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Developmental expression of *Pitx2c* in *Xenopus* trigeminal and profundal placodes

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ABSTRACT Cranial placodes are thickenings of the embryonic head ectoderm that contribute to the paired sense organs and to the cephalic peripheral nervous system. Here we report the spatio-temporal expression pattern of transcription factor *Pitx2c* during *Xenopus laevis* cranial placode formation, focusing more specifically on key stages of trigeminal and profundal placode development. We also compare its expression to five genes that have been associated with development of these sensory placodes, namely *Foxi1c, Islet1, NeuroD, Pax3,* and *Six1*. We show that while initially expressed in both the trigeminal and profundal placodes, *Pitx2c* is later restricted to the prospective profundal ganglion, where it is co-expressed with *Islet1, NeuroD* and *Pax3*. This combination of factors defines a molecular signature for the characterization of the profundal versus trigeminal ganglia in *Xenopus*.

KEY WORDS: *cranial placode, trigeminal, profundal, Pitx2c, Xenopus*

The cranial placodes are localized ectodermal thickenings in the head of vertebrate embryos that contribute to the specialized paired sense organs and sensory cranial ganglia. All placode progenitors arise from a common precursor field that borders the anterior neural plate known as the pre-placodal region or PPR (Schlosser, 2010; Grocott *et al*., 2012; Saint-Jeannet and Moody, 2014). The PPR is subsequently divided along the anterior-posterior axis into distinct domains in which cells will adopt fate characteristic for each sensory placode. The adenohypophyseal, olfactory and lens placodes arise from the anterior PPR, and the otic and epibranchial placodes from the posterior PPR, with the trigeminal placodes forming in between (Schlosser, 2010; Grocott *et al*., 2012; Saint-Jeannet and Moody, 2014).

Molecularly, the trigeminal placodes can be subdivided into two domains: the ophthalmic and maxillomandibular placodes, which are referred as profundal and trigeminal placodes in anamniotes. In most organisms, the neuroblasts delaminating from these placodes eventually coalesce into a single ganglion, and together with the neural crest cells give rise to the trigeminal ganglion complex of cranial nerve V, still this ganglion retains an ophthalmic and maxillomandibular subdivision. In *Xenopus*, the ganglia derived from the profundal and the trigeminal placodes are fused at their proximal ends but remain separated distally (Schlosser and Northcutt, 2000). The neurons of the trigeminal ganglia extend axons peripherally underneath the skin of the head, to detect mechanical, chemical, and thermal stimuli, and axons centrally to communicate these inputs to the brain (Baker and Bronner-Fraser, 2001).

Members of the Pitx family of homeobox transcription factors have been implicated in the regulation of many aspects of vertebrate development (Gage *et al*., 1999). In *Xenopus Pitx2c* is asymmetrically expressed in the lateral plate mesoderm and regulates proper looping of the heart and gut tubes (Ryan *et al*., 1998; Campione *et al*., 1999). *Pitx2c* is also expressed in several derivatives of the ectoderm (Schweickert *et al*., 2001). Here we describe the expression pattern of *Pitx2c* during profundal and trigeminal placodes development and compare its expression to other genes that have been associated with the development of these sensory placodes.

Results and Discussion

We analyzed by *in situ* hybridization the developmental expression of *Pitx2c* during cranial placode development, from neural plate (stages 14 and 17) through tail bud (stages 21-35) stages, and compared its expression to five genes (*Foxi1c, Islet1, NeuroD,*

Abbreviations used in this paper: PPR, pre-placodal region.

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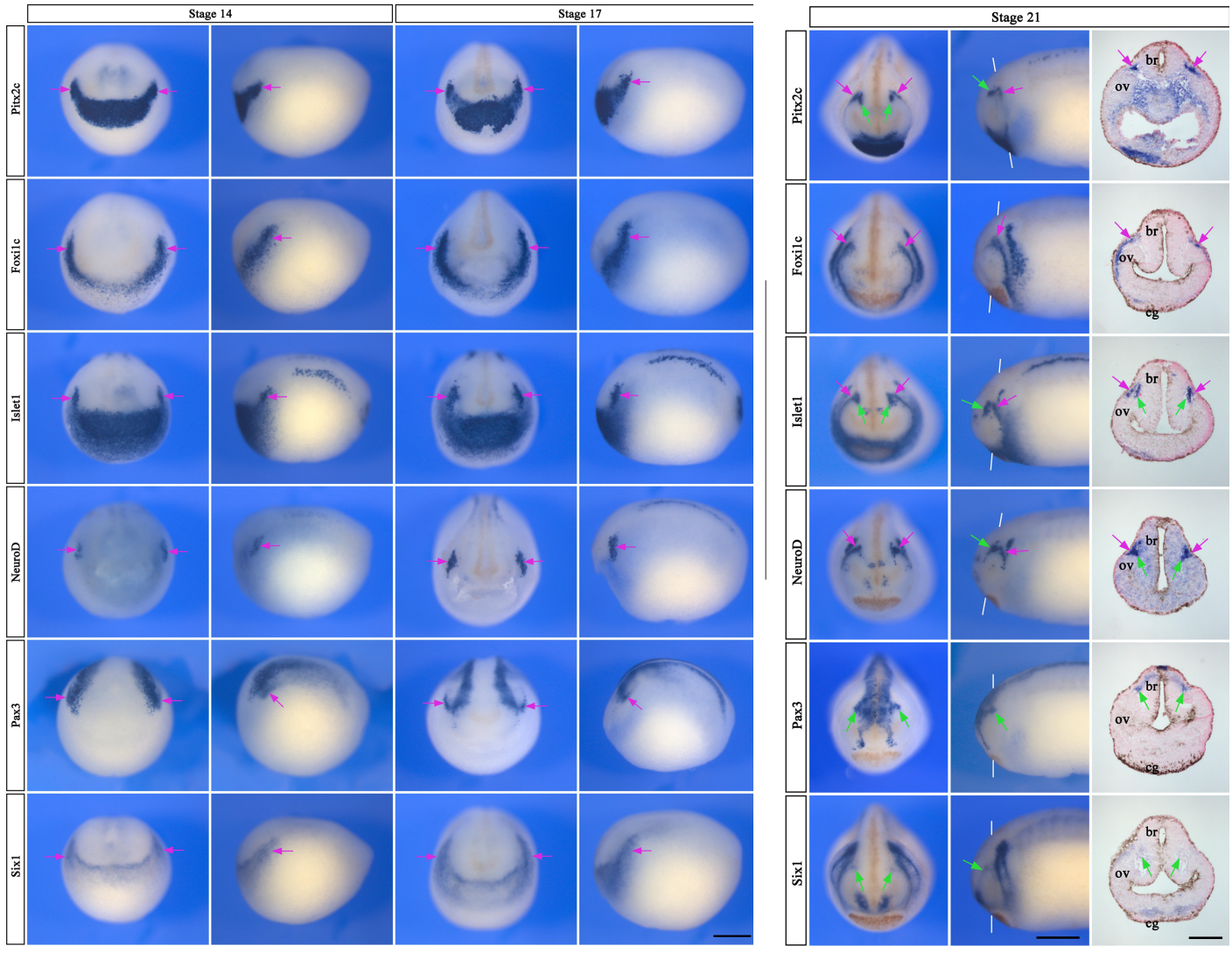

Fig. 1 (left). Whole-mount *in situ* hybridization of six placodal genes encoding transcription factors expressed at stage 14 (early neurula) and stage 17 (mid-neurula). *The position of the prospective trigeminal placode is indicated (magenta arrows). For each stage, left panels are frontal views, dorsal to top, and right panels are lateral views, anterior to left, dorsal to top. Scale bar, 500 μm.*

Fig. 2 (right). *In situ* hybridization of six placodal genes expressed at stage 21 (early tailbud). *Prospective trigeminal (magenta arrows) and profundal (green arrows) placodes are indicated. Left panels are frontal views, dorsal to top, and middle panels are lateral views, anterior to left, dorsal to top. Transverse sections (right panels) were performed at the level of the optic vesicles. A white line on each side of the embryo indicates the plane of section (middle panels). br, brain; cg, cement gland; ov, optic vesicle. Scale bar for whole embryos is 500 μm, and for histological sections is 200 μm.*

Pax3 and *Six1)* that have been associated with profundal and trigeminal placode development (Schlosser and Ahrens, 2004; Park and Saint-Jeannet, 2010).

At early neurula stage (stage 14; Fig 1), cranial placode progenitors originate from a narrow band of ectoderm anterior to the neural plate, the PPR. *Pitx2c* is expressed at the PPR, together with a few other transcription factors, including *Foxi1c*, *Six1* and *Islet1,* however *Pitx2c* is also more broadly expressed, extending ventrally to include the prospective cement gland, in a pattern very similar to that of *Islet1*. Interestingly, the posterior limit of *Pitx2c* and *Islet1* expression at the PPR does not extend as far posteriorly as *Foxi1c* and *Six1*, two genes that encompasses the entire PPR (Pandur and Moody, 2000; Schlosser and Arhrens, 2004). At this stage *Pax3* and *NeuroD* are confined to a subdomain of the PPR. *Pax3* is also detected in progenitors of the neural crest and hatching gland, which occupy a domain medial to the PPR (Hong and Saint-Jeannet, 2007). At mid-neurula stage (stage 17; Fig 1) *Pitx2c, Foxi1c*, *Six1* and *Islet1* are still broadly expressed at the PPR. The most posterior expression domain of *Islet1* is now more distinct, in a pattern similar to *NeuroD,* marking both the prospective profundal and trigeminal placodes. *Pax3* expression domain on the other hand appears more restricted to a subdomain of the placodal region expressing *Islet1* and *NeuroD,* which presumably correspond to the profundal placode.

Cranial placodes become visible as individual thickenings of the embryonic ectoderm around stage 21, the early tailbud stage

(Schlosser and Northcutt, 2000). At this stage, the trigeminal and profundal placodes can be seen as two separate entities, and the corresponding prospective ganglia can be traced based on their relationship to the optic vesicles. The profundal division of the trigeminal ganglion extends rostrally and dorsal to the optic vesicle, while the trigeminal branch extends ventrally along the posterior domain of the optic vesicle. *Pitx2c* is detected in both the trigeminal and profundal placodes, and appears to be more strongly expressed in the latter (Fig 2). *Islet1 and NeuroD* are also expressed in both placodes with variable intensity. *Foxi1c* is uniquely detected in the trigeminal placode, while *Pax3* and *Six1* are restricted to the profundal placode (Fig 2). At this stage *Pitx2c* is detected in the adenohypophyseal placode, as previously reported

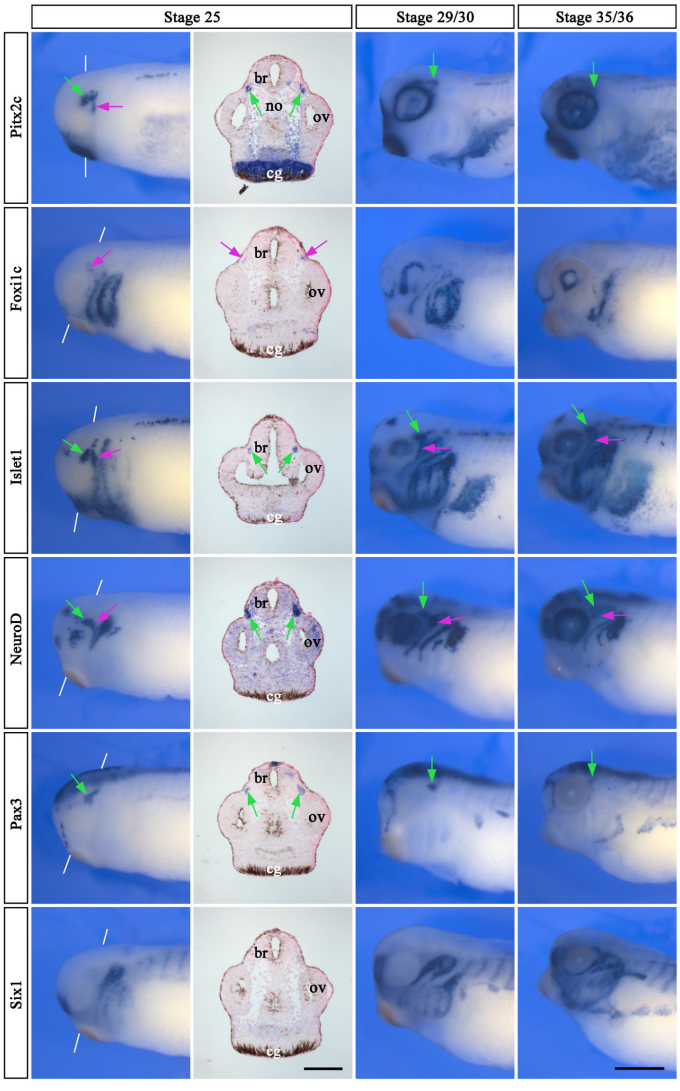

Fig. 3. *in situ* hybridization of six placodal genes expressed at the tailbud stages. *Trigeminal (magenta arrows) and profundal (green arrows) ganglia are indicated. For Whole-mount* in situ *hybridization, lateral views, anterior to left, dorsal to top. Transverse sections (stage 25) were performed at the level of the optic vesicles. A white line on each side of the embryo indicates the plane of section (left panels). br, brain; cg, cement gland; ov, optic vesicle. Scale bar for whole embryos is 500 μm, and for histological sections is 200 μm.*

TABLE 1

SUMMARY OF THE SPATIOTEMPORAL EXPRESSION OF SIX GENES IN THE TRIGEMINAL AND PROFUNDAL PLACODES AND GANGLIA

		Pitx2c	Foxi1c	Islet1	NeuroD	Pax3	Six1
St. 14	PPR	+	+	+	+	+	+
St. 17	PPR	+	+	+	+	+	+
St. 21	Profundal	+	–	+	+	+	+
	Trigeminal	+	+	+	+	–	–
St. 25	Profundal	+	–	+	+	+	–
	Trigeminal	+	+	+	+	–	–
St. 29/30	Profundal	+	–	+	+	+	–
	Trigeminal	–	–	+	+	–	–
St. 35/36	Profundal	+	–	+	+	+	–
	Trigeminal	–	–	+	+	–	–

"+" indicates gene expression, "–" indicates that the gene was not detected.

(Schweickert *et al.*, 2001).

At stage 25, *Pitx2c* expression is maintained in both the profundal and trigeminal ganglia, however by stage 29/30, *Pitx2c* is no longer expressed in the trigeminal ganglion (Fig 3). With the exception of *Six1* (stage 25) and *Foxi1c* (stage 29/30), which progressively become undetectable in their respective placodal domain, the other genes maintain their expression in the profundal (*Pax3*) and in the trigeminal and profundal (*Islet1* and *NeuroD*) ganglia throughout the tailbud stages (Fig 3). At the late tailbud stage (stage 35) the profundal ganglia can be visualized by the expression of *Pitx2c*, *Pax3*, *Islet1* and *NeuroD* while the trigeminal ganglia expresses both *Islet1* and *NeuroD.*

Here we described the expression of *Pitx2c* during cranial placode development. Our comparative analysis highlights a differential combinatorial expression of transcription factors in the profundal and trigeminal placodes and their derived ganglia (Fig 4; Table 1) suggesting that the formation of each placodal domain is independently regulated. In all vertebrates, including the lamprey, the profundal placode is characterized by *Pax3* expression (Modrell *et al.*, 2014). Moreover Pax3 is necessary for neurogenesis in the ophthalmic trigeminal placode in chicken (Dude *et al.*, 2009). We show that amongst the placodal genes analyzed, *Pitx2c* is only transiently expressed in the trigeminal placode, however like *Pax3* it is maintained in the profundal placode and its derived ganglion, suggesting an important role in the development of this structure.

Materials and Methods

Isolation of NeuroD and Pitx2c

Xenopus Pitx2c and *NeuroD* were amplified by PCR using specific primers for *Pitx2c* (F: ATCGATGCCACCATGAACTCTATGAAAGAGCC and R: CTCGAGCACGGGTCTGTTTA) and *NeuroD* (F: ATGACCAAATCGTATGGAGA and R: TTAATCATGAAAGATGGCAT) based on the published sequence of *Xenopus Pitx2c* (Ryan *et al.*, 1998; Campione *et al.*, 1999) and *NeuroD* (Lee *et al.*, 1995). The PCR products for Pitx2c (981 bp) and NeuroD (1057 bp) were ligated into pGEMT-easy and pGEMT (Promega), respectively, and sequenced.

In situ *hybridization*

Embryos were staged according to Nieuwkoop and Faber (1967). For whole-mount *in situ* hybridization, embryos were fixed with MEMFA and processed as previously described (Harland, 1991). For *in situ* hybridization on sections, after fixation in 4% paraformaldehyde solution in 1X PBS (pH 7.4) embryos were embedded in Paraplast+ and sectioned (12 μm)

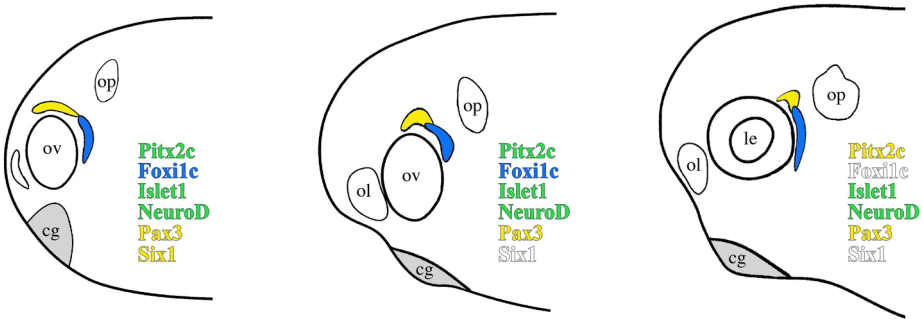

Fig. 4. Diagram summarizing the developmental expression of six placodal genes at the tailbud stages. *The position of the profundal placode/ganglion (yellow) and trigeminal placode/ganglion (blue) are indicated at stage 21 (placodes) and stage 25 and 29/30 (ganglia). Based on their expression in the profundal placode/ganglion, the trigeminal placode/ganglion or both, each gene name is highlighted in yellow, blue or green, respectively. White indicates no expression. cg, cement gland; le, lens; ol, olfactory placode; ov, optic vesicle; op, otic placode. The diagram of the embryos is modified from Schlosser and Northcutt (2000).*

on a Leica rotary microtome. The sections were hybridized according to the procedure described by Lemaire and Gurdon (1994) and briefly counterstained with Eosin. Antisense DIG-labeled probes (Genius Kit, Roche) were synthesized using template cDNA encoding *Pitx2c*, *NeuroD*, *Foxi1c* (Pohl and Knöchel, 2005), *Islet1* (Brade *et al.*, 2007), *Pax3* (Bang *et al.*, 1997), and *Six1* (Pandur and Moody, 2000).

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