Int. J. Dev. Biol. 51: 327-331 (2007)
doi: 10.1387/ijdb.062217al

Short Communication

Pax7 identifies neural crest, chromatophore lineages and pigment stem cells during zebrafish development

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ABSTRACT Using immunostaining during early zebrafish embryogenesis, we report that the cranial and trunk neural crest expresses the paired box protein Pax7, thus revealing a novel neural crest marker in zebrafish. In the head, we show that Pax7 is broadly expressed in the cranial crest cells, which indicates that duplication of the paralogous group Pax3/7 at the origin of vertebrates included the conserved expression of Pax7 in the head neural crest of all of the vertebrates species studied so far. In the trunk, Pax7 recognizes both premigratory and migratory neural crest cells. Notably, we observed the expression of Pax7 during the development of melanophore, xanthophore and iridophore precursor cells. In contrast to the case of melanocyte precursors in birds, Pax7 showed overlapping expression with early melanin pigment. Finally, during the larva to adult transition, we show that pigment stem cells recapitulate the expression of Pax7.

KEY WORDS: *Pax7, neural crest, chromatophore precursor, pigment stem cell*

To examine the cellular and molecular mechanisms of neural crest (NC) development, the zebrafish *Danio rerio* offers many advantages as an embryological and genetic model system (Kelsh and Raible, 2002; Quigley and Parichy, 2002).

Pax3 and *Pax7*, which are members of one of the four *Pax* subfamilies, are required to specify the NC in amniotes (Basch *et al.*, 2006; Relaix *et al.*, 2004). In a seminal paper by Seo *et al.* (1998), zebrafish *Pax3* and *Pax7* genes were analyzed and reported multiplicity of isoforms encoded by the paralogous *Pax7*. Since no expression of *Pax7* was detected in NC cells, they suggested the need for further study to reveal NC expression at the protein and RNA level.

Pax7 expression occurs in a large number of scattered cells throughout the body (Fig. 1A). With development, its expression progresses in an anterior-to-posterior wave. The large size of these Pax7 cells and its dynamic pattern of spatiotemporal distribution are consistent with a NC cell identity. Notably, Pax7 recognizes both trunk (Fig. 1) and cranial (Fig. 2) NC cells. Therefore, Pax7 is an evolutionary conserved NC marker, either of the head crest in mammals (Mansouri *et al.*, 1996) or throughout the crest in chick (Kawakami *et al.*, 1997; Lacosta *et al.*, 2005) and zebrafish.

Both in toto (Fig. 1A,B) and in sections (Fig. 1D), we found Pax7 expression in cells in a position dorsal and dorsolateral to the neural tube, which characterizes the premigratory phase of zebrafish NC development. To provide additional confirmation for the NC identity of these Pax7 positive cells, we compared the Pax7 staining pattern with that of several premigratory NC markers. As shown in Fig.1C, *crestin* (see Luo *et al.*, 2001) seems to precede Pax7 in the premigratory crest.

We also compared the immunostaining patterns of the Rohon-Beard (R-B) sensory neuron marker, HNK- 1 (Metcalfe *et al.*, 1990) and that of Pax7. Pax7 expression in the premigratory NC cells took place later than HNK-1 in the R-B (Fig. 1E). Therefore, these findings confirm the sequential steps of R-B neurogenesis and neurocristogenesis in the zebrafish (see Cornell and Eisen, 2000). In turn, this study identifies Pax7 as a suitable marker to study the specification of individual crest cells. Zebrafish *Pax3*, however, is the earliest identified marker of the NC domain and thus used to study the initial induction of NC (Lewis *et al.*, 2004). Hence, both *Pax3* and *Pax7* paralogs may help to dissect how NC cells become specified and differentiated.

Before the onset of ventral migration, the longitudinal migration of some NC cells occurs along the dorsal neural keel-tube (Raible *et al.*, 1992; Vaglia and Hall, 2000). Here, we confirm that pattern of migration through the trunk and tail of the zebrafish (Fig. 1B).

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† **Note:** Dedicated to the memory of Dr. Luis Dominguez who passed away recently.

0214-6282/2007/$30.00

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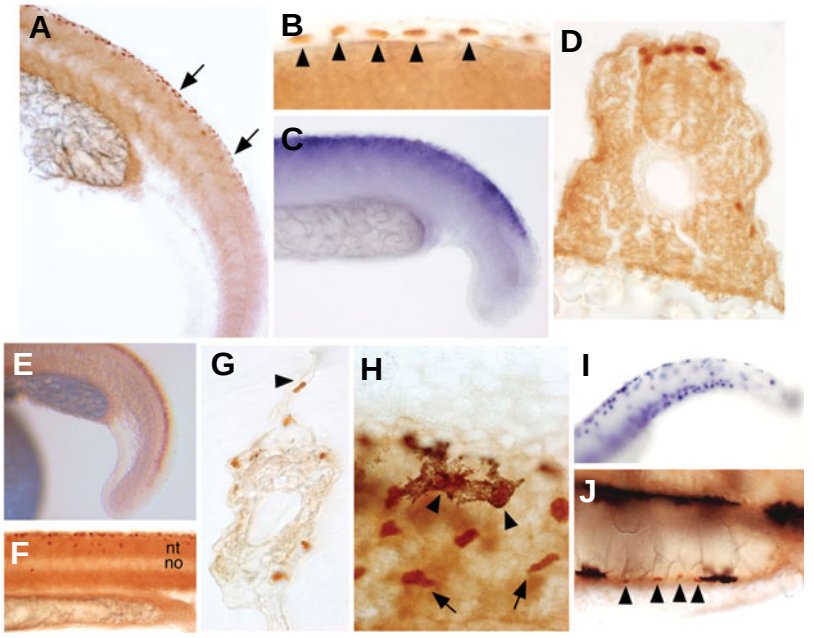


Fig. 1. Expression pattern of Pax7 in the trunk neural crest (NC). *Whole-mounts of 24 hpf (A,B,F), 21 hpf (C,E) and 48 hpf (H-J) embryos. Transverse sections of whole-mount 21 hpf (D) and 48 hpf (G) embryos.* **(A)** *Trunk and tail level showing Pax7 expression within the premigratory NC (arrows) and migrating NC cells (out of focus).* **(B)** *Higher magnification showing several Pax7-positive crest cells along the top of the neural tube (arrowheads).* **(C)** Crestin *expression is shown for comparison.* **(D)** *Expression of Pax7 in several premigratory NC cells located dorsal to the neural tube.* **(E)** *Embryo showing HNK-1 expression in the Rohon-Beard neurons.* **(F)** *Note several Pax7+ cells migrating in the medial pathway.* **(G)** *Many Pax7 cells are evident at different dorso-ventral levels of the lateral migratory pathway. Note one Pax7+ NC cell in the dorsal median fin (arrowhead). Focus is on the dorsalmost NC cell.* **(H)** *Whole-mount showing lightly pigmented melanin granules surrounding Pax7 expressing nuclei (arrowheads) in some melanophores within the dorsal stripe. Note also some Pax7+ cells on the lateral pathway (arrows), which are devoid of melanin and are likely xanthophore precursors.* **(I)** Dct in situ *RNA hybridization reveals many* dct*-positive cells. Focus is on the dorsal and ventral stripes. PTU treated embryo.* **(J)** *Yolk sac stripe at the level of the yolk extension. Note several Pax7 iridophore precursors (arrowheads). Heavily pigmented melanophores can also be seen in the yolk sac and ventral stripes. no, notochord; nt, neural tube; PTU, 1-phenyl-2-thiourea. Anterior to the left and dorsal uppermost in (A-C), (E-F) and (H-J).*

With development, Pax7 expression occurred in migrating trunk NC cells, which were located on the medial (Fig. 1F) and lateral migration routes (Fig. 1G). Additionally, because the NC frequently appears as streams of adjacent cells (Raible *et al.*, 1992; Vaglia and Hall, 2000), nuclear expression of the transcription factor Pax7 provides a reliable method for NC numbering. Cell counts of Pax7-positive early trunk NC cells are similar to other estimates of NC numbers (Raible *et al.*, 1992).

Comparison of Pax7 and the melanogenic enzyme dopachrome tautomerase (dct; Kelsh *et al.*, 2000) revealed coincident spatiotemporal distribution in some migrating cranial and trunk crest cells. In contrast to Pax7, however, premigratory NC cells lack *dct* (Fig. 1I; see also Kelsh *et al.*, 2000). Therefore, Pax7 precedes the expression of *dct* and thus may be involved in its regulation during zebrafish melanogenesis.

The spatial pattern of Pax7 downregulation closely follows the wave of melanogenesis. Thus, Pax7 downregulation initiated in melanoblasts located behind the otic vesicle and progressed anteriorly and posteriorly in 30 and 48 hpf embryos.

In 48 hpf embryos, at the dorsal melanophore stripe level (Fig. 1H), we found melanophore progenitor cells (revealed by their characteristic stellate shape, mostly flattened, with lightly or more heavily pigmented melanin granules) with a Pax7-positive nucleus or not.

Only iridophores and melanophores contribute to the yolk sac stripe (Kelsh, 2004; Kimmel *et al.*, 1995). Here, iridophores differentiate *in situ*, whereas melanophores become pigmented while migrating (Rawls *et al.*, 2001). As in the dorsal stripe, Pax7 positive cells, which are devoid of melanin, were also identified in the iridophore precursors (Fig. 1J). Pax7 was downregulated in the iridophore lineage at around 3 days post-fertilization.

In *albino* embryos (Kelsh *et al.*, 2000), with reduced and delayed melanin synthesis, distribution of Pax7 expressing cells was apparently similar (data not shown). This suggests that Pax7 acts upstream of *albino* during melanogenesis.

In contrast to trunk crest, there are no published reports of cranial NC cell counts. Here, immunocytochemical observations in toto and in sections permitted easy identification and counting of head NC cells by revealing the Pax7 transcription factor. That procedure even permitted the counting of overlapping NC nuclei. Crest cell counts made from the level of the first somite to the most rostral end in embryos aged between 24 hpf and 30 hpf showed an average of 182,10 ± 20,13 (n = 11) head NC cells.

Expression of Pax7 occurred in head NC cells located at every level of the embryonic brain, except at the most rostral telencephalic level (Fig. 2A), which supports their lack of specification in regions rostral to the diencephalon (Aybar and Mayor, 2002). To analyze whether Pax7 in the brain and the NC are correlated, we also studied younger embryos. As shown in Fig. 2B, Pax7 positive crest cells only segregate from a previous Pax7-expressing dorsal progenitor domain.

Pax7 immunostaining show positive cells with patterns of distribution that correspond to melanophore and xanthophore precursors (Fig. 2A). Strikingly, Pax7 displays a differential temporal expression in both pigment cell lineages (Fig. 2C,D). Thus, Pax7 is first downregulated in the melanophore lineage, while persisted longer in the xanthophore lineage, until about the 3dpf.

Dct and melanin as melanophore markers and xanthine dehydrogenase (xdh), GTP-cyclohidrolase I (gch) and the receptor fms as xanthophore markers (but see Parichy *et al.*, 2000) were also used to verify melanophore and xanthophore precursors identity (Fig. 2C-F).

From stage 20 hpf, we first noted some Pax7 cells over the optic cup (Fig. 2B). With development, their number increases, become located over the dorsal eye and later spread through the entire eye (Fig. 2G). At 3 dpf, Pax7 downregulate coinciding with the appear-

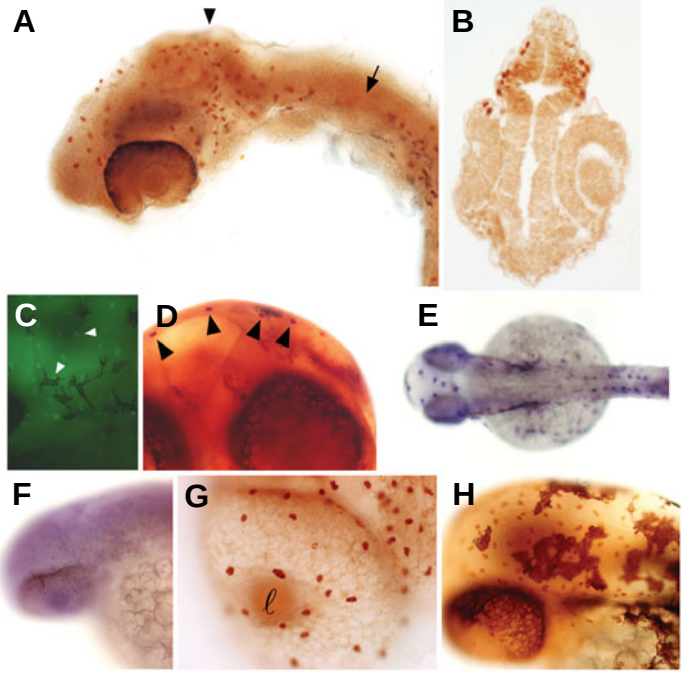
ance of the reflective platelets of the iridophores. Therefore, as in the trunk, the paired box protein Pax7 also identifies iridophore precursors in the head.

No NC cells occur at the level of the otic vesicle and, hence, Pax7-expressing cells were only located around the vesicle, within the second and third cranial NC streams (Fig. 2A).

Next, we examined whether Pax7 is also expressed by the pigment stem cells at metamorphosis. As previously reported (Mellgren and Johnson, 2006), an initial pigment pattern of scattered melanophores and xanthophores develops throughout the caudal fin at early-midlle metamorphosis (Fig. 3). Interestingly, we noted a distribution pattern of Pax7+ unpigmented precursors, mainly in the proximal area of the caudal (Fig. 3A,B,D,E) and anal (Fig. 3C) fins. In the caudal peduncle, we observed many Pax7+ cells adjacent to the hypurals (Fig. 3E). Together, these findings suggest a proximo-distal gradient of pigment cells development during caudal fin metamorphosis.

Here, we have observed a close association between Pax7 and the melanophore lineage that is similar to that noted between Pax7/Pax3 and the analogous melanocyte precursors of amniotes (Lacosta *et al.*, 2005; Lang *et al.*, 2005). In birds, however, Pax7 downregulation precedes the appearance of melanin pigment (Lacosta *et al.*, 2005), while in zebrafish it takes place after melanin deposition begins. Hence, there is a notable difference between Pax7 regulation and deposition of melanin pigment in both vertebrate pigmentation models. Together, these findings in zebrafish Pax7 open up new avenues to understand how different vertebrate pigmentation models orchestrate Pax3/Pax7 and other factors (see Hou *et al.*, 2006; Lang *et al.*, 2005) in controlling melanin formation at the proper time and place.

In contrast to melanophores, little is known of the mechanisms regulating the differentiation of the xanthophore and iridophore



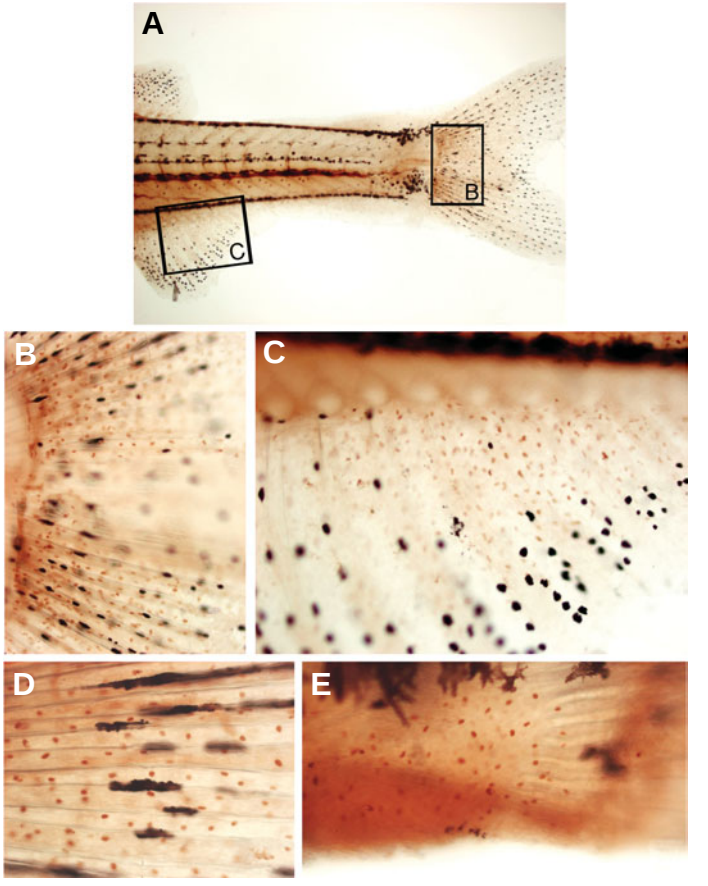


Fig. 2 (Left). Expression pattern of Pax7 in the developing head. (A) *Whole-mount of a 24 hpf embryo. Pax7 is expressed by head neural crest (NC) cells, except at its most rostral end and at the level of the otic vesicles (arrow). Note many Pax7-positive cells (arrowhead) at the mid-hindbrain.* **(B)** *Section through a 21 hpf embryo. Note that Pax7 positive NC cells of the first cranial stream are only generated caudal to the telencephalon.* **(C)** *Several dendritic melanophores and Pax7+ xanthophore precursors (arrowheads) showing close heterotypic interactions in the dorsal head of a stage 33 hpf embryo.* **(D)** *Whole-mount showing Pax7+ xanthophore precursors (arrowheads) and one melanophore at the rostral end of a stage 48 hpf embryo.* **(E)** *Typical distribution pattern of melanophores in the head is revealed by* dct in situ *RNA hybridisation. Stage 48 hpf. PTU treated embryo.* **(F)** *Broad distribution pattern of xanthophore precursors in the head is revealed by the xanthophore lineage marker* xdh. *Stage 48 hpf. PTU treated embryo.* **(G)** *Lateral view of a 48hpf embryo displaying Pax7+ iridophore precursors over the eye. Pax7 staining is also observed in adjacent xanthophore precursors. PTU treated embryo.* **(H)** *Pax3 immunolabeling reveals a similar spatio-temporal expression pattern in the three pigment cell precursors. Anterior to the left in (A) and (E-H). l, lens; PTU, 1-phenyl-2-thiourea.*

Fig. 3 (Right). Pax7 is expressed during pigment pattern metamorphosis. *Whole-mount staining for Pax7 of the caudal (A,B,D,E) and anal (C) fins.* **(A)** *A 7 mm stage showing many Pax7+ cells in the proximal end of the caudal and anal fins. Boxed areas are depicted in (B,C).* **(B)** *Detail showing numerous Pax7+ cells distributed throughout the proximal region of the caudal fin.* **(C)** *Detail of the anal fin. Most Pax7+ cells appear in the proximal area of the fin.* **(D)** *Detail of the caudal fin of a 10 mm larva. Numerous unpigmented Pax7+ cells are interspersed with mature pigment cells.* **(E)** *Higher magnification showing Pax7+ cells adjacent to the hypurals in the caudal peduncle.10 mm larva. Anterior to the left.*

lineages (Quigley and Parichy, 2002). Here we present evidence that Pax7 is a common transcription factor of the zebrafish cromathophore lineages. Of note, temporal control of Pax7 is closely related to the timing of differentiation of the three pigment cell precursors. Therefore, these findings are consistent with the tight association between downregulation of Pax genes and differentiation (Chen *et al.*, 2006).

In zebrafish, several studies have demonstrated that metamorphic and also regenerative pigment cells differentiate de novo from latent stem cells (Rawls *et al.*, 2001). It has been claimed the lack of molecular markers for pigment stem cells (Kelsh, 2004; Quigley and Parichy, 2002). Recent studies showed that Pax7 is a reliable skeletal muscle stem (satellite) cell marker across vertebrates (see Chen *et al.*, 2006 and references therein). Therefore, we evidence that the evolutionary conserved stem cell transcription factor Pax7 is also expressed in zebrafish pigment stem cells at metamorphic stages. Taken together, this study will provide the basis for future analysis of *Pax7* gene function during zebrafish development

Experimental Procedures

Zebrafish embryos and larvae were raised at 28.5°C and staged according to the number of hours or days post-fertilization (Kimmel *et al.*, 1995). Adult *albino* D. rerio were purchased at a local pet store. To reduce melanin formation, some embryos were treated with 0.003 % 1-Phenyl-2-thiourea (PTU, Sigma) in embryo medium beginning at 20-22 hpf. For whole-mount antibody staining, embryos or metamorphic larvae were anesthetized with MS222, fixed in 4% paraformaldehyde at 4°C for 5-24 h and incubated in primary antibodies overnight at 4°C. Embryos and larvae were incubated in horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Jackson Immuno Research) overnight at 4°C. To develop the peroxidase reaction product, embryos were incubated in 1.86 mg/ml DAB (Sigma) with 0.033% H_2O_2 in phosphate buffer. After the yolk cell was removed, some embryos were mounted on slides and photographed using an Olympus Microscope BX60. For sectioning, whole-mount Pax7 immunoreacted embryos were embedded in paraffin. Sections (10 μm) were mounted on slides and photographed as above.

For fluorescent detection, we used a biotin-conjugated anti-mouse antibody (1/200, Sigma) followed by AlexaFluor 488-conjugated Streptavidin (1/200, Molecular Probes) and viewed with epifluorescence optics.

We obtained the anti-Pax7 (used undiluted) (Kawakami, A.) and Zn-12/HNK-1 (1/20) (Trevarrow, B.) supernatant mAbs from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the NICHD and is maintained by the Department of Biological Sciences, University of Iowa. Pax3 (1/100) polyclonal Ab was a generous gift from Dr. Gerard C. Grosveld.

Whole-mount RNA *in situ* hybridization was performed essentially by the method of Thisse *et al.* (1993). Probes containing digoxigenin (Roche Biochemicals) were prepared for *crestin*, *Pax3* (Seo *et al.*, 1998), *dct* (Kelsh *et al.*, 2000), *fms*, *gch* and *xdh* (Parichy *et al.*, 2000). Detection of labeled antisense probes was performed by using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) and with BM Purple AP Substrate (Roche).

Acknowledgements

We are very grateful to Dr. E.M. González for helpful comments; Dr. M. Halpern for crestin, Dr. A. Fjose and Dr. H.C. Seo for Pax3 and Dr. R. Cornell for dct, xdh, gch and fms probes. We thank Dr. A. Kawakami for useful comments on Pax7 antibody; R. Puyo, S, Serrano and V. Melgarejo for technical assistance; and Dr. G.C. Grosveld for Pax3 antibody. We also thank Mr. K. Dobbie for the kind correction of the English manuscript and Dr. M.V. Robinson and Dr. B. McWhirter for editing a previous draft. This work was supported by grants from the Spanish Ministry of Science and Technology (PM99-0086) and from the Government of Aragón (PO68/2000).

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Received: 5th September 2006
Reviewed by Referees: 3rd October 2006
Modified by Authors and Accepted for Publication: 15th December 2006
Published Online: 16th May 2007

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