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Foxi transcription factors promote pharyngeal arch development by regulating formation of FGF signaling centers

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ABSTRACT

The bones of the vertebrate face develop from transient embryonic branchial arches that are populated by cranial neural crest cells. We have characterized a mouse mutant for the Forkhead family transcription factor *Foxi3*, which is expressed in branchial ectoderm and endoderm. *Foxi3* mutant mice are not viable and display severe branchial arch-derived facial skeleton defects, including absence of all but the most distal tip of the mandible and complete absence of the inner, middle and external ear structures. Although cranial neural crest cells of *Foxi3* mutants are able to migrate, populate the branchial arches, and display some elements of correct proximo-distal patterning, they succumb to apoptosis from embryonic day 9.75 onwards. We show this cell death correlates with a delay in expression of *Fgf8* in branchial arch ectoderm and a failure of neural crest cells in the arches to express FGF-responsive genes. Zebrafish *foxi1* is also expressed in branchial arch ectoderm and endoderm, and morpholino knock-down of *foxi1* also causes apoptosis of neural crest in the branchial arches. We show that heat shock induction of *fgf3* in zebrafish arch tissue can rescue cell death in *foxi1* morphants. Our results suggest that *Foxi3* may play a role in the establishment of signaling centers in the branchial arches that are required for neural crest survival, patterning and the subsequent development of branchial arch derivatives.

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Introduction

Skeletal elements of the middle ear and jaw are derived from the most anterior of five pairs of branchial arches (BA) that develop on the ventral side of the embryo at the level of the hindbrain (reviewed in Noden and Trainor, 2005; Szabo-Rogers et al., 2010). Initially, each arch comprises an ectodermal surface, a core of mesoderm, and an endodermal lining. As arch outgrowth progresses, cranial neural crest cells migrate laterally and ventrally from the hindbrain and posterior midbrain to populate the arch mesenchyme, eventually forming the bulk of the mesenchyme (Lumsden et al., 1991; Schilling and Kimmel, 1994; Serbedzija et al., 1992). Bones and cartilage of the lower face are mainly derived from these neural crest cells, whose descendants form the mandible, maxilla, tympanic ring, and bones of the middle ear, among other structures. Perturbations in proliferation, migration, patterning, and differentiation of cranial neural crest cells can therefore lead to malformations of craniofacial skeletal structures (Passos-Bueno et al., 2009; Szabo-Rogers et al., 2010).

As the branchial arches expand ventrally, their anterior and posterior borders are defined by a series of contact points between pharyngeal ectoderm and underlying pharyngeal endoderm, which form morphological barriers called pharyngeal pouches. The pouches isolate populations of mesenchyme in each arch, and are also points of communication between ectoderm and endoderm (Couly et al., 2002). They are sources of signaling molecules that promote neural crest cell survival and contribute to the

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establishment of proximal–distal and anterior–posterior axes within each arch (Brito et al., 2006; Couly et al., 2002; David et al., 2002).

We have previously identified a transcription factor, Forkhead Box i3 (*Foxi3*), that is expressed in the pharyngeal region of mouse embryos in a segmented pattern between the branchial arches (Ohyama and Groves, 2004). *Foxi3* is one of three Foxi transcription factors present in the mouse genome, all of which are closely related to the zebrafish *foxi1* transcription factor. Mouse *Foxi1* expression is limited to the dorsal otic vesicle, and *Foxi1* mutant mice exhibit only balance defects (Hulander et al., 1998, 2003). However, zebrafish *foxi1* is expressed in the pharyngeal epithelium during arch development (Solomon et al., 2003b). A zebrafish *foxi1* mutant, *hearsay*, lacks an otic vesicle and presents severe defects in skeletal structures of the face that develop from the anterior arches (Nissen et al., 2003; Solomon et al., 2003a). We have generated a mouse *Foxi3* mutant, and found a facial skeleton phenotype that is similar to zebrafish *hearsay* mutants. *Foxi3* mutants lack much of the lower jaw and other branchial arch derivatives, such as the entire middle and external ear apparatus. Here, we characterize the mechanism underlying the branchial arch defects of *Foxi3* mutants. We show that cranial neural crest cells emigrate normally from the brain of *Foxi3* mutants, but then undergo apoptosis as they populate the branchial arches. Since neural crest cells do not express *Foxi3*, this suggests that *Foxi3* may regulate the expression of trophic or survival factors in arch ectoderm or endoderm. We show that the activity of *Foxi3* in pharyngeal epithelia is required for early expression of *Fgf8* in arch ectoderm. We also show a conservation of this pathway in zebrafish; here, *fgf3* is expressed in branchial arch ectoderm and requires the expression of *foxi1.* We show that ectopic expression of *fgf3* in pharyngeal ectoderm can reduce neural crest cell death in zebrafish *foxi1* morphants. We propose that *Foxi1 and 3* expression is required for normal pharyngeal pouch morphology in zebrafish and mouse respectively, that it establishes signaling centers in the developing branchial arches necessary for crest survival, and that the craniofacial phenotype seen in *Foxi3* mutants is due to reduced FGF8 signaling in the pharyngeal region.

Materials and methods

Generation of Foxi3 mutant mice

The targeting vector for the mouse Foxi3-floxed-neo allele was constructed using BAC recombineering (Warming et al., 2005). Briefly, an approximately 11 kb genomic DNA fragment containing exon 2 of mouse Foxi3 was retrieved from a BAC clone bMQ 285H11 of 129Sv BAC genomic library obtained from the Wellcome Trust Sanger Institute (Adams et al., 2005) Using recombineering, a loxP site was inserted upstream of exon 2, and an Frt–PGKNeo–Frt–LoxP sequence as inserted downstream of exon 2 (Fig. 2A) (Meyers et al., 1998). Electroporation of the targeting vector into ES cells, screening of the targeted ES cells and blastocyst injection were performed by the transgenic core facility at the Norris Cancer Center of the University of Southern California. Germline Foxi3-floxed-neo founder mice were identified and confirmed by genomic Southern blotting to detect the extra EcoRV and NheI sites introduced by the Frt–PGKNeo–Frt–LoxP sequence (Fig. 2B). The Foxi3-del allele used in this study was generated by crossing the Foxi3-floxed-neo allele with CMV-Cre line (JAX Mice, stock #003465).

Mouse genotyping

The *Foxi3* deletion allele (Foxi3-del) was maintained by breeding heterozygous mice. Primers used to genotype embryos were f3G1 (5′-GGC CTT GTC TCA ACC AAC AG-3′), f3G2 (5′-GTT TCC TGT ATC CCT GGC TG-3′) and f3G3 (5′-CTT GGA ATG GGT TGA CTG AG-3′). f3G1 and f3G2 produce a 350 bp band corresponding to the wild-type allele and f3G1 and f3G3 yield a 600 bp band corresponding to the Foxi3-del allele.

Whole mount DAPI imaging

Embryos were fixed, washed in PBS with 1% Triton X-100, incubated for 5 min in DAPI solution, and washed in PBS with 0.1% Tween-20. Embryos were mounted in PBS in a depression slide and photographed on a confocal microscope using the methods described by Sandell et al. (2012).

Skeletal staining

Mouse embryos were deeply anesthetized in PBS on ice and decapitated. Heads were scalded in 70 °C water and skin was removed. Skulls were stained with Alizarin Red and Alcian Blue as described (Ovchinnikov, 2009). Briefly, skin was removed from the embryos and fixed in 95% ethanol, the heads were defatted overnight in acetone, and cartilage was stained overnight in 0.015% Alcian Blue in 80% ethanol:20% acetic acid. The heads were rinsed in 70% ethanol, cleared in 2% potassium hydroxide and counter-stained with 0.005% Alizarin Red in 1% potassium hydroxide. After staining, the embryos were cleared in 1% potassium hydroxide followed by stabilization and storage in a 1:1 solution of glycerol and ethanol.

Probe synthesis and in situ hybridization

Digoxygenin-labeled, cRNA probes were synthesized for whole mount in situ hybridization as described (Stern, 1998) using plasmid clones from the following sources: *Dlx2* (John Rubenstein), *Dlx5* (Jin Xian Liu), *Sox10* (Michael Wegner), *Erm* (Annette Neubuser), *Fgf8* and *Spry2* (Gail Martin), *Dlx3* (Maria Morasso), *Pax9* (Rudi Balling), *Gsc* (Richard Behringer), *Lhx7* (Maria Grigoriou), and *Pitx1* (Dan Bernard). The *Foxi3* probe was generated by our lab and previously described (Ohyama and Groves, 2004). Whole mount in situ hybridization was performed as recently described (Khatri and Groves, 2013). After in situ hybridization, stained embryos were equilibrated in 15% sucrose in PBS and embedded in 7.5% gelatin with 15% sucrose in PBS for sectioning.

Immunohistochemistry

Embryos were fixed and embedded in gelatin (7.5% gelatin and 15% sucrose in PBS). 14 μm thick sections were collected on Superfrost Plus slides. For cleaved caspase-3 detection, cleaved caspase-3 antibody (AF835, R&D Systems) was diluted 1:200 in blocking buffer (PBST with 0.1% Triton X-100 and 10% goat serum). Secondary antibody (AlexaFluor 488 goat anti-rabbit, Invitrogen) was diluted 1:1000 in blocking buffer. For AP2α detection, slides were boiled in 10 mM citric acid for 10 min prior to antibody application. The 3B5 AP2α monoclonal antibody developed by Trevor Williams was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242. AP2α antibody was diluted 1:100 in blocking buffer. Slides were incubated for 15 min at room temperature in 0.012% hydrogen peroxide prior to secondary antibody application. Staining was detected with biotinylated secondary antibody (Mouse Vectastain ABC kit) in conjunction with PerkinElmer TSA Plus Cyanine-3 System. All slides were mounted in Fluoromount G (Southern Biotech). For dephosphorylated-ERK, whole embryos were incubated with p44/p42 MAPK rabbit polyclonal (Cell Signaling #9101)

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