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 adenoehypophyseal,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, Pax3, and Six1).
Pax3 and Six1) that have been associated with profoundal 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 Ahrens, 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 profoundal 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 profoundal placode.

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

**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 profoundal (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.
At this stage, the trigeminal and profound 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 profound 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 profound 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 profound placode (Fig 2). At this stage Pitx2c is detected in the adenohypophyseal placode, as previously reported (Schlosser and Northcutt, 2000).

At stage 25, Pitx2c expression is maintained in both the profound 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 profound (Pax3) and in the trigeminal and profound (Islet1 and NeuroD) ganglia throughout the tailbud stages (Fig 3). At the late tailbud stage (stage 35) the profound 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 profound 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 profound placode is characterized by differential combinatorial expression of transcription factors in the placode development. Our comparative analysis highlights a difference in the expression of Islet1 and NeuroD.

### Materials and Methods

**Isolation of NeuroD and Pitx2c**

Xenopus Pitx2c and NeuroD were amplified by PCR using specific primers for Pitx2c (F: ATCGATGCCACCATGACTCAATGAGAGGCC and R: CTGAGGACGCTTCTCGTTTTA) and NeuroD (F: ATGACCAATCGTTGAGAAGAAGCAT and R: TTAATCGTAAAAGATGGCAT) 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. 3. *in situ* hybridization of six placodal genes expressed at the tailbud stages. Trigeminal (magenta arrows) and profound (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.](image-url)
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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