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Formation of posterior cranial placode derivatives requires the *Iroquois* transcription factor *irx4a*

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ABSTRACT

Members of the *Iroquois* (*Irx*) homeodomain transcription factor gene family have been implicated in a variety of early developmental processes, including neural pre-patterning, tissue differentiation, neural crest development and cranial placode formation. Here, we report that, in zebrafish, the *irx4a* gene participates in specification of a number of placode derivatives that arise from the posterior placodal field. Specifically, differentiation of the trigeminal, epibranchial and lateral line placodes are affected when *irx4a* function is interrupted using antisense morpholino oligonucleotides. We show that both in the trigeminal ganglion and in the lateral line, *irx4a* is involved in controlling the number of sensory cells that develop. Other phenotypes observed in morphant embryos include misspecification of the heart chambers and failure of retinal ganglion and photoreceptor cell differentiation, functions described previously for *Irx4* in other species. We also provide evidence that *irx4a* regulates the expression of the *sox2* gene, both in the neural plate and in progenitor cells of the lateral line system. Our results point to *irx4a* as a critical gene for numerous developmental processes and highlight its role in the formation of placodal derivatives in vertebrates.

Introduction

Ectodermal placodes consist of epithelial thickenings that form at characteristic positions anterior and lateral to the head of vertebrate embryos (reviewed in Schlosser, 2005, 2006). They are essential for the formation of the sensory nervous system and their derivatives include ciliated sensory receptors, sensory neurons, neuroendocrine and endocrine cells, the lens, glia and other supporting cells. Placodal cells give rise to the majority of neurons that form the cranial sensory nervous system and contribute to form the cranial ganglia and the specialized sense organs associated with hearing, balance, olfaction and vision. In fish and aquatic amphibians, the trigeminal, lateral line (anterior and posterior), otic and epibranchial placodes become distinct after neural tube closure. The latter three placodes derive from a single ectodermal domain termed the posterior placodal area (Schlosser and Ahrens, 2004). Signaling pathways that have been implicated in induction of these placodes are BMPs, Wnts, FGF, and retinoic acid (Garcia-Castro et al., 2002; Monsoro-Burq et al., 2003; Brugmann et al., 2004). It remains unclear how these signaling pathways are able to activate distinct developmental programs that specify each of the sensory systems. One family of transcription factors that has been linked to maintenance of the posterior placodal field and promotion of the neurogenic fate in specific placodes are the *Iroquois* (*Irx*) genes. Expression analysis in *Xenopus* has shown that the genes *Xiro1*, *Xiro2* and *Xiro3* are expressed in the trigeminal, lateral line, otic and epibranchial domains, both before and after these placodes become distinct (Gómez-Skarmeta et al., 1998; Bellefroid et al., 1998; Glavic et al., 2004; Schlosser and Ahrens, 2004). Inhibition of *Xiro1* activity by dominant negative variants induced at the late gastrula stage produces inhibition of general pre-placodal markers such as *six1*, as well as in the expression of specific placode markers such as *sox2* and *pax2*, without affecting neural plate genes (Glavic et al., 2004). In a similar way, in zebrafish, knockdown of *Irx* function revealed an essential role for this family of genes in the determination of neurons in the trigeminal placodes (Itoh et al., 2002). Expression of several *Irx* family members has been described in posterior placodes or their derived ganglia in zebrafish, including *irx1a*, *irx1b*, *irx2a*, *irx4a*, *irx4b*, *irx5b* and *irx7* (Lecaudey et al., 2005). It has thus been suggested that *Irx* genes, perhaps together with genes of the *sox1b* family, define a posterior equivalence group of placodes (Schossler, 2006).

The *Irx* genes encode a family of Homeoproteins, conserved during evolution, showing parallel functions between invertebrates and vertebrates. During early development, these genes define the identity of large territories and later they have a role in the subdivision of these territories into sub-domains (Cavodeassi et al., 2001; Gómez-Skarmeta and Modolell, 2002). There are three *Iroquois* genes in *Drosophila* (Gómez-Skarmeta and Modelell, 1996), six in the mouse

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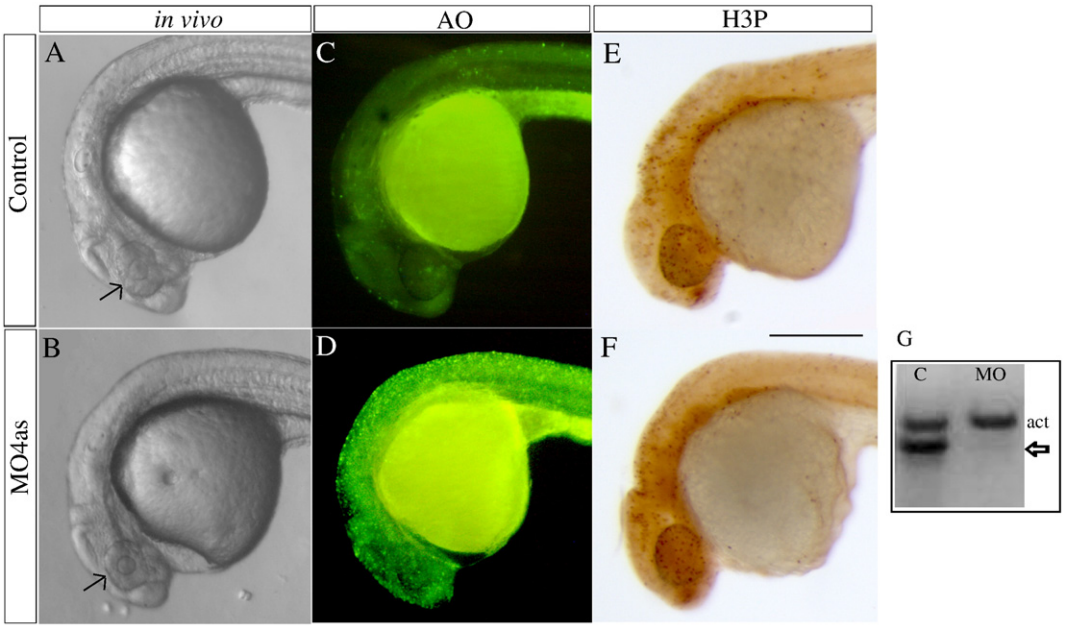

Fig. 1. *irx4a* morphant embryos show defects in head size and extensive cell death. (A, B) Images of live wild type (A) and morphant (B) embryos at 24 hpf; Compare the size of the eyes and head (arrows). (C, D) Acridine orange staining of wild type (C) and *irx4a* morphant (D) embryos viewed at 24 hpf. Note the increased number of labeled cells in the morphant embryo (E, F) MO4as injected embryos do not show substantial changes in cell proliferation as evidenced by anti-phospho-histone H3 (H3-P) immunostaining. (G) RT-PCR showing the effect of the MO4as on *irx4a* transcript splicing. RNA prepared from uninjected embryos ("C") shows the internal control band (actin, act) but the absence of unprocessed irx4a mRNA. The presence of this band in MO4as injected fish (arrow) indicates defective *irx4a* mRNA splicing. Panels A–F show lateral views of 24 hpf embryos; dorsal is up, anterior is left. Scale bar in F: 70 μm.

(Bosse et al., 2000; Peters et al., 2000) and eleven in zebrafish (Feijóo et al., 2004; Dildrop and Rüther, 2004). In the genome, the *Iroquois* genes are arranged in clusters, the *Iro* complex (Iro-C) in *Drosophila* and the *Irx* complexes in vertebrates (Gómez-Skarmeta and Modolell, 1996; Peters et al; 2000; Dildrop and Rüther, 2004; Feijóo et al., 2004). Interestingly, the expression patterns of genes within a cluster are highly similar, suggestive of conserved regulatory sequences in the surrounding genomic region (Gómez-Skarmeta and Modelell, 1996, Lecaudey et al., 2005; de la Calle-Mustienes et al., 2005). Moreover, the orthologs of different *Irx* genes among different vertebrates show equivalent expression patterns in several tissues.

In the present study, we have investigated the function of the zebrafish *Irx* family member, *irx4a*. We focus on the role of this gene in specification and neurogenesis during development of the hindbrain

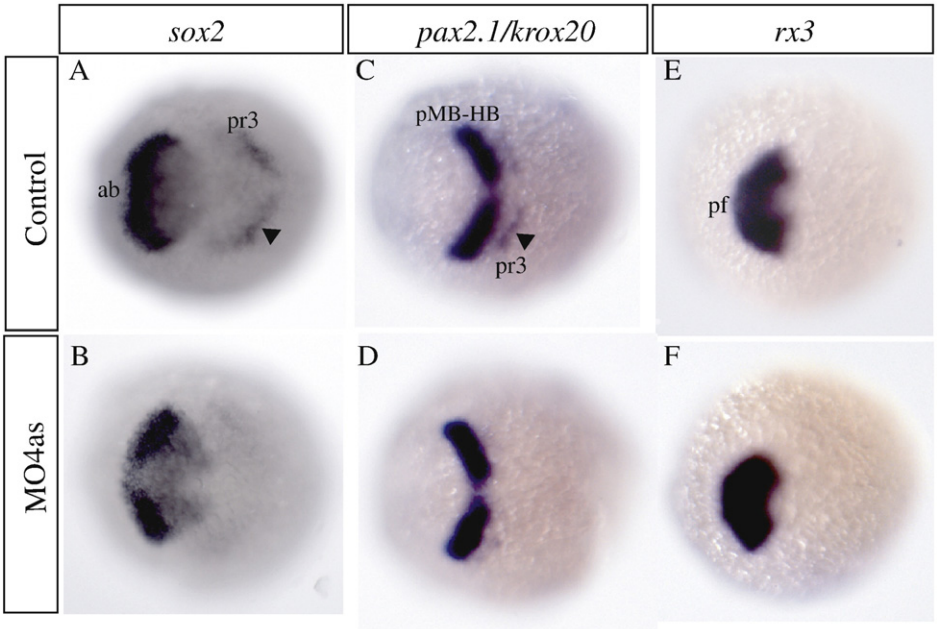

Fig. 2. *irx4a* regulates sox2 expression in the neural plate. *In situ* hybridization of brain markers was carried out on control and *irx4a* morphant embryos at 90% epiboly. (A, B) The neural precursor marker *sox2* appears diminished in the anterior brain (ab) and hindbrain (presumptive rhombomere 3, arrowhead, pr3) in *irx4a* morphant embryos (B) compared to uninjected controls (A). (C, D) The expression pattern of *pax2.1* in the midbrain–hindbrain boundary (pMB-HB) appears unaffected but the expression of the rhombomere 3 marker *krox20* (arrowhead) is diminished in morphant embryos. (E, F) *rx3* expression in the presumptive forebrain (pf) is unchanged in morphant compared to control embryos. All views are dorsal, anterior is towards the left.