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

Isabel Bahm¹, Elias H Barriga¹,³, Antonina Frolov², Eric Theveneau¹,⁶, Paul Frankel², Roberto Mayor¹,*

¹Department of Cell and Developmental Biology, University College London, London WC1E 6BT, UK
²Centre for Cardiovascular Biology and Medicine, Division of Medicine, University College London, London WC1E 6JJ, United Kingdom
³London Centre for Nanotechnology, University College London, London WC1H 0AH, United Kingdom.
⁴Present address: Centre de Biologie du Développement–UMR5547, Centre National de la Recherche Scientifique and Université Paul Sabatier, Toulouse 31400, France
*Author for correspondence (r.mayor@ucl.ac.uk)

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Summary statement:

PDGFRα controls neural crest migration by promoting epithelial to mesenchymal transition (EMT) and contact inhibition of locomotion (CIL) via regulation of N-cadherin
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 amphibian 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 PDGFRα and its ligand PDGF-A are co-expressed in migrating cranial NC. Inhibition of PDGF-A/PDGFRα blocks NC migration by inhibiting N-cadherin and, consequently impairing CIL. Moreover, we find PI3K/AKT as a downstream effector of the PDGFRα cellular response during CIL. Our results lead us to propose PDGF-A/PDGFRα signalling as a tissue-autonomous regulator of CIL by controlling N-cadherin upregulation during EMT. Finally, we show that once NC have undergone EMT, the same PDGF-A/PDGFRα works as NC chemoattractant guiding their directional migration.
Introduction

One of the most migratory cell types during early vertebrate development are cells of the neural crest (NC). NC is a transient cell population, induced between the neural tube and surface ectoderm, which eventually gives rise to many different tissues such as craniofacial cartilages and bone, cells of the peripheral nervous system, smooth muscle cells, tendons, and pigment cells. Disruption of NC cell migration during development can lead to pathologies including craniofacial abnormalities, heart malformation and colonic aganglionosis (Hirschprungs disease) generally called neurocristopathies (Feiner et al., 2001; Miyagawa-Tomita et al., 1991; Van de Putte et al., 2007).

A defining characteristic of NC cells is the epithelial-to-mesenchymal transition (EMT) they undergo to segregate from the neural tube and start their migration (Theveneau and Mayor, 2012). EMT is a cellular process that converts non-motile epithelial cells to motile mesenchymal cells, defined by a change in cell-cell adhesion, polarity, and the acquisition of migratory properties (Thiery et al., 2009). One of the migratory properties acquired during NC cell EMT is Contact inhibition of Locomotion (CIL) (Scarpa et al., 2015). This cellular process is characterised by a change of direction of migration upon cell-cell contact and is associated with embryonic processes such as neuronal cell and macrophage dispersion, and collective 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-cadherin to N-cadherin (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; Theveneau et al., 2013). However the mechanism of N-cadherin upregulation during NC migration remains unknown. The receptor tyrosine kinase pathway Platelet-derived growth factor (PDGF) 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 et al., 2003). Furthermore, evidence suggest that the involvement of 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 derivated 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/Rα, PDGFRβ/Rβ, PDGFRα/Rβ), leading to the subsequent
activation of downstream signalling cascades (Hoch and Soriano, 2003). 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 PDGF-A and PDGF-C activate 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; Fantauzzo and Soriano, 2016). PDGFRα signalling, together with its ligand PDGF-A, has been suggested to work as a chemotactic 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 developmental role of PDGFRα signalling in NC development towards its craniofacial targets (Eberhart et al., 2008; He and Soriano, 2013; Morrison-Graham et al., 1992; Soriano, 1997; Tallquist et al., 2003). In contrast, PDGFRβ signalling does not seem to be essentially 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 mutant exhibit a more severe craniofacial phenotype, than either mutation alone (Fantauzzo and Soriano, 2016). Analysis of 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 neural crest 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 the migratory, but not pre-migratory NC cells. Analysis of this pre-migratory phenotype shows that, inhibition of PDGF-a/PDGFRα blocks cell dispersion due to a 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 require downstream activity of the PI3K/AKT signalling pathway.
Results

PDGF-A and PDGFRα are co-expressed in NC and 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 the comparison with the specific NC markers slug and twist (Fig. 1 A-F). While the expression of PDGF-A was found in pre-migratory NC (Fig. 1G) and later on 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 dissecting from stage 18 embryos (pre-migratory), finding a clear expression of PDGF-A in the dissected tissue (Fig 1J). To test for non-NC tissue contamination, we also performed RT-PCR for neural plate marker (Sox2), ectoderm marker (e-Keratin) mesoderm marker (Brachyury) and for a 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.1 J). The expression of PDGFRα in the NC was further confirmed by immunostaining (Fig.1 K) and western blot (Fig.1 L, M; control lane). 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 neural crest migration, we developed an anti-sense Morpholino (PDGFRα MO), which showed high efficiency to reduce the protein levels (Fig. 1 L, M). In addition, we used previously published tools, such as a MO against the ligand (PDGF-A MO; Nagel et al., 2004) and a dominant negative form of the PDGFRα (PDGFRαw37 mRNA; Ataliotis et al., 1995). Depletion of the ligand or the receptor led to a significant inhibition of NC cell migration in vivo (Fig. 2 A, B), without affecting neural crest specification (Fig. 2 C, D), suggesting a specific mechanism during migration without affecting NC cell induction. To verify the specificity of both receptor and ligand Morpholinos, we co-injected mouse mRNA, which does not hybridize with the Xenopus laevis target sequence in the MOs (see methods) and analysed the effect on NC migration. For both Morpholinos (PDGF-A MO and PDGFRα MO) co-injection of their respective mRNAs rescued NC migration back to wild type levels (Fig 2 E-H).

To investigate possible changes in NC cell motility due to 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 PDGFRα MO and control cells (Fig 2 I, J), suggesting that inhibition of PDGFRα does not affect cell 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 chemotaxis 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 the deficiency of NC migration after PDGFRα inhibition is due to inhibition of chemotaxis towards PDGF-A, we used a previously published in vitro chemotaxis bead assay (Theveneau and Mayor, 2011; Theveneau et al., 2010). Control and PDGFRα 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 3 A, B; Movie 1). Furthermore, depletion of PDGFRα inhibited chemotaxis towards the PDGF-A bead (Fig 3 A, B; Movie 1), indicating that PDGF-A might work as a chemoattractant. To test whether the inhibition of chemotaxis in PDGFRα Morpholino injected NC is due to a loss of transmission of the chemotaxing signal and not caused by a loss of migratory behaviour, we investigated whether PDGFRα depleted explants are still able to chemotax towards the known NC chemoattractant SDF-1 (Belmadani, 2005; Olesnicky Killian et al., 2009; Theveneau et al., 2010). No difference in the migration towards the SDF-1 source between the PDGFRα depleted explants and control NC explants was observed (Fig. 3 A, B; Movie 1), indicating that the effect of PDGF-A/PDGFRα chemotaxis is independent of the role of SDF-1 in neural crest migration. Taken together, this suggests that PDGF-A might work as a chemoattractant in Xenopus neural crest cells.

As PDGFRα is expressed at pre-migratory and migratory stages we performed a temporal analysis of the chemotaxis response. We found that while migratory NC (stage 22) exhibited a strong chemotaxis response (Fig. 3C, mig), pre-migratory NC (stage 18) did not chemotax towards PDGF-A (Fig. 3 C, 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. 3 C, D; Movie 2). Taken together these data indicate that chemotaxis of NC towards PDGF-A is present only in migrating NC, and its absence in pre-migratory cells suggest a role of PDGF-A/PDGFRα at these early stages different to 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 non-chemotactic 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 due to defects in EMT. To assess the potential influence of PDGF signalling on EMT, NC cell dispersion was analysed in pre-migratory NC (stage 18). Nuclear fluorescently labelled NC cell clusters were monitored by time-lapse microscopy and cell dispersion was quantified by measuring the distance between the nearest neighbour, using Delaunay triangulation of the NC cell nuclei (Carmona-Fontaine et al., 2011). Inhibition of PDGF-A (Fig. 4 C, G) and PDGFRα (Fig. 3 E, G) drastically reduced NC cell dispersion compared to control explants (Fig. 3 A, G), confirming our previous observation that both PDGFRα 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. 3 B, G; Movie 3) or cells depleted of PDGFA (Fig. 3 D), but it was unable to promote dispersion in cells lacking PDGFRα (Fig. 3 F, G; Movie 3), showing again the specificity of the inhibition by PDGF-A and PDGFRα Morpholinos. This suggest that PDGF-A/PDGFRα signalling regulates NC dispersion at these 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 we showed that depletion of PDGFA/PDGFRα inhibits cell dispersion without affecting cell motility, we decided to analyse cell-cell adhesion. A key cell-cell adhesion molecule is the cadherin protein family and it has been shown that 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 PDGFRα signalling on N-cadherin and E-cadherin levels by western blot at the pre-migratory stages. We observed a reduction of N-cadherin protein levels in PDGFRα MO injected NC cells (Fig. 4 J, K), but no change in E-cadherin levels (Fig. S1 A, B). The specific decrease in N-cadherin but not E-cadherin was further confirmed by immunofluorescence in PDGFRα depleted NC cells (Fig. 4 H, I and Fig. S1 C, D). Additionally, we did observe a similar decrease in N-cadherin staining in PDGF-A depletes cells (Fig. 4 H, I, PDGF-A MO) and an opposite increase after PDGF-A mRNA injection (Fig. 4 H, I, PDGF-A mRNA). These data suggest that PDGF-A/PDGFRα signalling controls NC EMT and cell dispersion at these 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 decreases the levels of N-cadherin protein at the cell-cell contact, but at the same time reduces cell dispersion. How can this decrease in N-
It is known that blocking N-cadherin leads to a loss of CIL behaviour in NC (Theveneau et al., 2010). Hence, we hypothesized that the decreased dispersion in PDGFRα MO explants could be due to a N-cadherin dependent loss of CIL. To address this, we used three different assays to analyse CIL (Carmona-Fontaine et al., 2008b; Moore et al., 2013; Scarpa et al., 2015). All these assays were performed using premigratory 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. We measured the time that pairs of colliding cells remain together as an outcome of CIL (Stramer and Mayor, 2016). Our results show that cells injected with the PDGFRα MO remain in contact significantly longer compared to control cells (Fig. 5B), with some PDGFRα MO cells never separating even after 10h of culture, indicating an impairment in the CIL response. Second, when two cells explants that exhibit CIL are confronted they do not overlap, while an overlapping between adjacent explants is an indication of CIL impairment (Stramer and Mayor, 2016). Two NC explants fluorescently labelled with two distinct colours, Fluorescein-Dextran and Rhodamine-Dextran, were cultured at a short distance and the overlapping area between them was analysed. Our result show that while control explants did not overlap, a clear overlapping was observed in explants in which the PDGFRα was inhibited, indicating a clear reduction in CIL response (Fig. 5 C, D). Third, a direct consequence of CIL is the acquisition of cell polarity, where cells extend larger protrusion away from the contact and they 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 protrusion area away from the cell contact and cell circularity. We found a significant decrease of protrusion area and higher circularity in PDGFRα MO injected cells compared to control cells (Fig. 5 E, F), suggesting a change in polarity. Overall, this data supports the idea that PDGFRα is controlling CIL via N-cadherin regulation.

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

It is known that PDGFR can activate several signalling pathways, such as PI3K, MAPK, PKC, JAK-STAT, and Src (Demoulin and Essaghir, 2014). Therefore, we were interested in identifying the pathways involved in controlling CIL/N-cadherin in the pre-migratory NC cells. Studies in mouse and zebrafish during cranio-facial NC migration, suggested PI3K 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 PI3K as a downstream component of PDGFRα signalling we expressed an 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 the 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). The PH-AKT-GFP has a high and specific affinity for PIP3 and therefore translocates to the plasma membrane upon binding PIP3. Thus, a GFP intensity change from the cytosolic to membrane bound form is used as a read-out of PI3K pathway activation. The treatment of PH-AKT-GFP expressing cells with PDGF-A protein induced a clear increase in the membrane GFP intensity, compared to neural crest not treated with PDGF-A protein (Fig. 6 A, B, D-F; Movie 4). As a positive control we co-injected with PI3K-CAAX mRNA, a dominant active form of PI3K, which resulted in a strong membrane GFP response without PDGF-A protein addition (Fig. 6 C, D).

To further examine the role of PI3K/AKT signalling we tested for endogenous pathway activation by western blot against phosphorylated AKT (pAKT). Treatment with PDGF-A protein led to an increase in AKT phosphorylation in NC cells (Fig. 6 G, I), suggesting an involvement of PI3K/AKT signalling downstream of PDGF-A/PDGFRα. We were unable to observe a decrease on AKT phosphorylation in PDGFRα MO cells, probably because the basal levels of AKT are already too low in control cells.

To investigate the role and specificity of the PI3K/AKT signalling in NC migration we used pharmacological inhibitors (Fig. 7 A), against PDGFR's (AG1296), PI3K (LY294002), AKT (MK2206) and MEK (UO126). In vivo treatment of PDGFR, PI3K and AKT inhibitors from pre-migratory stage led to inhibition of NC migration (Fig. 7 B-F and G) supporting our earlier findings. No significant effect of MAPK inhibition on NC migration was detected (Fig. 7 F, G). Next, we investigated the effect of PI3K/AKT on pre-migratory NC dispersion in vitro that is considered as a CIL assay (Stramer and Mayor, 2016). PI3K (Fig. 7 I, P; black bars) and AKT (Fig. 7 J, P; black bars) inhibitor treatment led to an inhibition of NC dispersion compared with control NC (Fig. 7 H, P; black bars). Remarkably, treatment with the MAPK inhibitor also showed a reduction in NC cell dispersion (Fig. 7 K, P; black bars) despite showing no effect NC cell migration in vivo detected (Fig. 7 F, G). As some of these inhibitors could also have non-specific or off target effects on other pathways present in the NC, different to the one activated by PDGF-A, we proceeded to use the inhibitors after treating the cell clusters with PDGF-A protein. As previously shown, treatment of NC cell with PDGF-A leads to cell dispersion (Fig. 7 L, P; grey bar). However, this dispersion was dramatically impaired when PDGF-A treated cells were co-incubated with PI3K (Fig. 7 M, P; grey bars) and AKT inhibitors (Fig. 7 N, P; grey bars), but not with the MAPK inhibitor (Fig. 7 O, P; grey bars). These results show that PDGF-A promotes cell
dispersion in a PI3K/AKT manner, but independent of MAPK, which is consistent with the effect of the inhibitors of these pathways on NC migration in vivo. The results using the UO126 inhibitor suggest some off-target effects in vitro, but not in vivo, possible 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 PDGFR inhibitor treatment (Fig. 7Q, T), similar to inhibitor treatment of PI3K (Fig. 7 R, U) and AKT (Fig. 7 S, V). Taken together, this data show that PDGF-A/PDGFRα controls NC migration and dispersion via the PI3K/AKT signalling pathway.

**N-cadherin dependent CIL is regulated via PI3K/AKT signalling downstream of PDGF-A/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; Villar-Cerviño et al., 2013), conversely the inhibition of CIL causes an impairment on dispersion, as observed in PDGF-A/ PDGFRα MO explants (Fig. 3 E-K; Movie 3). To assess whether this inhibition of dispersion is due to a lack of N-cadherin, we co-injected N-cadherin mRNA together with the PDGFRα MO. Interestingly, co-injection of PDGFRα MO/N-cadherin mRNA was sufficient to rescue the inhibition of dispersion induced by PDGFRα MO (Fig. 8 A-E; Movie 4). This experiment indicates that PDGF-A/PDGFRα signalling controls N-cadherin dependent CIL in NC cells.

Finally, to investigate whether the N-cadherin regulation that is controlled by PDGF-A/PDGFRα (Fig. 4 H-K) is also PDGFR-PI3K/AKT dependent, we treated embryos with PDGFR or PI3K inhibitor and analysed N-cadherin levels by western blot at pre-migratory stages. Both inhibitor treatments decreased N-cadherin protein levels (Fig. 8 F-I). In conclusion, these results demonstrate that the tissue-intrinsic PDGF-A/PDGFRα-PI3K-AKT signalling controls N-cadherin levels, which in turn are required for CIL during NC EMT.
Discussion

PDGF-A and PDGFRα functionally linked in NC

Our results point to a double role of PDGF-A/PDGFRα in Xenopus NC development. At early migratory stages (Fig. 8H) NC expresses the receptor PDGFRα and the ligand PDGF-A, which in a tissue autonomous manner activates the PI3K/AKT pathway leading to up-regulation of N-cadherin at the cell contact; this increased in N-cadherin is sufficient to promote CIL and cell dispersion during EMT (Theveneau et al., 2010; Scarpa et al., 2015). Once EMT has started and the NC are migrating (Fig. 8J), the NC senses PDGF-A from the surrounding tissues, which induces chemotaxis and promotes directional NC migration as it has been described for NC from 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, different to our finding that PDGF-A is expressed in Xenopus cranial neural crest, the expression PDGF-A in other animal models has so far been attributed only to the NC cell 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 where the only possible source of PDGF are the NC cells. So far, analysis of PDGF-A expression in migratory NC of Xenopus has only been performed using radioactive in situ hybridization that can be easily confused with background signal (Ho et al., 1994). We confirmed our results concerning the co-expression of PDGFRα and PDGF-A by RT-PCR, a much more sensitive technique than in situ hybridization. Our data suggests to revisit the studies of PDGF-A expression during early cephalic NC migration in other model organisms with more recent and sensitive approaches.

Various interactions between different ligands (PDGF-A, PDGF-AB, PDGF-BB, PDGF-CC, PDGF-DD) and receptors (PDGFRα/Rα, PDGFRβ/Rβ, PDGFRα/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 far not been described for Xenopus laevis development. Although PDGFRβ expression has yet to be determined during Xenopus laevis development. PDGFRα and PDGFRβ can from 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 have to look at the potential role of PDGFRβ during NC migration in Xenopus.
We showed that loss-of-function of both PDGF-A and the receptor PDGFRα inhibits NC cell migration in vivo using Morpholinos against PDGF-A and PDGFRα, a dominant negative form of the PDGFRα and pharmacological inhibition of PDGFR phosphorylation, AG1296. NC specification was not affected using the Morpholinos and dominant negative form of PDGFRα, excluding that the inhibition of NC migration is due to a defect in NC specification. In line with our data, mouse and zebrafish PDGFRα knock-down studies have shown defects in cranial and cardiac NC derived tissues (Eberhart et al., 2008; Soriano, 1997; Tallquist et al., 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 NC, 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 PDGFRα conditional knockdown in 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 of cell-cell adhesion molecules, E-cadherin to N-cadherin (Scarpa et al., 2015). In line with this data, we demonstrate that PDGFRα signalling controls NC cell-cell adhesion by regulating N-cadherin levels. Importantly, we were able to rescue the inhibition of dispersion by overexpression of N-cadherin mRNA, strongly suggesting that PDGFRα 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, a-catenin, and b-catenin is transiently assembled upon cell-cell interactions in cranial NC (Kuriyama et al., 2014; Theveneau 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 PDGFRα MO injected NC were not able to undergo efficient CIL (Fig 5A–H), supporting the hypothesis that PDGFRα is regulating CIL. N-cadherin expression promotes polarisation of RAC1 activity towards the leading edge during CIL and N-cadherin depleted cells display a reduction in protrusion size (Theveneau et al., 2010). As expected, due to their reduction in N-cadherin PDGFRα MO injected NC cell explants displayed a decrease in protrusion area, indicating a loss in polarity. Taken together this suggests that PDGFRα signalling controls EMT in a CIL dependent manner by regulation of N-cadherin levels. NC cell EMT and migration does have many similar characteristic with malignant cancer invasion. In line with this and our data, PDGF
signalling has been implicated in EMT during cancer invasion (Eckert et al., 2011; Jechlinger et al., 2006; Thiery and Sleeman, 2006). Furthermore, mouse studies reported that conditional PDGFα KO in NC, using Wnt1-Cre1 driver, showed platogenesis defects, linked to delayed migration of NC in the frontal-nasal prominence (He and Soriano, 2013; Tallquist et al., 2003). This delayed migration in mouse could be consistent with the observed phenotype of CIL reduction in PDGFα depleted NC.

**Downstream signalling of PDGF in NC**

Using a biosensor and pharmacological inhibition, we were able to link the 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 shows abnormalities in craniofacial development (Klinghoffer et al., 2002). Also the increase of PI3K signalling in zebrafish is able to rescue craniofacial development in a PDGFα knock down background (McCarthy et al., 2013). Albeit these studies focus on the later, frontonasal migration of cranial NC cells, the PI3K/AKT cytoplasmic signalling appears to be conserved for NC development.

Spatial PI3K activation in the leading edge of chemotaxing 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 localisation 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 localisation.

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/PI3K/AKT axis indeed leads 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; Theveneau et al. 2010; Rogers et al., 2013; Scarpa el 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 PDGFRα signalling leads to an inhibition of NC migration. Our data suggests that at these early stages, inhibition NC migration by PDGFRα depletion is due to a N-cadherin dependent impairment of CIL. Furthermore our data suggests PI3K/AKT pathway as that the downstream effector of PDGF signalling during NC EMT.

**Materials and Methods**

**Embryos and microinjections and micromanipulation**

Animal were used following the instruction from the Home Office (UK), where animal licences were required. Xenopus laevis embryos were obtained and staged as described previously (Nieuwkoop and Faber, 1967) and embryos were injected at the 8- 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 μg/ml or 50 μg/ml fibronectin (Sigma) for plastic or glass dishes, respectively in DFA medium as described previously (Theveneau et al., 2010). For *in vivo* experiments, Fluorescein-dextran (Invitrogen, D1821; 3 μg) or Rhodamine-dextran (Invitrogen, D1824; 3 μg) were used as tracers, 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 DMSO were used as controls.

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

Cells were dissociation was performed by incubation in Ca²⁺-Mg²⁺-free DFA for 3-5min before transferring them back to normal DFA medium. Cells were tracked using the ImageJ Manual Tracking plugin (US National Institutes of Health; 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 (Theveneau et al., 2010) Heparin-acrylic beads (Sigma H5263) were incubated over night at 4°C in a 1 mg ml⁻¹ Sdf1 or 1 mg ml⁻¹ PDGF-AA (Peprotech AF-100-13A) solution in PBS. To measure dispersion, NC cells from embryos injected with H2B-mCherry were imaged for 12hr, nuclei triangulation was analysed using the ImageJ Delaunay Triangulation plugin (Carmona-Fontaine et al., 2011). For small molecule inhibitor treatment inhibitors were incubated 1h before PDGF-A (50ng/ml, Peprotech) protein addition. To study CIL explant confrontation assay was performed as described by (Carmona-Fontaine et al., 2008a). For single cell confrontation assay single cell CIL time was measured by first frame of contact t=0 until last frame of contact t=end. Protrusion area was analysed as previously described (Law et al., 2013).
RNAs, Morpholinos, Inhibitors

Morpholinos against PDGF-A (PDGF-A MO; 8ng, 5’-AGAATCCAAGCCCAGATCATTG-3’; Nagel et al, 2004) and new designed Morpholino against PDGFRα (16ng, 5’-TGCCCTCATGGCAGGATCATGGAC-3’) were obtained by GeneTools. Mouse mRNA mismatches are underlined. Plasmids were linearized and mRNA transcribed using the mMessenger mMachine Transcription Kits (Thermo Scientific). mRNA constructs injected were: membrane GFP (300pg), nuclear RFP (H2B-RFP, 300pg), PDGFRα-37 (300pg;Ataliotis et al, 1995), ph-AKT-GFP (500pg;Montero et al, 2003) PI3K-p110CAAX (300pg;Montero et al, 2003), mouse PDGF-A (200pg, Fruttiger et al, 1999), mouse PDGFRα (300pg, IMAGE ID 5704645) and N-cadherin (300pg, Scarpa et al, 2015)). For mouse PDGF-A mRNA transcription mouse PDGF-A pGEM-1 was linearized with PvuII and transcribed with SP6 polymerase. For mouse transcription PDGFRα IMAGE consortium Vector was linearized with PacI and transcribed using T7 polymerase. Pharmacological inhibitors were all solubilized in DMSO (Sigma) and appropriate DMSO controls were used for all experiments. AG1296 (Merck Millipore, 658551, in vivo 20μM), LY294002 (Cell Signaling, 9901, in vivo 40μM, in vitro 5μM), MK-2206 (Axon, 1684, in vivo 100μM, in vitro 25μM), UO126 (Cell Signaling, 9903, in vivo 100μM, in vitro 25μM).

Semi quantitative RT-PCR

RNAs were extracted from NC or ventral non-NC tissue using RNeasy Mini kit (Qiagen). cDNA were reverse-transcribed using Promega Reverse transcription (Promega). PCR cycles were analysed in pilot experiments. For a primer list and annealing temperatures see Table 1.

Western blotting, In situ hybridisation and Immunostaining

For immunoblotting, cells were lysed, 25 NC per lane, in a lysis buffer containing 100mM Tris-HCl (pH 8.0), 1% Triton X-100, 0.01%SDS, complete Mini protease inhibitor (Roche) and PhosStop phosphatase inhibitor (Roche). Protein fraction were isolated by centrifugation (13,200rpm, 4°C) in two rounds for whole embryo lysates. NC lysates were applied to SDS-Gels without purification step. Protein lysates were analysed by SDS-PAGE using 4 to 12% Bis-Tris gels (NuPAGE; Invitrogen), and subsequent transfer onto Invitrolon polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked with 5% non-fat dry milk and 0.1% Tween-20 in PBS for 1 hour at room temperature, before being probed with the primary antibody by overnight incubation at 4 °C, followed by incubation for 1 hour at room temperature with a horseradish peroxidase-linked secondary antibody (Santa-Cruz) and detection using ECL.
reagent (Luminata Forte Western HRP Substrate, Milipore). Band intensity was measured by
scanning of films and analysis by densitometry using Image J (NIH). *In situ* hybridisation was
performed as described (Harland, 1991). NC was labelled with digoxigenin-labelled RNA probes
against Slug (Mayor et al., 1995) or Twist (Hopwood et al., 1989) in Xenopus. Immunostaining
was performed according to standard procedure. Following antibodies were used: PDGFRα (Cell
Signaling, 3164, pAKT Ser437 (Cell Signaling, 9271, 1:2000), total (pan) AKT (Cell Signaling,
4691, 1:2000), GAPDH-HRP (Santa Cruz, sc20357, 1:2000). N-cadherin (DHSB, MNCD-2,
western blot 380ng ml\(^{-1}\), Immunostaining 6μg ml\(^{-1}\)), E-cadherin (DHSB, 5D3, western blot 60ng
ml\(^{-1}\), Immunostaining 120μg ml\(^{-1}\)). If required DAPI was applied with the secondary antibody
(Sigma, D9542; 20 μg/ml).

**Statistical analysis**

Significant differences between two data sets, using contingency tables as previously described
(Carmona-Fontaine et al., 2008b), were considered different (null hypothesis rejected if
\(T>3.841\) (\(\alpha=0.05\)), \(T>6.635\) (\(\alpha=0.01\)) or \(T>10.83\) (\(\alpha=0.001\)). Data sets (expect Western blot data)
were analysed as follows: Normality was evaluated by Kolmogorov-Smirnov’s test, d’Agostino
and Pearson’s test using. Data set were treated normal distributed if found so by all two tests.
Normal distributed data was compared using Student’s t-test (two-tailed, unequal variances) or
using one-way ANOVA with a Dunnett’s multiple comparisons post-test. Data sets that were
found not to follow normal distribution were compared using Mann-Whitney’s test or a non-
parametric ANOVA in Prism5 (GraphPad). Normalised western blot data was analysed by one-
way ANOVA followed by Student Newman-Keuls test for multiple comparison differences. All
analysis was performed in Prism5 (GraphPad).

**Abbreviations**

| Abbreviation | Description                   |
|--------------|-------------------------------|
| NC           | Neural Crest                  |
| PI3K         | phosphatidylinositol-3-kinase |
| PDGF-A       | platelet derived growth factor A |
| PDGFRα       | platelet derived growth factor receptor alpha |
| MO           | Morpholino                    |
| EMT          | epithelial to mesenchymal transition |
| CIL          | contact inhibition of promotion |
| tAKT         | total AKT                     |
| pAKT         | phosphorylated AKT            |
Competing interests

The authors declare no competing financial interests.

Author contributions

I. Bahm and R. Mayor performed and designed most of the experiments. E. H. B helped with some chemotaxis experiments. A.F. helped with some of the western blot experiments. P.F. helped with western blot experiment design and statistical analysis; E.T. performed preliminary experiments. The manuscript was written by I. Bahm and R. Mayor. All authors commented on the manuscript.

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Figure 1. NC cells express both PDGF-A and PDGFRα. (A-I) Whole mount in situ hybridisation of Xenopus embryos. (A, D, G) Lateral view of stage 18 embryos showing expression of slug (A), pdgfα (D) and pdgf-a (G). Scale bar 500μm. (B, E, H) lateral view of stage 24 embryos, showing migrating neural crest expressing twist (B) pdgfrα (E) and pdgf-a (H). Scale bar 1mm. Grey asterisk marks the eye. (C, F, I) Sections of embryos shown in B, E and H, respectively. Line in (B) indicates level of section. Yellow lines outline cephalic NC streams. (J) RT-PCR analysis of PDGF-A and PDGFRα expression in NC dissected from stage 18 embryo, Sox9 (NC marker), Sox2 (neural plate marker), e-keratin (epidermis marker), Brachyury (mesoderm marker), ODC (control). (K) Immunostaining on NC explants against PDGFRα (green), Phalloidin (red), DAPI (blue). Scale bars 20μm. (L) Western blot analysis of PDGFRα using NC dissected from control embryos or embryos treated with PDGF-AA or injected with PDGFRα MO. (M) Band intensity normalized to loading control, bar graph shows mean and s.d. of three independent experiments, AU=arbitrary units, nsp>0.5, ***p<0.001.
Figure 2. PDGF-A/PDGFRα are required for NC migration in vivo. (A-D) PDGF signalling depletion affects NC migration, but not specification. (A) In situ hybridisation of twist in stage 24 embryos after different treatments as indicated, scale bar 1mm. (B) Diagram of quantification of NC migration, distance of 2nd (hyoid) NC stream on injected side normalized to un.injected control side; graph of NC migration for control n=30, dominant negative mRNA (PDGFRαw37) n=51, PDGF-A MO n=47, PDGFRα MO n=28 embryos, from three independent experiments. (C) In situ hybridisation of Slug in stage 18 embryos after different treatments as indicated. Dorsal view, yellow arrow site of injection, scale bar 500μm. (D) Analysis of NC induction. (E) In situ hybridisation of twist in stage 24 embryos after different treatments as indicated, scale bar 1mm. (F) Analysis of NC migration for control n=20, PDGFRα MO n=36, mouse PDGFRα mRNA n=22, PDGFRα MO and mouse PDGFRα mRNA n=41 embryos. (G) In situ hybridisation of twist in stage 24 embryos after different treatments as indicated, scale bar 1mm. (H) Analysis of NC migration for control n=30, PDGF-A MO n=24, mouse PDGF-A mRNA=28, PDGF-A MO and mouse PDGF-A mRNA n=27. All Bar graphs indicate mean and s.e.m. (I) Analysis of NC motility (Speed (μm sec-1)) in control and PDGFRα MO injected single cells, control n=202, PDGFRα MO=208 cells from three independent experiments, scatter blot median and interquartile range. (J) Images showing representative tracks of nuclear RFP injected cells over 5h, scale bar 50μm. ns p>0.05, **p> 0.01, ***p>0.001.
Figure 3. Only migratory NC chemotax towards PDGF-A. (A) In vitro chemotaxis assay towards PDGF-A and SDF-1 protein coated beads, representative images of nuclear-fluorescence labeled NC (cyan), NC clusters with time-coded tracks from t=0min to t=300min. (B) Chemotaxis Index (CI) of migratory NC with PDGF-A protein (control n=50, PDGFRα MO n=50) and SDF-1 protein beads (control n=47, PDGFRα MO n=50). (C) In vitro chemotaxis assay of pre-migratory (premig., stage 18) and migratory (mig., stage 22) NC clusters towards PDGF-A protein coated beads, representative images of nuclear-fluorescence marked (cyan) NC clusters with time-coded tracks from t=0min to t=300min. (D) Chemotaxis Index (CI) with PDGF-A protein (pre-mig. n=131, mig n=115) and SDF-1 protein beads (pre-mig. n=100, mig. n=100); box and whisker: box and median are ± 25th/75thpercentile, whiskers are min and max, scale bar 100µm, ns p>0.05, ****p>0.0001.
Figure 4. PDGF signalling affects NC dispersion and N-cadherin levels. (A-F) Nuclear fluorescence (nuclear RFP) of NC explants cultured in vitro for the indicated time and treated as indicated, scale bar 100µm. Bottom row shows analysis of cell dispersion using Delaunay triangulation at 720min. (G) NC dispersion analysis based on average Delaunay triangulation area (μm²) of control=43, PDGF-A protein (50ng ml⁻¹) n=31, PDGF-A MO n=18, PDGF-A MO+PDGF-A protein n=13, PDGFRα MO n=30.
PDGFRα MO+PDGF-A protein n=29 explant clusters from at least three independent experiments; box and whisker: box and median are ± 25th/75th percentile, whiskers are min and max. (H) Analysis of immunostaining against N-cadherin, pixel intensity across cell-cell contact (0μm), normalized to average cell background level; control n=43, PDGF-A MO n=50, PDGFRα MO n=61, PDGF-A mRNA n=50, data points represent mean with s.e.m. (I) Representative projections of confocal images of immunostaining against N-cadherin (green), DAPI (blue) of control, PDGF-A MO, PDGFRα MO and PDGF-A mRNA injected NC cells. Scale bar 20μM. (J) Western blot against N-cadherin from NC explant taken from control embryos or embryos injected with PDGFRα MO. (J) Normalized levels of N-Cadherin protein levels. Bars indicate mean with s.d. of three independent experiments. AU=arbitrary units, ns p>0.05, **p=<0.01, *** p<0.001.
**Figure 5. PDGF signalling controls CIL.** (A,B) Single cell collision assay representing time between first contact and separation, PDGFRα MO explants have impaired CIL, control-control CIL events $n=58$ and PDGFRα MO-PDGFRα MO events $n=63$, from three independent experiments, error bar represent s.d. (C) Thresholded images of explant invasion assay, scale bar 100µm. (D) Overlap percentage between two NC explants of control explants $n=52$, PDGFRα MO explants $n=59$, from three independent experiments. Scatter blot represents median with interquartile range. (E) Protrusions formed away from the cell contact (labelled in white), scale bar 30µM. (F) Protrusion area analysis (µm²) of control $n=51$, PDGF-A (50ng ml⁻¹) $n=34$, PDGFRα MO $n=41$ cells, scatter blot represents median with interquartile range. (G) Circularity index. Representative examples of control and PDGFRα MO, used to measure cell circularity. (H) Circularity of control ($n=79$) and PDGFRα MO ($n=73$) injected NC cells. ns $p>0.05$, *$p<0.05$, ***$p<0.001$. 
Figure 6. AKT signalling downstream of PDGFRα. (A, B, C) Confocal images of Ph-AKT-GFP mRNA injected NC cells for t=0sec and t=425sec. Scale bar 20μM. (A,E) Control, no PDGF-A protein. (B,F) PDGF-A protein (50ng ml⁻¹) at 300sec. (C) PI3K-CAAX mRNA injected NC. (D) Analysis of ph-AKT-GFP pixel intensity at membrane normalized to cytoplasmic ph-AKT-GFP level over time. From three independent experiments, PDGF-A protein addition control n=12, no PDGF-A addition control n=7, PI3K-CAAX n=10 cells; line graph represents mean and s.d.. (E, F) Time course (10min) of images at cell membrane showing membrane localization of ph-AKT-GFP after PDGF-A addition. (G) Western blot analysis of pAKT (Ser437) using NC explants lysates control, treated with PDGF-A protein (50ng ml⁻¹ for 60min). (I) Band intensity normalized to loading control, bar graph shows mean and s.d., three independent experiments. AU=arbitrary units, ns p>0.05, *p=<0.05.
Figure 7. Small molecule inhibition of PI3K/AKT signalling. (A) Scheme illustrating PDGF-PI3K-AKT signalling axis. Signalling is activated upon PDGF ligand/PDGF receptor binding, inducing receptor dimerization and subsequent autophosphorylation. PI3K gets 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 gets activated. (B-F) Twist in situ of stage 24 embryos treated with the indicated inhibitors from stage 14 until stage 24. (B) DMSO control. (C) AG1296 (20μM) (D) LY294002 (40μM). (E) MK-2206 (100μM) (F) UO126 (100μM), scale bar 100μm. (G) NC migration normalised to control average of each experiment. DMSO control n=256, AG1296 n=101, LY294002 n=40, MK-2206 n=106, UO126 n=68 embryos, from three independent experiments. Bar graph represents mean and sem., scale bar 1mm. (H-O) Dispersion analysis of NC cultured in vitro for 720min, using Delaunay triangulation. Treatments as indicated. (P) Analysis of NC dispersion, showing average Delaunay triangulation area normalized to control of each experiment, control n=92, control and PDGF-A protein n=55, LY294002 5μM n=21, MK-2206 5μM n=16, UO126 25μM n=14, LY294002 5μM and PDGF-A n=25, MK-2206 5μM and PDGF-A protein n=35, UO126 25μM and PDGF-A n=19 explants, PDGF-A concentration used for all conditions 50ng ml⁻¹. Bar graphs indicate mean and s.d. (Q-S) Western blot against pAKT using lysates of whole embryos treated with small molecule inhibitors as indicated. (T-V) Quantification of western blot shown in Q-S respectively; intensity of pAKT normalized to total AKT control, bar graph represents mean and s.d. of three independent experiments. AU=arbitrary units, ns p>0.05, **p=<0.01, *** p<0.001.
Figure 8. PDGFRα controls dispersion via PI3K/AKT in a N-cadherin dependent manner. (A-D) Nuclear fluorescence (nuclear RFP) of NC explants cultured in vitro for the indicated time and treated as indicated, scale bar 100µm. Bottom row shows analysis of cell dispersion using Delaunay triangulation at 720min. (E) NC dispersion analysis based on average Delaunay triangulation area (µm2) of control=41, PDGFRα MO n=19, N-cadherin-GFP mRNA n=23, N-cadherin-GFP mRNA and PDGFRα MO n=23 explants, box and whisker: box and median are ± 25th/75th percentile, whiskers are min and max. (F, G) Western blot against N-cadherin using lysates of NC cells treated with DMSO, AG1296 (20 μM, n= 3 blots) or LY294002 (40μM, n=3 blots). (H, I) Band intensity of N-cadherin normalized to loading control, bar graph represents mean and s.d. of three independent experiments. AU=arbitrary units, ns p>0.05, **p=<0.01, *** p<0.001. (J,K) Model of effect of PDGFRα/PDGFA on early (J) and mid (K) neural crest migration.
| Name       | Primer forward/reverse                        | Cycle     | Reference                      |
|------------|-----------------------------------------------|-----------|--------------------------------|
| PDGF-A     | 5’-GGAATGACACGTACAGCAA-3’<br>5’-CGGGAATGAAACATGCCAGTA-3’ | 60°C, 32x | Damm and Winkelbauer 2011      |
| PDGFRα     | 5’-CTCGCAAATGCCACGACGAA-3’<br>5’-CCACAAGGCTGTACATTGTC-3’ | 60°C, 30x |                                 |
| ODC        | 5’-GTCAATGATGGAGTGATGGATC-3’<br>5’-TCCATTCCGCTCTCTGACGACAC-3’ | 60°C, 25x |                                 |
| Sox9       | 5’-AACAGGAGTTCCATCAATCCC-3’<br>5’-CTTTTGTAAACCCCGTGTCAC-3’ | 55°C, 30x | Monsoro-Burq et al., 2003      |
| Sox2       | 5’-CCACACGCCGCTCGGTG-3’<br>5’-TCAGCCCCACGCTCTGAC-3’ | 55°C, 27x | Sugiura et al., 2004          |
| e-Keratin  | 5’-GAGATCGTTATCACCTCTG-3’<br>5’-GTGATCGCTGTACGACG-3’ | 55°C, 27x | Wilson and Melton, 1994        |
| Brachyury  | 5’-GGAATGACACGTACAGCAA-3’<br>5’-CGGGAATGAAACATGCCAGTA-3’ | 60°C, 32x |                                 |
|            | 5’-CTCGCAAATGCCACGACGAA-3’<br>5’-CCACAAGGCTGTACATTGTC-3’ | 60°C, 30x |                                 |
|            | 5’-GTCAATGATGGAGTGATGGATC-3’<br>5’-TCCATTCCGCTCTCTGACGACAC-3’ | 60°C, 25x |                                 |
|            | 5’-AACAGGAGTTCCATCAATCCC-3’<br>5’-CTTTTGTAAACCCCGTGTCAC-3’ | 55°C, 30x | Monsoro-Burq et al., 2003      |
|            | 5’-CCACACGCCGCTCGGTG-3’<br>5’-TCAGCCCCACGCTCTGAC-3’ | 55°C, 27x | Sugiura et al., 2004          |
|            | 5’-GAGATCGTTATCACCTCTG-3’<br>5’-GTGATCGCTGTACGACG-3’ | 55°C, 27x | Wilson and Melton, 1994        |
|            | 5’-GGAATGACACGTACAGCAA-3’<br>5’-CGGGAATGAAACATGCCAGTA-3’ | 60°C, 32x |                                 |
|            | 5’-CTCGCAAATGCCACGACGAA-3’<br>5’-CCACAAGGCTGTACATTGTC-3’ | 60°C, 30x |                                 |
|            | 5’-GTCAATGATGGAGTGATGGATC-3’<br>5’-TCCATTCCGCTCTCTGACGACAC-3’ | 60°C, 25x |                                 |
|            | 5’-AACAGGAGTTCCATCAATCCC-3’<br>5’-CTTTTGTAAACCCCGTGTCAC-3’ | 55°C, 30x | Monsoro-Burq et al., 2003      |
|            | 5’-CCACACGCCGCTCGGTG-3’<br>5’-TCAGCCCCACGCTCTGAC-3’ | 55°C, 27x | Sugiura et al., 2004          |
|            | 5’-GAGATCGTTATCACCTCTG-3’<br>5’-GTGATCGCTGTACGACG-3’ | 55°C, 27x | Wilson and Melton, 1994        |
Figure S1. PDGFRα MO does not affect E-cadherin levels

(A) Western blot against E-cadherin from NC explant taken from control embryos and embryos injected with PDGFRα MO. (B) Normalized levels of E-Cadherin proteins. Error bar is s.d. of three independent experiments. (C) Immunostaining against E-cadherin (green), DAPI (blue) of control, PDGF-A protein treated (50ng ml⁻¹ for 180min) and PDGFRα MO injected NC cells. Scale bar 20μM. (D) Analysis of pixel intensity across cell-cell contact (0μm), normalized to average cell background level. Data points represent mean with s.e.m. Control n=445, PDGFRα MO n=137, PDGF-A protein n=256. AU=arbitrary units, nsp>0.05, **p<0.01, ***p<0.001.
Movies

**Movie 1 PDGF-A chemotaxis assay**

Control explants migrate chemotax towards PDGF-A protein coated beads and the control SDF-1 protein coated beads. Chemotaxis toward PDGF-A beads was impaired by PDGFRα MO injection. Scale bar 50 μM (5min frame⁻¹).
Movie 2 premigratory vs migratory PDGF-A chemotaxis

Premigratory (premig.) NC explants do not chemotax towards PDGF-A protein beads compared to their migratory (mig.) counterpart. Both premigratory and migratory NC explants chemotax towards SDF-1 protein coated bead. Scale bar 25 μM (5min frame⁻¹).
Movie 3 PDGF-A/PDGFRa dispersion assay
PDGF-A MO and PDGFRa MO explants have decreased dispersion compared to control NC cluster. Addition of PDGF-AA protein can rescue PDGF-A MO, but PDGFRa MO dispersion, scale bar 150μM (25min frame⁻¹).
**Movie 4 ph-AKT-GFP with and without PDGF-A protein**
Ph-AKT-GFP mRNA injected NC cells with and without PDGF-A protein (50ng ml\(^{-1}\) at t=300sec) addition. PH-AKT-GFP translocates to the Plasma membrane after protein addition, no effect was observed in untreated NC cells, scale bar 10μM (15sec frame\(^{-1}\)).

**Movie 5 PDGFRa MO N-cadherin dispersion assay**
N-cadherin mRNA can rescue PDGFRa MO inhibition of dispersion, scale bar 150μM (10min frame\(^{-1}\)).