PDGF-B: the Missing Piece in the Mosaic of PDGF Family Role in Craniofacial Development

Debora Corsinovi[1#], Kety Giannetti[1#], Alessia Cericola[1], Valentina Naef[1], Michela Ori[1,2] *

1 Department of Biology, University of Pisa, Pisa, Italy
2 Inter-University Center for the Promotion of the 3Rs Principles in Teaching & Research (Centro 3R), Italy

# These authors contributed equally to this work.

* Corresponding Author

Correspondence to: Michela Ori, PhD. Unit of Cellular and Developmental Biology, Dep. of Biology, University of Pisa, Italy.

Ph. +390502211484
Fax +390502211495
Email: michela.ori@unipi.it
ORCID ID: 0000-0002-6777-0208

Running title: *Pdgf-b role in craniofacial development*

Keywords: neural crest cells, cadherin, *Xenopus laevis, cdh6, cdh11*.

Abstract

Background: The PDGF family consists of four ligands (PDGF-A to -D) and two tyrosine kinase receptors (PDGFR-α and -β). In vertebrates, PDGF signaling influences cell proliferation, migration, matrix deposition and its up-regulation is implicated in cancer progression. Despite this evidence, the role of each family member during embryogenesis is still incomplete and partially controversial. In particular, study of the role of pdgf signaling during craniofacial development has been focused on pdgf-a, c while the role of pdgf-b is almost unknown due to the lethal phenotypes of pdgf-b null mice.

Results: By using a *pdgf-b* splice-blocking morpholino approach, we highlighted impairment of NCC migration in *Xenopus laevis* morphants, leading to alteration of NCC derivatives formation, such as cranial nerves and cartilages. We also uncovered a possible link between pdgf-b and the expression of cadherin superfamily members *cdh6* and *cdh11*, which mediate cell-cell adhesion promoting NCC migration.

Conclusions: Our results suggested that pdgf-b signaling is involved in cranial NCC migration and it is required for proper formation of craniofacial NCC derivatives. Taken together, these data unveiled a new role for pdgf-b during vertebrate development, contributing to complete the picture of pdgf signaling role in craniofacial development.

Introduction

Platelet-Derived Growth Factor (PDGF) family comprises four ligands (PDGF-A, -B, -C, and -D), that form homodimers or the heterodimer AB, and two tyrosine kinase receptors (PDGFR-α and -β) (Demoulin & Essaghir, 2014). The active ligand-receptor complex consists of two receptor chains associated with one dimeric ligand; while PDGFR-α binds to all PDGF isoforms except for PDGF-DD, PDGFR-β binds only to PDGF-BB and -DD; also a heterodimeric αβ receptor has been reported that binds to PDGF-AB, -BB and possibly -CC and -DD (Demoulin & Essaghir, 2014; Muhl *et al.*, 2017). The ligand-receptor interaction could also be cell specific. For example in mouse embryonic fibroblasts, the heterodimeric αβ receptor could bind exclusively the PDGF-BB and -DD (Fantauzzo & Soriano, 2016).

From their first identification in the ‘70s, the members of the PDGF family have been extensively studied in embryogenesis, development, adult homeostasis and disease (Hoch & Soriano, 2003; Andrae *et al.*, 2008; Heldin, 2013; Heldin, 2014).

Most of the studies aimed to elucidate the roles of PDGF signaling during embryonic development were performed in transgenic mice models (Hoch & Soriano, 2003; Andrae *et al.*, 2008) while more recently new functions for PDGF signaling in early development emerged from studies on zebrafish and *Xenopus* embryos (Eberhart *et al.*, 2008; Wiens *et al.*, 2010; Damm & Winklbauer, 2011; McCarthy *et al.*, 2016; Bahm *et al.*, 2017).

Regarding the very early phases of embryonic development, it was shown in *Xenopus* that pdgf-a and pdgfr-α are essential for gastrulation (Ataliotis *et al.*, 1995), during which pdgf-a controls mesoderm cells orientation and migration (Damm & Winklbauer, 2011). Pdgfr-α signaling has also an essential role during the formation of Neural Crest Cells (NCC) derived structures both in mouse and in zebrafish. Knock-out mice for *pdgfr-α*, or with NCC-conditional loss of *pdgfr-α*, and zebrafish hypomorphic *pdgfr-α* mutants exhibit, in fact, cleft face and palate (Tallquist & Soriano, 2003; Eberhart *et al.*, 2008).

These phenotypes were not observed in *pdgf-a* (Boström *et al.*, 1996) or *pdgfr-β* (Soriano, 1994) or *pdgf-d* (Gladh *et al.*, 2016) null mice, while in *pdgf-c* null mice similar palate formation defects were observed (Ding *et al.*, 2004), suggesting a possible role for this ligand in palate structures formation. In comparison with *pdgf-c* null mice, double *pdgf-a* and *pdgf-c* knock-out mutants phenotypes better correlate with *pdgfr-α* knock-out phenotypes, leading to hypothesize that pdgf-a and pdgf-c are the principal ligands for pdgfr-α in mice during NCC development (Ding *et al.*, 2004). More recently, the development of a tissue-specific knock-out mouse line in which *pdgfr-β* was deleted in NCC, revealed defects in the nasal septum and a delay in the palatal shelf development suggesting that also *pdgfr-β* could have a role in craniofacial development (Fantauzzo & Soriano, 2016). Furthermore, in mouse and zebrafish, double knockdown mutants for *pdgfr-α* and *pdgfr-β* exhibited a more severe craniofacial phenotype than those with either mutation alone (Fantauzzo & Soriano, 2016; McCarthy *et al*., 2016). During mammalian development, the homodimers PDGF-AA and PDGF-CC have been shown to exclusively activate PDGFR-α signaling (Boström *et al.*, 1996; Soriano, 1997; Ding *et al.*, 2004), while PDGF-BB induces the formation of both PDGFR-β homodimers and PDGFR-α/β heterodimers (Fantauzzo & Soriano, 2016).

Pdgf-b knock-out embryos showed different penetrance of the phenotypes: one fourth of the embryos died perinatally (E17-18,5) while the remaining embryos died immediately

after birth due to several anatomical abnormalities including enlarged heart and arteries, abnormal kidney development and the development of fatal hemorrhages already present prior to birth (Levéen *et al*., 1994). In these embryos, severely compromised, the NCC derivates in the ear and craniofacial region have not been investigated and the *pdgf-b* expression profile during mouse embryonic development was not described. For all these reasons, the role of pdgf-b during embryonic life remains elusive and, so far, not fully understood. A new hint emerged from our recent analysis of the *pdgf-b* gene expression profile in *Xenopus* embryos (Giannetti *et al.*, 2016). We showed that *pdgf-b* mRNA was not expressed in NCC but in adjacent territories, indicating that pdgf-b could contribute to the early phases of NCC migration, given that these cells express, at least, pdgfr-α. In *Xenopus*, cranial NCC migrate from the mesencephalic and rhombencephalic regions of the CNS towards the branchial pouches, starting at late neurula stage (stage 19). At the same stage, *pdgf-b* mRNA reaches its maximum expression level. At tailbud stages (stage 24-25), *pdgf-b* mRNA was still present in distinct territories of the hindbrain and in the facial placode. *Pdgf-b* expression domain remained in close proximity to NCC, that are still migrating at this stage but never overlaps them. The presence of *pdgf-b* in the facial placode suggested that it might contribute to the correct formation of the facial nerve that is composed both by an ectodermal derivative, the placode, and NCC (Giannetti *et al.*, 2016).

As *pdgf-b* is expressed in specific embryonic territories adjacent to *pdgfr-α* expressing NCC we hypothesize that also this ligand could participate in the orchestration of NCC development. To test our hypothesis, we aimed to perform gene loss of function experiments in *Xenopus* embryos. This animal model offers the unique opportunity to manipulate gene function just in one side of the embryo and the morphological and molecular events that govern the specification, migration and differentiation of NCC are conserved and well described (Casini *et al.*, 2012; Gouignard *et al.*, 2018).

Our results unveiled a new role for pdgf-b in embryonic development showing its requirement for the correct cranial NCC development interfering with the expression of key cell-cell interaction molecules such as cadherins.

Results and Discussion

In order to study the possible role of *pdgf-b* during cranial NCC development, we used a splice-blocking morpholino approach. Splice-blocking morpholinos are morpholino-modified antisense oligonucleotides designed to anneal with the pre-mRNA at the intron-exon junction; when the spliceosome starts the maturation of pre-mRNA, that splice site is not recognized, leading to an exon skipping or the inclusion of an intron on the matured mRNA. We chose this strategy because the splice-blocking morpholino efficiency can be easily verified by RT-PCR and subsequent sequencing. We injected the splice-blocking morpholino (*pdgf-b* MO) in one dorsal blastomere of the *Xenopus* embryo in order to selectively target the central nervous system (Fig. 1A) (Naef *et al.*, 2018). *Pdgf-b* MO was designed to target exon 2 because it contained a base pair number that is not a multiple of three to increase the likelihood of a frameshift mutation after exon excision (Fig. 1B). After the microinjection of the *pdgf-b* MO in *Xenopus* embryos, we performed RT-PCR on *pdgf-b* morphants (Fig. 1C) and we sequenced the PCR product obtained. As expected, cDNA from control embryos gave only the band corresponding to the predicted amplicon (647 bp), while the amplification of cDNA from morphants showed two bands: one corresponding to the wild type *pdgf-b* mRNA and a lower band (556 bp). From sequencing analysis, we defined that the lower band corresponded to the sequence of *pdgf-b* lacking 4 bases from exon1 and total deletion of exon2. This deletion caused a frameshift mutation leading to an altered translation of exon 3 codons and a premature stop codon formation. Thus, the resulting protein product could lack both the Cysteine residues needed for dimerization and the receptor binding domain, therefore being not functional.

We reared *pdgf-b* morphants till neurula stage and we analyzed possible effects on NCC performing whole mount *in situ* hybridization (WISH) with specific NCC markers such as *twist* and *sox10*. Since the injection was performed in only one side, effects could be easily and reliably discriminated by comparing the injected to the uninjected side. The analysis revealed that, in *pdgf-b* morphants, the formation of NCC pre-migratory streams is affected (Fig. 2A, 2C), NCC streams appeared in fact fused and the onset of migration was delayed. *Pdgf-b* morphants also showed an alteration of NCC migration pattern later on. Indeed, at tailbud stage (stage 25) a significant delay of NCC migration was observed in the injected side (Fig. 2A, 2C), while no effects were detectable in the embryos injected with the control morpholino, neither at neurula nor at tailbud stages (Fig. 2A, 2C). The statistical significance of these results is reported in Fig. 2B and Fig. 2D. In order to verify

the specificity of *pdgf-b* MO, we performed functional rescue experiments by co-injecting *pdgf-b* MO with *PDGF-B* mRNA and we analyzed the effects on NCC migration at both neurula and tailbud stages (Fig. 2E, 2F). We observed and confirmed the rescued phenotype in three different rounds of experiments but it was not possible to reach a complete functional rescue. We defined the injected doses of *PDGF-B mRNA* to obtain a reasonable compromise between maximal efficacy and minimal side effects. Higher doses of *PDGF*-B mRNA caused in fact gastrulation defects (data not shown) that alter the possibility to correctly interpret the data.

We then evaluated if and how the abrogation of pdgf-b could impact the formation of cranial NCC derivatives such as cranial nerves and cartilages. In *pdgf-b* morphants we visualized cranial nerves by using the 3A10 antibody (Fig. 3) and cranial skeletal elements by histological alcian blue staining (Fig. 4). NCC deriving from the posterior mesencephalon and from rhombomeres 1 and 2 contribute to the formation of trigeminal nerve (V), while NCC migrating from rhombomere 4 and 6 are necessary for guiding the sensory neurons deriving from epibranchial placods (VII, IX and X) in the correct final position, as well as for establishing proper connection with the central nervous system (Steventon *et al.*, 2014). *Pdgf-b* is expressed by rhombomere 1 and 3 and also by the facial placodes, suggesting that these territories could provide a source of pdgf-b for those NCC that contribute to cranial nerves formation. This idea is supported by recent findings that indicated pdgf signaling as an important guiding cue for NCC migration (Bahm *et al.*, 2017). To test this hypothesis we performed whole mount immunostaining with 3A10 antibody to visualized cranial nerves in *pdgf-b* morphants at larval stages (stage 45) (Fig. 3). Comparing the control side and the injected side of each morphant, a lack of facial nerve (VII) formation was observed from dorsal, ventral and lateral view of the larval head (Fig. 3A, red arrowhead). In addition, the branching pattern of the trigeminal nerve (V) appeared less complex in the injected side with respect to the control side of the embryo (Fig. 3A, blue arrowhead). These phenotypes were statistically significant and were most likely not due to toxic effect of the morpholino or the injection procedure as showed by the comparison with control embryos injected with the control morpholino (Fig. 3A, 3B). Cranial NCC also contribute to craniofacial skeletal elements (Casini *et al.*, 2012). Interestingly, the knock-down of *pdgf-b* specifically affected skeletal structure deriving from the mesencephalic NCC such as the ethmoidal plate and the subocular cartilage (Sadaghiani & Thiébaud, 1987) and caused a slight reduction of the NCC emerging from rhombomere 4 and 6 contributing to the ceratohyal cartilage and the branchial basket, as

revealed by histological alcian blue staining (Fig. 4A, 4B). The low percentage of phenotypes in morpholino-injected embryos could be due to the morpholino dilution in daughter cells during cell divisions and the progressive degradation of morpholino during the time. The skeletal analysis is in fact performed 15-20 days after the morpholino injection. This observation could also suggest that pdgf-b is needed also at late stages of NCC development and that the morphological changes in the skeletal elements are not simply due to the early perturbation of NCC development.

In order to elucidate possible molecular mechanisms altered in *pdgf-b* morphants, we analyzed the gene expression pattern of known key regulators of NCC development such as cadherins. This choice was supported by the recent demonstration that pdgf-a/pdgfr-α inhibition cause a downregulation of N-cadherin (*NCad*) in *Xenopus* NCC during epithelial-to-mesenchimal transition (EMT) (Bahm *et al.*, 2017) and further supported by the observation that overexpression of PDGF-B, causing high-grade glioma on murine neural progenitor cells, can regulate the expression level of *cdh4* (also known as retinal cadherin) (Turtoi *et al.*, 2012; Appolloni *et al.*, 2015). We, therefore, evaluated the expression of the *Ecad* and *Ncad*, key players of the EMT both in NCC development and glioma cells migration in brain parenchyma (Appolloni *et al.*, 2015; Gouignard *et al.*, 2018). In *pdgf-b* morphants at stage 18-20 we found no significant alteration of *Ecad* and *Ncad* by qRT-PCR (Fig. 5A). On the other hand, we observed a significant decrease of *cdh6* and *cdh11* expression in *pdgf-b* morphants analyzed by qRT-PCR and whole-mount *in situ* hybridization (Fig. 5A-E). The downregulation of these two cadherins, specifically involved in NCC migration process (Inoue *et al.*, 1997; Coles *et al.*, 2007; Kashef *et al.*, 2009; Becker *et al.*, 2013; Clay & Halloran, 2014; Taneyhill & Schiffmacher, 2017), is still visible at tailbud stages when NCC are completing their migration in the pharyngeal pouches (Fig. 5B, 5D). These findings might suggest that during NCC development the pdgf-b, secreted by rhombomere 1 and 3 and present in the facial ganglia could act on NCC expressing the pdgfr-α, modulating the expression of cadherins involved in NCC migration. We suggested that pdgf-b signaling is, therefore, necessary for the formation of the trigeminal and facial nerves and the cartilages derived by mesenchephalic NCC.

Functional rescue experiments showed that the co-injection of *pdgf-b* MO and *PDGF-B* mRNA was able to induce restoration of *cdh6* and *cdh11* expression (Fig. 6A, 6B).

An insidious problem with the use of morpholinos is that they may have "off-target" effects, also resulting in non-specific cell death induction (Eisen & Smith, 2008). In order to assess whether *pdgf-b* MO injection could cause cell apoptosis, we performed a TUNEL assay on

morphants at neurula (stage 17) and tailbud (stage 26) stages (Fig. 6C). No evident differences in TUNEL positive cells number were detected between injected and control sides of the embryos, suggesting that *pdgf-b* MO injection did not exert any evident toxic effects.

Conclusion

Our data suggested a new developmental role of pdgf-b ligand that could finally complete the picture of the pdgf family function during the craniofacial development in vertebrates. The requirement of pdgf-b during the formation of craniofacial structures derived from mesencephalic NCC could provide additional information to clarify why the pdgf-c null mice showed a phenotype of palate cleft less severe than the one observed in the pdgfr-α null mice. Our hypothesis is, in fact, that pdgf-b could reinforce, in mesencephalic NCC, the pdgf signaling contributing to the correct migration of the NCC and the subsequent formation of the appropriate craniofacial structures. Furthermore, knowing that differently from Pdgf-aa, Pdgf-bb induces, in mouse embryonic fibroblasts, the formation of both PDGFR-β homodimers and PDGFR-α/β heterodimers, which have distinct signal molecule-binding properties (Fantauzzo & Soriano, 2016), it can be speculated that the presence of pdgf-b, alongside with pdgf-a and -c, might act not redundantly during NCC migration in vertebrates to finely regulate gene expression in EMT and migration steps. Indeed, that could explain the fact that the abrogation of *pdgf-a* leads to a downregulation of *Ncad* in *Xenopus* (Bahm *et al.*, 2017), while the abrogation of *pdgf-b* did not alter *Ncad* expression but seemed to modulate *cdh6* and *cdh11* mRNA levels, involved in the early and late phases of migration. It is worth of note that *pdgf-b* morphants showed altered branching and navigation of cranial nerves, in particular the trigeminal and the facial nerve that has never been investigated in vertebrates. The localized expression of *pdgf-b* and the specificity of the morphants molecular and morphological phenotype suggested an intriguing hypothesis of the existence of a “pdgf code”. The fine regulation of the expression and activity of the different combination of pdgf ligands and receptors in space and time could define, not redundantly, the cadherin expression timing during cranial NCC migration and differentiation steps contributing to defining the development of specific craniofacial structure.

Experimental procedures

Embryos preparation

Animal procedures were approved by Italian Ministry of Public Health and by the local Ethical Committee of the University of Pisa (authorization 99/2012-A, 19.04.2012). *Xenopus laevis* embryos were obtained by hormone-induced laying and *in vitro* fertilization, then reared till the desired stage according to Nieuwkoop and Faber (1967).

Splice-blocking morpholino, constructs generation and microinjections

Pdgf-b splice-blocking morpholino oligonucleotide (*pdgf-b* MO) and a standard control morpholino (Co MO) were synthesized (Gene Tools, Philomath, OR, USA). *pdgf-b* MO sequence: 5'-TCCTCTTCATCTGCAAAAGACACAA-3'; Co MO sequence: 5'-CCTCTTACCTCAGTTACAATTTATA-3'. Embryos were injected at 4-cell stage into one dorsal blastomere, as previously reported (Naef *et al.*, 2018). Injected MO amounts were: 6 ng *pdgf-b* MO and 6 ng Co MO per embryo. 250 pg of *GFP* capped mRNA were co-injected to verify successful injections using a fluorescence microscope. The uninjected side of each embryo represented an internal control. To perform rescue experiments, the open reading frame of human *PDGF-B* (a gift from Prof. P. Malatesta) was subcloned into pCS2+ vector. Capped mRNA encoding the full coding sequence of *PDGF-B* was synthesized using mMESSAGE mMACHINE™ SP6 transcription kit (Thermo Fisher Scientific), following manufacturer's instructions. For rescue experiments, 6 ng of *pdgf-b* MO and 150 pg of *PDGF-B* mRNA were co-injected.

Whole mount *in situ* hybridization (WISH)

Whole mount *in situ* hybridization was carried out using a standard protocol with minor modifications (Harland, 1991). Antisense RNA probes were generated via *in vitro* transcription as reported below: *twist*-PCR2.1-TOPO (HindIII/T7), *sox10*-pBSK (EcoRI/T3), *cdh6*-pGEM-T (NcoI/SP6), *cdh11*-pCMV-SPORT6 (StuI/T7). The color reaction was carried out using the BM Purple (Roche). After color development, embryos were post-fixed and bleached under light to remove the pigment.

Whole mount immunohistochemistry

Whole mount antibody staining was performed following standard procedures. Tadpoles (stage 45) were stained with the neurofilament-specific 3A10 antibody (Developmental Studies Hybridoma Bank), diluted 1:100. Horseradish peroxidase-conjugated secondary antibody goat anti-mouse (1:500 dilution, Invitrogen) was used to detect the primary antibody, and DAB (Roche) was used as a substrate for peroxidase.

Alcian blue staining

Stage 45 tadpoles were fixed overnight in 4% PFA in PBS and processed for Alcian blue staining as described (Casini et al., 2012). After staining, specimens were manually dissected as described (Reisoli et al, 2010).

Reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (qRT-PCR)

Total RNA was extracted from embryos (20 per each experimental group, morphants and control embryos) at stage 20 using Nucleospin® RNA (Macherey-Nagel). cDNA was synthesized from total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). PCR was performed by using DreamTaq™ Green PCR Master Mix (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed using GoTaq® qPCR Master Mix (Promega). Ct values were obtained for each gene and normalized to the housekeeping gene ornithine decarboxylase (ODC). Fold change was calculated relative to control embryos expression level using the $2^{-\Delta\Delta Ct}$ method. The following primers were used for PCR: *pdgf-b* (Forward: 5'-GAGATGTTCAAGAAGATCTCAG-3'; Reverse: 5'-CACTACGTGACCAAAAGTTCTC-3'). The following primers were used for qPCR: *Ecad* (Forward: 5'-CGACCTTTGGACAGAGAAGC-3'; Reverse: 5'-GCACAGAGCCTTCAAAGACC-3'), *Ncad* (Forward: 5'-CAGGAAGAAGGGTTGTTTGC-3', Reverse: 5'-CGGGATCTGAAAGTTTGGAG-3'), *cdh6* (Forward: 5'-CGGAAACAGTGCAAGAGTTG-3'; Reverse: 5'-TCTGTCCATGTTTGGCAATG-3'), *cdh11* (Forward: 5'-ATGATAATCCCCCGGAGTTC-3', Reverse: 5'-

ACAAGCTTAGCGCTGTTTCC-3'), *odc* (Forward: 5'-ACATGGCATTCTCCCTGAAG-3', Reverse: 5'-TGGTCCCAAGGCTAAAGTTG-3').

TUNEL assay

Apoptosis was detected using the DeadEnd™ Fluorometric TUNEL System kit (Promega) with some modifications. *Xenopus* embryos were fixed in 1X MEMFA at the desired developmental stages and stored in 100% methanol at -20°C. After gradual rehydration in methanol (70% - 50% - 30% in 1X PBS), embryos were washed in PBT and permeabilized through digestion with proteinase K (PK, final solution at 5 µg/ml). Incubation time in PK varied according to the embryo developmental stage: 5 minutes for stage 20 embryos, 10 minutes for stage 25 embryos. Embryos were post-fixed in 4% PFA in 1X PBS for 20 minutes to stop the PK digestion, then washed in PBT and incubated overnight at room temperature in the rTdT (Terminal Deoxynucleotidyl Transferase) Incubation Buffer according to the manufacturer's instructions. Fluorescein-12-dUTP provided in the kit was used as the substrate for the TdT and detection was accomplished as for *in situ* hybridization. Alkaline phosphatase-conjugated anti-fluorescein antibody (Roche) was diluted 1:2000 in blocking solution containing 20% lamb serum and 2% Blocking Reagent (Roche). The color reaction was carried out using the BM Purple (Roche) as a substrate for alkaline phosphatase. Embryos were post-fixed in 1X MEMFA and bleached under light to remove the pigment.

Statistical analysis

All results are presented as mean and standard deviation of at least three independent experiments. For qRT-PCR experiments, graphs are representative of three independent experiments with three technical replicates each. In all experiments, *pdgf-b* MO-injected embryos were compared with Co MO-injected embryos at the same developmental stage. Data were statistically analyzed applying Student's *t*-test and visualized using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant when $p < 0.05$ and represented as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Acknowledgments

We thank Prof. Paolo Malatesta for *PDGF-B* cDNA, Prof. Doris Wedlich for *cdh11* cDNA and Prof. Jean-Pierre Saint-Jeannet for *sox10* cDNA. We are grateful to Guglielma De Matienzo for technical support and Salvatore Di Maria for frog care. This work was supported by funding from the University of Pisa (Michela Ori).

References

Andrae J, Gallini R, Betsholtz C. 2008. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22: 1276-312.

Appolloni I, Barilari M, Caviglia S, Gambini E, Reisoli E, Malatesta P. 2015. A cadherin switch underlies malignancy in high-grade gliomas. Oncogene 34:1991-2002.

Ataliotis P, Symes K, Cho MM, Ho L, Mercola M. 1995. PDGF signalling is required for gastrulation of Xenopus laevis. Development 121: 3099-110.

Bahm I, Barriga EH, Frolov A, Theveneau E, Frankel P, Mayor R. 2017. PDGF controls contact inhibition of locomotion by regulating N-cadherin during neural crest migration. Development 144: 2456-2468.

Becker SF, Mayor R, Kashef J. 2013. Cadherin-11 mediates contact inhibition of locomotion during Xenopus neural crest cell migration. PLoS One 8(12): e85717.

Boström H, Willetts K, Pekny M, Levéen P, Lindahl P, Hedstrand H, Pekna M, Hellström M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Törnell J, Heath JK, Betsholtz C. 1996. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. Cell 85: 863-73.

Casini P, Nardi I, Ori M. 2012. Hyaluronan is required for cranial neural crest cells migration and craniofacial development. Dev Dyn 241: 294-302.

Clay MR, Halloran MC. 2014. Cadherin 6 promotes neural crest cell detachment via F-actin regulation and influences active Rho distribution during epithelial-to-mesenchymal transition. Development 141: 2506-15.

Coles EG, Taneyhill LA, Bronner-Fraser M. 2007. A critical role for Cadherin6B in regulating avian neural crest emigration. Dev Biol 312: 533-44.

Damm EW, Winklbauer R. 2011. PDGF-A controls mesoderm cell orientation and radial intercalation during Xenopus gastrulation. Development 138: 565-75.

Demoulin JB, Essaghir A. 2014. PDGF receptor signaling networks in normal and cancer cells. Cytokine Growth Factor Rev 25: 273-83.

Ding H, Wu X, Boström H, Kim I, Wong N, Tsoi B, O'Rourke M, Koh GY, Soriano P, Betsholtz C, Hart TC, Marazita ML, Field LL, Tam PP, Nagy A. 2004. A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. Nat Genet 36: 1111-6.

Eberhart JK, He X, Swartz ME, Yan YL, Song H, Boling TC, Kunerth AK, Walker MB, Kimmel CB, Postlethwait JH. 2008. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. Nat Genet 40: 290-8.

Eisen JS, Smith JC. 2008. Controlling morpholino experiments: don't stop making antisense. Development 135: 1735-43.

Fantauzzo KA, Soriano P. 2016. PDGFRβ regulates craniofacial development through homodimers and functional heterodimers with PDGFRα. Genes Dev 30: 2443-2458.

Giannetti K, Corsinovi D, Rossino C, Appolloni I, Malatesta P, Ori M. 2016. Platelet derived growth factor B gene expression in the Xenopus laevis developing central nervous system. Int J Dev Biol 60: 175-9.

Gladh H, Folestad EB, Muhl L, Ehnman M, Tannenberq P, Lawrence AL, Betsholtz C, Eriksson U. 2016. Mice Lacking Platelet-Derived Growth Factor D Display a Mild Vascular Phenotype. PLoS One 11: e0152276.

Gouignard N, Andrieu C, Theveneau E. 2018. Neural crest delamination and migration: Looking forward to the next 150 years. Genesis 56: e23107.

Harland RM. 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol 36: 685-695.

Heldin CH. 2013. Targeting the pdgf signaling pathway in tumor treatment. Cell Commun Signal 11: 97.

Heldin CH. 2014. Targeting the pdgf signaling pathway in the treatment of non-malignant diseases. J Neuroimmune Pharmacol 9: 69-79.

Hoch RV, Soriano P. 2003. Roles of PDGF in animal development. Development 130: 4769-84.

Inoue T, Chisaka O, Matsunami H, Takeichi M. 1997. Cadherin-6 expression transiently delineates specific rhombomeres, other neural tube subdivisions, and neural crest subpopulations in mouse embryos. Dev Biol 183: 183-94.

Kashef J, Köhler A, Kuriyama S, Alfandari D, Mayor R, Wedlich D. 2009. Cadherin-11 regulates protrusive activity in Xenopus cranial neural crest cells upstream of Trio and the small GTPases. Genes Dev 23: 1393-8.

Levéen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. 1994. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 8: 1875-87.

McCarthy N, Liu JS, Richarte AM, Eskiocak B, Lovely CB, Tallquist MD, Eberhart JK. 2016. Pdgfra and Pdgfrb genetically interact during craniofacial development. Dev Dyn 245: 641-52.

Muhl L, Folestad EB, Gladh H, Wang Y, Moessinger C, Jakobsson L, Eriksson U. 2017. Neuropilin 1 binds PDGF-D and is a co-receptor in PDGF-D-PDGFRβ signaling. J Cell Sci 130: 1365-1378.

Naef V, Monticelli S, Corsinovi D, Mazzetto MT, Cellerino A, Ori M. 2018. The age-regulated zinc finger factor ZNF367 is a new modulator of neuroblast proliferation during embryonic neurogenesis. Sci Rep 8: 11836.

Nieuwkoop PD, Faber J. 1967. Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Elsevier: Amsterdam, The Netherlands.

Reisoli E, De Lucchini S, Nardi I, Ori M. 2010. Serotonin 2B receptor signaling is required for craniofacial morphogenesis and jaw joint formation in *Xenopus*. Development 137: 2927-37.

Sadaghiani B, Thiébaud CH. 1987. Neural crest development in the *Xenopus laevis* embryo, studied by interspecific transplantation and scanning electron microscopy. Dev Biol 124: 91-110.

Soriano P. 1994. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev 8: 1888-96.

Soriano P. 1997. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 124: 2691-700.

Steventon B, Mayor R, Streit A. 2014. Neural crest and placode interaction during the development of the cranial sensory system. Dev Biol 389: 28-38.

Tallquist MD, Soriano P. 2003. Cell autonomous requirement for PDGFRalpha in populations of cranial and cardiac neural crest cells. Development 130: 507-18.

Taneyhill LA, Schiffmacher AT. 2017. Should I stay or should I go? Cadherin function and regulation in the neural crest. Genesis 55(6).

Turtoi A, Musmeci D, Naccarato AG, Scatena C, Ortenzi V, Kiss R, Murtas D, Patsos G, Mazzucchelli G, De Pauw E, Bevilacqua G, Castronovo V. 2012. Sparc-like protein 1 is a new marker of human glioma progression. J Proteome Res 11: 5011-21.

Wiens KM, Lee HL, Shimada H, Metcalf AE, Chao MY, Lien CL. 2010. Platelet-derived growth factor receptor beta is critical for zebrafish intersegmental vessel formation. PloS One 5: e11324.

Figure legends

Figure 1. Splice-blocking morpholino microinjection. (**A**) Schematic representation of the microinjection procedure. Embryos were injected with morpholino oligos and *GFP* mRNA at 4-cell stage in one dorsal blastomere in order to specifically target the central nervous system. The green dashed line highlights the neural plate on the injected side of a neurula stage embryo in bright field. The same embryo shows GFP fluorescence in the injected side. (**B**) Graphic representation of *pdgf-b* splice-blocking morpholino (*pdgf-b* MO) action on RNA maturation. (**C**) RT-PCR on Co MO and *pdgf-b* MO-injected embryos. Co MO-injected embryos show the band corresponding to the full *pdgf-b* coding sequence, while *pdgf-b* MO-injected embryos present both the wild type band and a lower one, corresponding to the PCR product obtained after exon2 excision.

Figure 2. *pdgf-b* downregulation affects NCC migration. (**A**) WISH on *pdgf-b* MO and Co MO-injected embryos showing expression of *twist*. Black dashed line shows the middle line of the embryo. (**B**) Graph reporting the percentage of embryos with altered migration of NCC expressing *twist* (stage 20: Co MO 12%, *pdgf-b* MO 84%; stage 25: Co MO 5%, *pdgf-b* MO 66%). (**C**) WISH on *pdgf-b* MO and Co MO-injected embryos showing expression of *sox10*. (**D**) Graph reporting the percentage of embryos with impaired migration of NCC expressing *sox10* (stage 20: Co MO 10%, *pdgf-b* MO 83%; stage 25: Co MO 5%, *pdgf-b* MO 60%). (**E**) Rescue experiment on embryos co-injected with *pdgf-b* MO

and *PDGF-B* mRNA showing expression of *twist* and *sox10*. (**F**) Graph indicating the mean percentage of stage 20 embryos with NCC migration defects (*twist*: *pdgf-b* MO 84%, *pdgf-b* MO + *PDGF-B* mRNA 52%; *sox10*: *pdgf-b* MO 83%, *pdgf-b* MO + *PDGF-B* mRNA 56%). Abbreviations: MCS, mandibular crest stream (branchial arch I); HCS, hyoid crest stream (branchial arch II); BCS, branchial crest stream (branchial arches III-IV); N, number of independent experiments; n, number of embryos. The injected side is marked with an asterisk (*). Arrowheads indicate impaired NCC migration.

Figure 3. *pdgf-b* depletion affects cranial nerves development. (**A**) Whole mount immunostaining on stage 45 tadpoles labeled with the neurofilament-specific 3A10 antibody revealing cranial nerves. Co MO-injected tadpoles display a normal development of the same nerves on both sides, whereas *pdgf-b* morphant tadpoles lack the most anterior portion of the VII cranial nerve on the injected side, detectable in dorsal, ventral and lateral view (red arrowhead). V cranial nerve (blue arrowhead) appears thinner on the injected side than on the control one and its branching pattern is severely disrupted, as we can observe in dorsal and lateral view. (**B**) Statistical analysis of data shown in **A**; graph reporting the percentage of tadpoles with altered cranial nerves (Co MO 4%, *pdgf-b* MO 49%). Abbreviations: V, trigeminal nerve; VII, facial nerve; N, number of independent experiments; n, number of tadpoles. The injected side is marked with an asterisk (*). Black dashed lines show the middle line of the tadpoles.

Figure 4. *pdgf-b* depletion alters craniofacial cartilages development. (**A**) Dorsal and ventral view of *pdgf-b* MO and Co MO-injected tadpoles after dissection. The ethmoidal plate and the subocular cartilage are mainly affected on the injected side of *pdgf-b* morphants (black arrowhead), but also a slight reduction of the ceratohyal and the branchial cartilages (gills) size is detectable in ventral view. Skeletal elements of the Co MO-injected tadpoles are well developed and bilaterally symmetric. (**B**) Statistical analysis of data shown in **A**; graph reporting the percentage of tadpoles with altered craniofacial cartilages (Co MO 4%, *pdgf-b* MO 49%). Abbreviations: C, ceratohyal; Et, ethmoidal plate; G, gills; M, Meckel's cartilage; Q, quadrate; So, subocular arc; N, number of independent experiments; n, number of tadpoles. The injected side is marked with an asterisk (*). Black dashed lines show the middle line of the tadpoles.

Figure 5. *pdgf-b* knockdown causes a reduction of *cdh6* and *cdh11* expression levels. (**A**) qRT-PCR analysis on stage 20 embryos revealing no effect of *pdgf-b* downregulation on *Ecad* and *Ncad* expression levels and a reduction of *cdh6* and *cdh11* expression levels in *pdgf-b* morphants compared to Co MO-injected embryos. (**B**) WISH on *pdgf-b* MO and Co MO-injected embryos showing *cdh6* expression. Black dashed line shows the middle line of the embryo. Arrowheads indicate a strong reduction of *cdh6* expression on the injected side of *pdgf-b* morphants. (**C**) Statistical analysis of data shown in **B**; graph reporting the percentage of embryos with reduced *cdh6* expression (stage 20: Co MO 11%, *pdgf-b* MO 88%; stage 25: Co MO 5%, *pdgf-b* MO 80%). (**D**) WISH on *pdgf-b* MO and Co MO-injected embryos showing *cdh11* expression. Arrowheads indicate reduced *cdh11* expression. (**E**) Statistical analysis of data shown in **D**; graph reporting the percentage of embryos with reduced *cdh11* expression (stage 20: Co MO 11%, *pdgf-b* MO 82%; stage 25: Co MO 4%, *pdgf-b* MO 75%). Abbreviations: N, number of independent experiments; n, number of embryos. The injected side is marked with an asterisk (*).

Figure 6. Control and functional rescue experiments to verify *pdgf-b* MO specificity. (**A**) Rescue experiment on embryos co-injected with *pdgf-b* MO and *PDGF-B* mRNA showing expression of *cdh6* and *cdh11* at neurula and tailbud stages. Black dashed line shows the middle line of the embryo. *pdgf-b* MO and mRNA co-injection restored cadherins expression. (**B**) Graph indicating the mean percentage of stage 20 embryos with reduced cadherins expression level (*cdh6*: *pdgf-b* MO 88%, *pdgf-b* MO + *PDGF-B* mRNA 45%; *cdh11*: *pdgf-b* MO 82%, *pdgf-b* MO + *PDGF-B* mRNA 47%). Rescue percentage is indicated. (**C**) TUNEL assay performed on *pdgf-b* morphants at neurula (n=61, N=3) and tailbud (n=65, N=3) stages. n, total number of analyzed embryos; N, number of independent experiments. The injected side is marked with an asterisk (*).