as a regulator of CIL controlled by PI3K/AKT signalling. A remaining question for further studies will be the link between AKT and N-cadherin regulation.

The requirement of N-cadherin for proper NC migration has been shown in chick, mouse, *Xenopus* and zebrafish embryos (Nakagawa and Takeichi, 1998; Xu et al., 2001; Luo et al., 2006; Shoval et al., 2007; Théveneau et al., 2010; Rogers et al., 2013; Scarpa et al., 2015; Broders-Bondon et al., 2016). Here, we show that the regulation of N-cadherin at the pre-migratory NC stages is PDGF-A/PDGFRα dependent and that loss of N-cadherin by depletion of PDGFα signalling leads to an inhibition of NC migration. Our data suggest that, at these early stages, inhibition of NC migration by PDGFα depletion is due to N-cadherin-dependent impairment of CIL. Furthermore, they suggest that the PI3K/AKT pathway is the downstream effector of PDGF signalling during NC EMT.

**MATERIALS AND METHODS**

Embryos, microinjections and micromanipulation

Animals were used according to instructions from the Home Office of the United Kingdom, where animal licences are required. *Xenopus laevis* embryos were obtained and staged as described previously (Nieuwkoop and Faber, 1967), and the embryos were injected at the eight- to 16-cell stage as previously described (Carmona-Fontaine et al., 2008b). Explants were dissected at stage 17 for *in vitro* experiments and plated on a fibronectin-coated dish using 10 or 50 μg/ml fibropectin (Sigma-Aldrich) for plastic or glass dishes, respectively, in DFM medium as described previously (Théveneau et al., 2010). For *in vivo* experiments, Fluorescein-Dextran (3 μg, D1821, Invitrogen) or Rhodamine-Dextran (3 μg, D1824, Invitrogen) were used as tracers, and embryos were fixed at stage 24 to perform *twist in situ* hybridization. Embryos were treated from stage 14/15 to stage 24 with small molecule inhibitors, and equal amounts of DMSO were used as controls.

**Single-cell migration, chemotaxis, cell dispersion and CIL**

Cell dissociation was performed by incubating in Ca2+/Mg2+-free DFM for 3-5 min before transferring to normal DFM medium. Cells were tracked using the ImageJ Manual Tracking plugin (http://rsb.info.nih.gov/ij). Track speed and persistence were determined using the ImageJ Chemotaxis Tool plugin. Chemotaxis assay was performed as described previously (Théveneau et al., 2010). Heparin-acrylic beads (H5263, Sigma-Aldrich) were incubated overnight at 4°C in a 1 mg/ml SDF1 or 1 mg/ml PDGF-AA (AF-100-13A, PeproTech) solution in PBS. To measure dispersion, NC cells from embryos injected with H2B-mCherry (Carmona-Fontaine et al., 2008b) were imaged for 12 h, and nuclei triangulation was analysed using the ImageJ Delaunay Triangulation plugin (Carmona-Fontaine et al., 2011). For small molecule inhibitor treatment, inhibitors were incubated 1 h before addition of PDGF-A protein (50 ng/ml, PeproTech). To study CIL, an explant confrontation assay was performed as described by Carmona-Fontaine et al. (2008a). For the single-cell confrontation assay, single-cell CIL time was measured from the first frame of contact (t=0) until the last frame of contact (t=end). Protrusion area was analysed as previously described (Law et al., 2013).

**RNAs, morpholinos and inhibitors**

PDGF-A MO (8 ng, 5′-AGAATCCAGGCCAGATCTCATGT-3′) (Nagel et al., 2004) and the newly designed PDGFα MO (16 ng, 5′-TGGCTTCTAGGCGGATCATGAC-3′) were obtained from Gene Tools. Mouse mRNA mismatches are underlined. Plasmids were linearized from stage 14/15 to stage 24 with small molecule inhibitors, and equal amounts of DMSO were used as controls.
Vector was linearized with PstI (Promega) and transcribed using T7 polymerase (Promega). Pharmacological inhibitors were all solubilized in DMSO (Sigma-Aldrich) and appropriate DMSO controls were used for all experiments. AG1296 (in vivo 20 μM, 658551, Merck Millipore), LY294002 (in vivo 40 μM, in vitro 5 μM, 9901, Cell Signaling Technology), MK-2206 (in vivo 100 μM, in vitro 5 μM, 1684, Axon Medchem), U0126 (in vivo 100 μM, in vitro 25 μM, 9903, Cell Signaling Technology).

**Semi-quantitative RT-PCR**

RNAs were extracted from NC or ventral non-NC tissue using a RNeasy Mini Kit (Qiagen). cDNA were reverse transcribed using an ImProm-II Reverse Transcription System (Promega). PCR cycles were analysed in pilot experiments. For a primer list and annealing temperatures see Table S1.

**Western blotting, in situ hybridization and immunostaining**

For immunoblotting, NC cells were lysed (25 cells/ lane) in a lysis buffer containing 100 mM Tris-HCl (pH 8.0), 1% Triton X-100, 0.01% SDS, cOmplete Mini Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche). Protein fractions were isolated by centrifugation (19,500 g, 4°C) in two rounds for whole embryo lysates. NC lysates were applied to SDS gels without the purification step. Protein lysates were analysed by SDS-PAGE using 4-12% NuPAGE Bis-Tris gels (Invitrogen), and subsequently transferred onto Invitrogon polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with 5% nonfat dry milk and 0.1% Tween-20 in PBS for 1 h at room temperature, before being probed with the primary antibody by overnight incubation at 4°C, followed by incubation for 1 h at room temperature, with a horseradish peroxidase-linked secondary antibody (sc-2030; 1:3000; Santa Cruz Biotechnology) and detection using an ECL reagent (Luminata Forte Western HRP Substrate, Millipore). Band intensity was measured by scanning films and by densitometry using ImageJ.

**Statistical analysis**

Significant differences between two data sets were determined using contingency tables as previously described (Carmona-Fontaine et al., 2008b) [the null hypothesis was rejected if T=3.841 (α=0.05), T=6.365 (α=0.01) or T>10.83 (α=0.001)]. Data sets (western blot data) were analysed as follows: normality was evaluated by the Kolmogorov-Smirnov test and d’Agostino–Pearson test; data sets were treated as normal distributed if found so by the two tests. Normal distributed data were compared using Student’s t-test (two-tailed, unequal variances) or one-way ANOVA with a Dunnett’s multiple comparisons post-test. Data sets that were found not to follow a normal distribution were compared using Mann–Whitney’s test or a nonparametric ANOVA in Prism 5 (GraphPad). Normalized western blot data were analysed by one-way ANOVA followed by Student Newman–Keuls test for multiple comparison differences. All analyses were performed in Prism 5 (GraphPad).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

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