

Platelet derived growth factor B gene expression in the *Xenopus laevis* developing central nervous system

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ABSTRACT Platelet-derived growth factor B (PDGF-B) belongs to the mitogen and growth factor family and like the other members it has many roles in cell differentiation, proliferation and migration during development, adult life and in pathological conditions. Among them it has been observed that aberrant PDGF signalling is frequently linked to glioma development and progression, and *Pdgf-b* over-expression in mouse neural progenitors leads to the formation of gliomas. Despite this evidence, the mechanisms underlying PDGF-B driven tumorigenesis and its role during brain development are not fully understood. In order to contribute to clarifying possible new roles of *pdgf-b* signalling, we present here the embryonic gene expression pattern of *pdgf-b*, so far unknown in early vertebrate development. By using *Xenopus laevis* as a model system we performed qRT-PCR and whole mount *in situ* hybridization. *Pdgf-b* mRNA is expressed in discrete regions of the developing central nervous system, in the cranial nerve placodes and in the notochord. We also compared the gene expression of *pdgf-b* with that of its receptor *pdgfr-α* suggesting so far unsuspected roles for this signalling pathway during the development of specific embryonic structures.

KEY WORDS: PDGF-B, PDGF receptor α , central nervous system, neural crest, *Xenopus laevis*

Platelet-derived growth factor (PDGF) family comprises two tyrosine kinase receptors (PDGFR- α and - β) and four ligands (PDGF-A, -B, -C, and -D) that form homodimers or the heterodimer AB (Demoulin and 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 (Fig.1). Also a heterodimeric $\alpha\beta$ receptor has been reported that binds to PDGF-AB, -BB and possibly -CC and -DD (Demoulin and Essaghir, 2014). The members of this family have been extensively studied for more than 30 years in development, adult homeostasis and disease (Heldin, 2013, Heldin, 2014, Hoch and Soriano, 2003). *Pdgf-a* expression was recently characterized in mouse tissues and in early embryonic development, showing its involvement in developmental processes including gastrulation and craniofacial development (Andrae *et al.*, 2014, Eberhart *et al.*, 2008). On the contrary, for *pdgf-b* the only information available mainly concern its role during embryonic angiogenesis (Hoch and Soriano, 2003, Leveen *et al.*, 1994), and a complete gene expression pattern in vertebrate embryogenesis is still lacking. Functional studies using a knock out mouse for *pdgf-b* showed abnormal kidney glomeruli,

heart and blood vessel dilation, anemia, thrombocytopenia, haemorrhages and perinatal death (Betsholtz, 1995, Leveen *et al.*, 1994). However, in this mutant line, BB homodimer and AB heterodimer were simultaneously abrogated, making difficult to precisely define the specific role of *pdgf-b* alone during embryogenesis. Furthermore, due to extensive perinatal haemorrhages, other possible phenotypes caused by the lack of *pdgf-b* in specific tissues could not be deeply investigated.

It is known that *pdgf-b* possesses a C-terminal basic retention motif, which binds to extracellular matrix components. The presence of this motif does not prevent binding to the receptor but limits the *pdgf-b* diffusion. When the retention motif is removed by intracellular or extracellular proteases, a soluble and diffusible *pdgf-b* form is released. Recently, the study of mutant mice harboring a *pdgf-b* gene that lacks the retention motif shed light on its importance, showing that the mutant mice present an intermediary phenotype between wild-type and *pdgf-b* knock-out animals (Demoulin and

Abbreviations used in this paper: PDGF, platelet derived growth factor; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; WISH, whole mount *in situ* hybridization; *Xenopus*, *Xenopus laevis*.

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Essaghir, 2014). The interplay between pdgf-bb and the extracellular matrix has been also demonstrated in many cell types, for instance, in dermal fibroblasts pdgf-b stimulates the synthesis of hyaluronan, which in turn is required for the mitogenic activity of pdgf-b, via the activation of CD44 receptor (Li *et al.*, 2007).

Another interesting aspect of studying PDGF family function relies on the fact that over activity of PDGF signalling is associated with several malignant and non-malignant diseases (Heldin, 2013, Heldin, 2014). Among malignant ones it was shown that in gliomas, a group of heterogeneous and aggressive brain tumours, alteration of PDGF signalling is commonly observed (Calzolari and Malatesta, 2010); interestingly *PDGF-B* over-expression alone was shown inducing oligodendroglioma, a specific subclass of glioma, from mouse neural progenitors, suggesting a crucial role for this molecule in tumorigenesis (Appolloni *et al.*, 2009). Moreover recent data about the cellular mechanisms of PDGF-B driven glioma progression and maintenance indicate that PDGF may be required to override cell contact inhibition and promote glioma cell infiltration (Calzolari and Malatesta, 2010). Nevertheless not much is known about the molecular mechanisms activated downstream PDGF-B signalling. A deeper comprehension of pdgf-b role during development might help in shedding light on its mechanisms of action in modulating different aspects of cell behaviour also involved in tumorigenesis and tumour progression.

Results and Discussion

PDGF-b s expressed during *Xenopus laevis* development

The aim of this work was to describe the gene expression pattern of *pdgf-b* during embryogenesis using *Xenopus laevis* as a model. *Xenopus* is a tetrapod from the same evolutionary branch

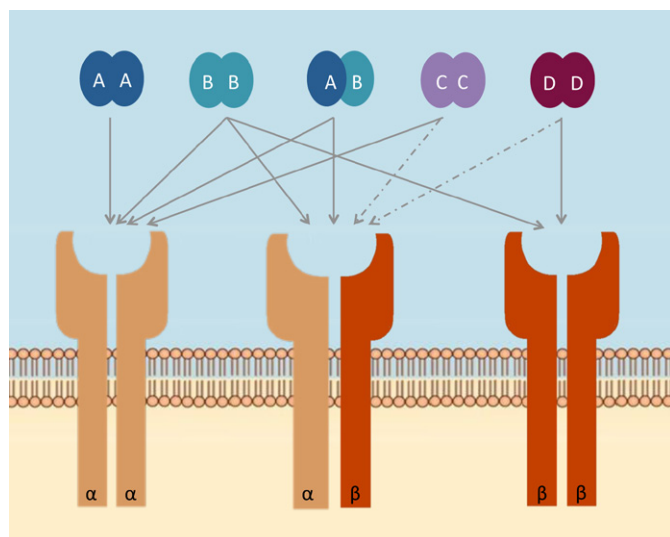


Fig. 1. Platelet derived growth factor (PDGF) family members and their interactions. PDGF family is composed by the four ligands A, B, C and D that need to dimerize in order to be active and bind to the receptor. They can homodimerize and the only known heterodimer is AB. The two receptors, α and β , are tyrosine kinases and have different affinity for the ligands. When the ligand-receptor complex forms, the intracellular domains of the receptors trans-phosphorylate each other, triggering the signal. Arrows indicate interaction, dashed arrows indicate hypothetical interactions.

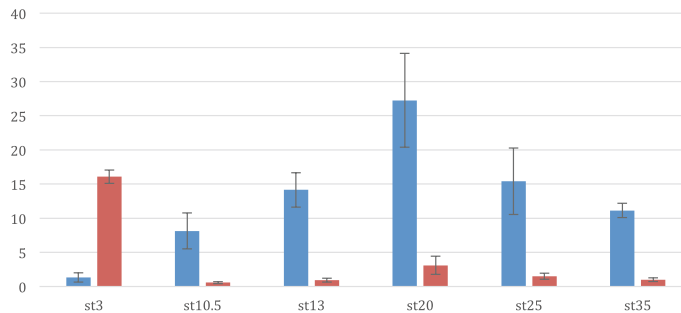


Fig. 2. The temporal expression pattern of *pdgf-b* revealed by qRT-PCR.

Expression levels (y axis) of *pdgf-b* (red) and its receptor *pdgfr- α* (blue) at different developmental stages (x axis) were analyzed by qRT-PCR. The data are an average of three independent experiments, the values were normalized to *odc* and presented as mean \pm s.d. Expression of *pdgf-b* mRNA reaches its maximum level as maternal transcript (stage 3), then drastically decreases during gastrulation (stage 10,5). A slight increase is observed during early neurulation (stage 13), however the expression becomes more robust at late neurula stage (stage 20) to decrease again in tailbud stages. *Pdgfr- α* , is barely detectable at stage 3 and then rise firmly already during gastrulation, reaching its maximum level at stage 20.

of mammals and because of its external embryonic development, it represents a suitable model to study in details the expression pattern of a specific gene from the very early steps of embryogenesis. We firstly performed qRT-PCR comparing the relative expression level of *pdgf-b* with that of its receptor *pdgfr- α* already described (Bae *et al.*, 2014) (Fig. 2). *Pdgf-b* mRNA is present as a maternal transcript within the blastula at 4 cells stage (stage 3 according to Nieuwkoop and Faber, 1967). After the start of the zygotic transcription (stage 8), *pdgf-b* mRNA was barely detectable and it showed a low expression level both during gastrulation (stage 10.5) and neurulation (stage 13); on the contrary, *pdgfr- α* was abundantly expressed at these stages. During gastrulation in *Xenopus*, a crucial role for *pdgf-a* and *pdgfr- α* has been demonstrated (Damm and Winklbauer, 2011). As *pdgf-b* mRNA is extremely poorly expressed during gastrulation, this data corroborates the idea that in the early phases of development, the main role of PDGF signalling is played by the homodimer *pdgf-aa* via *pdgfr- α* . This is in line with the observation that in *pdgf-a* morphants the prechordal mesoderm fails to spread during gastrulation and no compensatory mechanisms occurs (Damm and Winklbauer, 2011). At the end of neurulation (Stage 20), *pdgf-b* mRNA expression level rose and then decreased again at late tailbud stages (stage 35) (Fig. 2).

In order to define the localization of *pdgf-b* mRNA, we performed whole mount *in situ* hybridization (WISH) using a digoxigenin labelled *pdgf-b* antisense RNA probe on wild type *Xenopus* embryos at different stages of development (Figs. 3-4). We confirmed the presence of *pdgf-b* as a maternal transcript at stage 3, when the mRNA is localized in the animal pole (Fig. 3A). In the gastrulating embryo, *pdgf-b* was faintly expressed in the animal pole in the deep layer of the ectodermal blastocoel roof (Fig. 3 B-B'). In these early phases of development the riboprobes could sometimes generate false positive signals due to the unspecific labelling of the blastocoel cavity. We therefore confirmed the specificity of *pdgf-b* mRNA localization performing the same WISH experiment using a *pdgf-b* sense riboprobe (Fig. 3 A*-B*-B'*). At the end of neurulation (stage 20-21) the gene expression pattern of *pdgf-b* resulted

more robust and localized (Fig. 3C). At this stage, two stripes in the midbrain–hindbrain region of the central nervous system (CNS) are clearly defined and the placode of the facial nerve is labelled. We also observed a strongly labelled domain in anterior region of the neuroectoderm, possibly corresponding to the future telencephalon (Fig. 3 C-H). At the same stage, *pdgf-b* mRNA is also localized in the notochord (Fig. 3C inset). We then performed double WISH to better define *pdgf-b* expression territories in the CNS by using specific molecular markers to label respectively the midbrain-hindbrain boundary (*engrailed2*) and rhombomeres 3 and 5 (*krox20*). Our data showed that *pdgf-b* mRNA localized in rhombomere 1 and rhombomere 3 of the hindbrain (Fig. 3 D-E). In order to define the identity of the most anterior *pdgf-b* expression domain, we also performed double WISH with the retinal marker

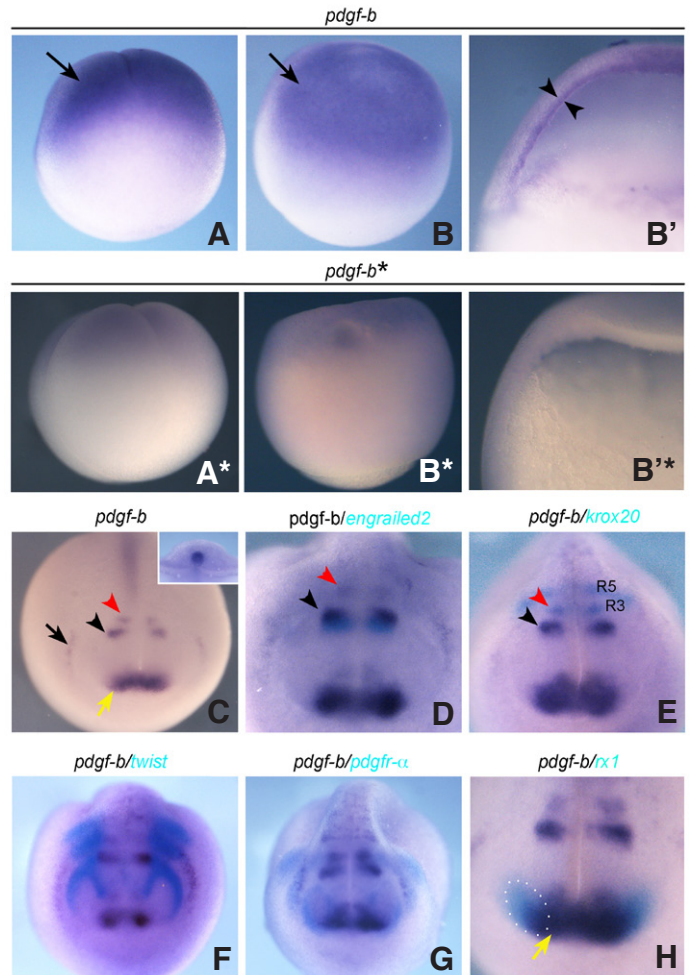


Fig. 3 (right). *Pdgf-b* spatial expression pattern during early stages of development in *Xenopus laevis* embryos. (A) *Pdgf-b* maternal transcript is distributed in the animal pole (arrow) at four cell stage (stage 3), lateral view. (B) Lateral view of a stage 10,5 embryo showing the *pdgf-b* mRNA localization in the animal pole of the embryo (arrow). (B') Bisected gastrula showing the *pdgf-b* expression confined within the deep layer of the ectodermal blastocoel roof (arrowheads). (A*) WISH performed using a *pdgf-b* sense riboprobe on a stage 3 embryo, lateral view. (B*) Stage 10,5 lateral view WISH using the *pdgf-b* sense riboprobe: no signal is detected in the animal pole. (B'*) Bisected gastrula showing no unspecific labelling of the blastocoel cavity using the *pdgf-b* sense riboprobe. (C) Late neurula, (stage 20) embryo frontal view. *Pdgf-b* mRNA is localized in the CNS (midbrain–hindbrain region: black and red arrowheads: the most ventral anterior region of neural tube: yellow arrow), the placode of the facial nerve (black arrow) and the notochord (inset). (D) Stage 20 frontal view double WISH: *pdgf-b* signal revealed in purple (black and red arrowheads), *engrailed2* labelling the midbrain-hindbrain boundary and rhombomere 1, in light blue. The two signals overlaps at the level of rhombomere 1. (E) Stage 20 frontal view double WISH: *pdgf-b* signal in purple (black and red arrowheads), *krox20* labelling rhombomeres 3 (R3) and 5 (R5), in light blue. The two signals overlaps faintly at the level of rhombomere 3. (F) Double WISH using *twist*, a NCC marker in light blue. NCC are in close proximity of the *pdgf-b* expression domain but never overlap it. (G) Double WISH using *pdgfr-α* in light blue. *Pdgfr-α* is expressed in migrating NCC. (H) Frontal view of an embryo processed with double WISH using *rx1*, a marker for the eyefield (dashed oval), revealing a partial overlap of the *pdgf-b* signal (yellow arrow) with an anterior and ventral domain of the eyefield.

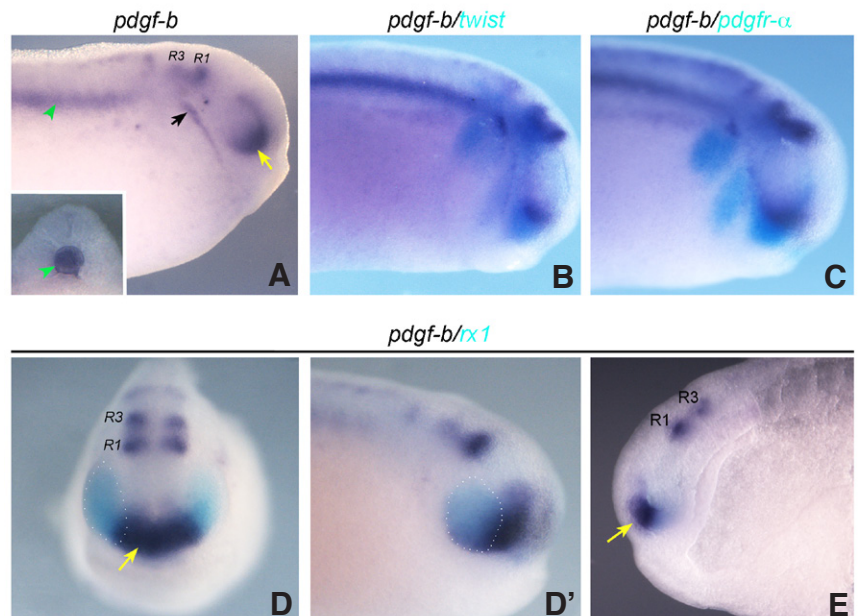


Fig. 4. *Pdgf-b* expression spatial distribution in *Xenopus laevis* embryo at tailbud stage. (A) *Pdgf-b* mRNA is present in the hindbrain (R1 and R3), the most ventral anterior region of the developing CNS (yellow arrow), the placode of the facial nerve (black arrow) and the notochord (inset, green arrowhead). (B-E) Double WISH was performed using different markers (the color code is described in Fig. 3) at tailbud stage (stage 25). (B) Lateral view of an embryo processed by double WISH using *twist* as NCC marker. (C) *Pdgfr-α* is expressed by NCC, in close proximity with the *pdgf-b* mRNA expression domain. (D-D') Frontal and lateral views of the same embryo labelled to visualize *rx1*, a retinal marker (dashed oval identifies the eyefield) and *pdgf-b*. (E) A sagittal view of a half embryo bisected along the antero-posterior axis shows the expression of *pdgf-b* in the ventral telencephalon.

rx1, observing that *pdgf-b* partially overlapped *rx1* expression domain in the most medial domain of the eyefield (Fig. 3H). These data established the presence of *pdgf-b* mRNA within precise and confined areas of the developing CNS, suggesting a potential role for *pdgf-b* in CNS development. This idea is supported also by previous data showing that the activity of the PDGF pathway is necessary to properly regulate oligodendrocyte progenitor cells (OPC) migration and proliferation (McKinnon et al., 2005) and that *pdgf-b* overexpression *in vitro* is sufficient to induce an OPC identity in neural progenitor cells (Appolloni et al., 2009). In addition, mutations in *PDGF-B* gene are responsible for causing primary familial brain calcification disease in humans (Keller et al., 2013).

Different studies demonstrated that PDGF signalling is implicated in Neural Crest Cells (NCC) development, but the mechanism of action remains controversial (Smith and Tallquist, 2010). In particular, the studies described so far were mainly focused on the role of *pdgf-a* and *pdgfr- α* during cranial NCC formation and migration (Eberhart et al., 2008, Ho et al., 1994), while a role for *pdgf-b* was supposed to be limited to a sub-population of cardiac NCC (Van den Akker et al., 2008). Cranial NCC are known to express *pdgfr- α* (Ho et al., 1994), that can activate an intracellular signalling cascade after the binding of *pdgf-aa* or *pdgf-bb* homodimers or the *pdgf-ab* heterodimer. In order to analyze the spatial relation between *pdgf-b* expression domain and NCC, we performed double WISH using *twist*, a NCC marker (Fig. 3F), or *pdgfr- α* (Fig. 3G). These analyses showed that *pdgf-b* mRNA was not expressed in NCC but in adjacent territories that can represent a source of *pdgf-b* during the early phases of NCC migration. In *Xenopus*, in fact, cranial NCC migrate from the mesencephalic and rhombencephalic regions of the CNS towards the branchial arches starting from late neurula (from stage 19). At the same stage, *pdgf-b* mRNA reaches its maximum expression level (Fig. 2). At tailbud stages (Stage 24-25) *pdgf-b* mRNA was still present in the same CNS territories described before, in the notochord and in the facial placode (Fig. 4A and the inset). *Pdgf-b* expression domain remained in close proximity to NCC, that are still migrating at this stage, but never overlaps them as shown by the double WISH performed with *twist* or *pdgfr- α* (Fig. 4 B-C). 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. In order to better define the most anterior expression domain of *pdgf-b* in the CNS we repeated a double WISH at tailbud stage using the *rx1* probe (Fig. 4 D-E). *Pdgf-b* mRNA expression domain partially overlaps with the most anterior-medial *rx1* retinal expression domain (Fig. 4 D-D') and in a medial-sagittal view of an embryo at the same stage, a clear *pdgf-b* labelling was visible also in the ventral telencephalon (Fig. 4E).

Taken together, these data unveiled the presence of *pdgf-b* mRNA in neuroectoderm derived structures during embryogenesis. On the basis of *pdgf-b* mRNA localization in a region adjacent to NCC expressing *pdgfr- α* , it is tempting to speculate that *pdgf-b* might also contribute to NCC development (Fig. 5). In this context it is interesting to remind that *pdgf-b* can bind to extracellular matrix components and that it can also stimulate hyaluronan synthesis,

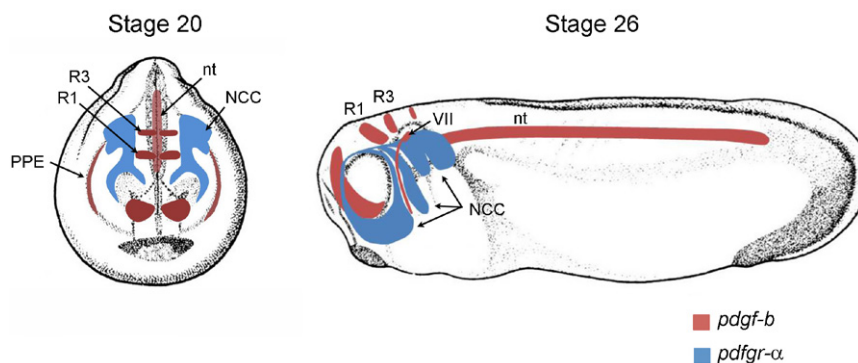


Fig. 5. The cartoon represents the spatial relation between *pdgf-b* (red) and *pdgfr- α* (blue) mRNAs at neurula (stage 20) and tailbud (stage 26) stages. NCC: neural crest cells; nt: notochord; PPE: preplacodal ectoderm; R1 and R3: rhombomere 1 and 3; VII: facial nerve.

the most abundant glycosaminoglycan in the ECM surrounding cranial NCC. The developing NCC in *Xenopus*, in fact, produce hyaluronan and express the hyaluronan receptor CD44 (Casini et al., 2012). As the inhibition of the hyaluronan receptor CD44 could in turn inhibit *pdgf-b* induced cell growth (Li et al., 2007), it is tempting to hypothesize a possible role of *pdgf-b* in the activation of CD44 to instruct cranial NCC to keep on dividing during their migration. In the future, it will be interesting to investigate by functional experiments whether *pdgf-b* signalling could be required to control these two key phenomena in NCC development: cell division and migration.

Conclusions

The final result of this study was to unveil the gene expression pattern of *pdgf-b* during embryonic development. The possibility to have a vertebrate model such as *Xenopus*, in which the *pdgf-b* expression pattern is characterized, will allow future functional experiments aimed to clarify *pdgf-b* functions during brain and NCC development so far unsuspected. Moreover, overexpressing the PDGF-B mRNA in *Xenopus* embryos it will be possible to generate an alternative and useful model to study *in vivo* the pathogenic cellular mechanisms triggered by PDGF-B overexpression.

Materials and Methods

Embryo preparation

Animal procedures were performed in strict compliance with protocols approved by Italian Ministry of Public Health and of the local Ethical Committee of University of Pisa (authorization n.99/2012-A, 19.04.2012). *Xenopus laevis* embryos were obtained by hormone-induced laying and *in vitro* fertilization then reared in 0.1 X Marc's Modified Ringer's Solution (MMR 1x: 0.1 M NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES pH 7.5) till the desired stage according to Nieuwkoop and Faber (1967).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 10 wild type embryos for each developmental stage examined with Nucleospin® RNA (Macherey-Nagel) according to the manufacturer's instruction. cDNA was prepared by using iScript™ cDNA Synthesis Kit (Bio-Rad) and quantitative real-time PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad) according to the manufacturer's instruction. mRNA levels were normalized to the housekeeping gene ornithine decarboxylase (*odc*). Three different couple of primers were used: *odc*

and *pdgfr-α* sequences were reported in (Bae *et al.*, 2014), while primers for *pdgf-b* were as follow: Forward 5'- GCTCTTCAATGCTCCTTTCC -3', Reverse 5'- TGTGCAATAAGAGGGCAGTG -3'.

Whole mount in situ hybridization (WISH)

Xenopus pdgf-b sequence was obtained via RT-PCR using the following primers: forward, 5'- GAGATGTTCAAGAAGATCTCAG-3'; reverse, 5'- CACTACGTGACCAAAAAGTTCTC-3'. PCR product sequence was aligned to *pdgf-b* sequence (Gene Bank: NM_001094466) and then cloned into pGEM-T easy vector (Promega). Digoxigenin (DIG) (Roche) labelled sense and antisense RNA probes were generated for *pdgf-b* and Fluorescein (FLUO) (Roche) labelled antisense RNA probes were generated for *twist*, *pdgfr-α*, *engrailed2*, *krox20* and *rx1*. WISH was performed as described (Reisoli *et al.*, 2010). We first revealed the DIG probe signal with BM Purple (Roche) and then the FLUO probe signal was visualized using BCIP substrate (Roche). After colour development, embryos were post-fixed and bleached under fluorescent light to remove the pigment.

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