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barx1 is necessary for ectomesenchyme proliferation and osteochondroprogenitor condensation in the zebrafish pharyngeal arches

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ABSTRACT

Barx1 modulates cellular adhesion molecule expression and participates in specification of tooth-types, but little is understood of its role in patterning the pharyngeal arches. We examined *barx1* expression during zebrafish craniofacial development and performed a functional analysis using antisense morpholino oligonucleotides. *Barx1* is expressed in the rhombencephalic neural crest, the pharyngeal arches, the pectoral fin buds and the gut in contrast to its paralogue *barx2*, which is most prominently expressed in the arch epithelium. Additionally, *barx1* transient expression was observed in the posterior lateral line ganglia and developing trunk/tail. We show that Barx1 is necessary for proliferation of the arch osteochondrogenic progenitors, and that morphants exhibit diminished and dysmorphic arch cartilage elements due to reductions in chondrocyte differentiation and condensation. Attenuation of Barx1 results in lost arch expression of osteochondrogenic markers *col2a1*, *runx2a* and *chondromodulin*, as well as odontogenic marker *dlx2b*. Further, loss of *barx1* positively influenced *gdf5* and *chordin*, markers of jaw joint patterning. FGF signaling is required for maintaining *barx1* expression, and that ectopic BMP4 induces expression of *barx1* in the intermediate region of the second pharyngeal arch. Together, these results indicate an essential role for *barx1* at early stages of chondrogenesis within the developing zebrafish viscerocranium.

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Introduction

The viscerocranium consists of highly adapted skeletal elements derived from the embryonic pharyngeal arch ectomesenchyme that function in concert but allow for diverse pharyngolaryngeal activities. The viscerocranium is composed of membranous and endochondral bones. Endochondral bone formation within the pharyngeal arches is a multi-step process that requires the migration of the cranial neural crest into the facial prominences and their subsequent epitheliomesenchymal interactions; these interactions are necessary for fate determination, aggregation of the cells into discrete condensations, and their terminal differentiation (Hall and Miyake, 2000; Helms and Schneider, 2003; Goldring et al., 2006). Signaling factors that include Bone Morphogenic Proteins (BMPs) and Fibroblast Growth Factors (FGFs), through reciprocal epitheliomesenchymal interactions, influence the expression of downstream factors that pattern the chondrogenic elements (de Crombrugghe et al., 2000; Hall and Miyake, 2000; Tuan, 2004; Goldring et al., 2006). The precise mechanism of patterning osteochondrogenic progenitors is not well understood.

The Barx family of homeodomain-containing transcription factors participates in the formation of mesenchymal condensations through the modulation of cellular adhesion molecules (CAMs), and regulation of fibril-forming type II collagen (*Col2a1*) (Jones et al., 1997; Edelman et al., 2000; Meech et al., 2005). The regulation of CAMs and *Col2a1*, a major component of the cartilage extracellular matrix, by *Barx1* indicates a direct link between expression of a tissue-specific transcription factor and changes in cartilage morphology. In the mouse, *Barx1* expression is located at sites of mesenchymal condensation that include the pharyngeal arches, the limb buds, developing joints, molar tooth papillae and the stomach wall (Tissier-Seta et al., 1995; Jones et al., 1997; Barlow et al., 1999; Kim et al., 2005). Ectopic *Barx1* gene expression in mouse mandible cultures results in the alteration of incisor teeth to an unusual molariform shape (Tucker et al., 1998; Miletich et al., 2005), while attenuation of *Barx1* by RNA interference results in arrest of molar mesenchyme at the bud stage (Song et al., 2006). Manipulation of the closely related *Barx2* gene expression in limb bud cultures affects cellular aggregation and chondrocyte differentiation, indicating a familial role in patterning chondrocytes (Meech et al., 2005). The function of *Barx1* in craniofacial development has yet to be fully examined. In humans, rare duplications and deletions of the BARX1 locus result in craniofacial and joint anomalies, but no disease-causing mutations have been associated directly with this gene (Stalker et al., 1993; Gould and Walter, 2000;

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Gould and Walter, 2004; Redon et al., 2006). To explore the relationship of this gene with craniofacial development we characterized zebrafish *barx1* and the phenotype on the *barx1* morphant.

Here we show that the zebrafish *barx1* gene is expressed in the migrating cranial neural crest and subsequently in the developing cartilage elements of the pharyngeal arches. Transient expression is observed in the posterior lateral line ganglia and in the trunk and tail. In contrast to *barx1*, the *barx2* paralogue is expressed predominantly in the proximal aspects of the fin buds and in oral and aboral epithelium of the first and second pharyngeal arches. Attenuation of *barx1* expression using antisense morpholinos results in a loss of arch cartilage tissue and micrognathia without an increase in apoptosis, but with a reduction in Phosphohistone-H3 stained nuclei, indicating a role for *barx1* in cellular proliferation and chondrocyte differentiation. As FGF and BMP signaling are known to pattern arch development, we examined the influence of these factors on *barx1* expression. Examination of mutant embryos deficient in *fgf3* (*lia*) and *fgf8* (*ace*), as well as embryos treated with an FGF receptor antagonist (SU5402), show that FGF signaling is necessary for maintaining and patterning *barx1* expression in the arches. In contrast, ectopic BMP4 signaling induces misspecification of *barx1* patterning within the intermediate aspect of the second pharyngeal arch. This work provides insight into *barx1* function and patterning during prechondrogenic condensation events in the developing zebrafish viscerocranium.

Materials and methods

Animal maintenance and transgenic zebrafish

Zebrafish embryo lines, EK wild-type, *fli1:GFP* transgenics (Motoike et al., 2000), and the *fgf8/ace* mutant, were maintained according to Westerfield (1995). Fixed wild-type and sibling *fgf3/lia* embryos were a gift from the Hammerschmidt laboratory (Germany). Embryos and larvae were fixed in 4% paraformaldehyde/phosphate buffered saline and dehydrated in methanol for storage at −20 °C. FGF inhibition was performed by soaking 24 hour post-fertilization (hpf) embryos in 10 μm SU5402 (Pfizer) in 5% DMSO.

In vitro transcription of synthetic RNA

The zebrafish *barx1* (NM001024949) and the *barx2* predicted open reading frame (XM001342008) were amplified from cDNA produced by reverse transcription from 96 hpf RNA and cloned into pCRII-TOPO (Invitrogen). For capped sense mRNA, the *barx1* open reading frame was cloned into pCS2+ plasmid, digested with Not I, and transcribed with SP6 Polymerase. Capping was performed with 7′ methylguanosine GTP analogue cap (NEB).

Attenuation of barx1 expression

Morpholinos (MO) complementary to the translational start site of *barx1* (*Bx MO*) and the splice acceptor (*Sa MO*) of the second exon (Fig. 1A) were microinjected into one to two cell-stage embryos. Lissamine conjugated MOs (Gene-Tools) used are as follows: *Bx MO*, [5′ CCCCAATCTCCAAAGGATGTTGCAT3′], *Sa MO* [5′GCCTTCAGAACTGGAATG AAATAAG3′], and a standard control [5′CCTCTTACCTCAGTTA-CAATTTATA3′]. MOs were diluted in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes, pH 7.6) and 0.01% Phenol Red. Embryos were injected with approximately 1 nl of MO at a concentration of 4 ng/nl. For rescue, the MOs were co-injected with 25 ng/μl of *barx1* mRNA containing five base pair mismatches within the ATG MO target site. To test inhibition of translation, a *barx1:eGFP* fusion construct was injected together with the MO, and proteins extracted from 24 hpf embryos were blotted with anti-*gfp* (1/1000; Santa Cruz) and anti-α-*tubulin* (1/1000; CalBiochem) antibodies, and detected by a Dura Chemiluminescence Kit (Pierce). To test the effectiveness of the Sa MO, total RNA was extracted from 24 hpf morphant and control embryos, DNase I treated and column purified (Qiagen). RT-PCR, using random hexamer primers was performed on 1 μg of RNA for each sample (Superscript III, Invitrogen). Forward (5′-AATGCAACATCCTTTGGAGATT-3′) and reverse (5′-ATCCCGTTTATTCCTCTTGGTT-3′) primers were used to test for properly spliced *barx1* mRNA. β-*actin* amplification was used as a control.

Whole-mount in situ hybridization and histological characterization

The following antisense riboprobes were used: *chondromodulin* (AF322374) (Sachdev et al., 2001); *col2a1* (U23822) (Yan et al., 1995); *crestin* (AF195881) (Luo et al., 2001); *dlx2a* (NM131311) (Akimenko et al., 1994); *dlx2b* (NM131297) (Jackman et al., 2004); *crestin* (Rubinstein et al., 2000; Luo et al., 2001); *goosecoid* (*gsc*) (NM131017) (Stachel et

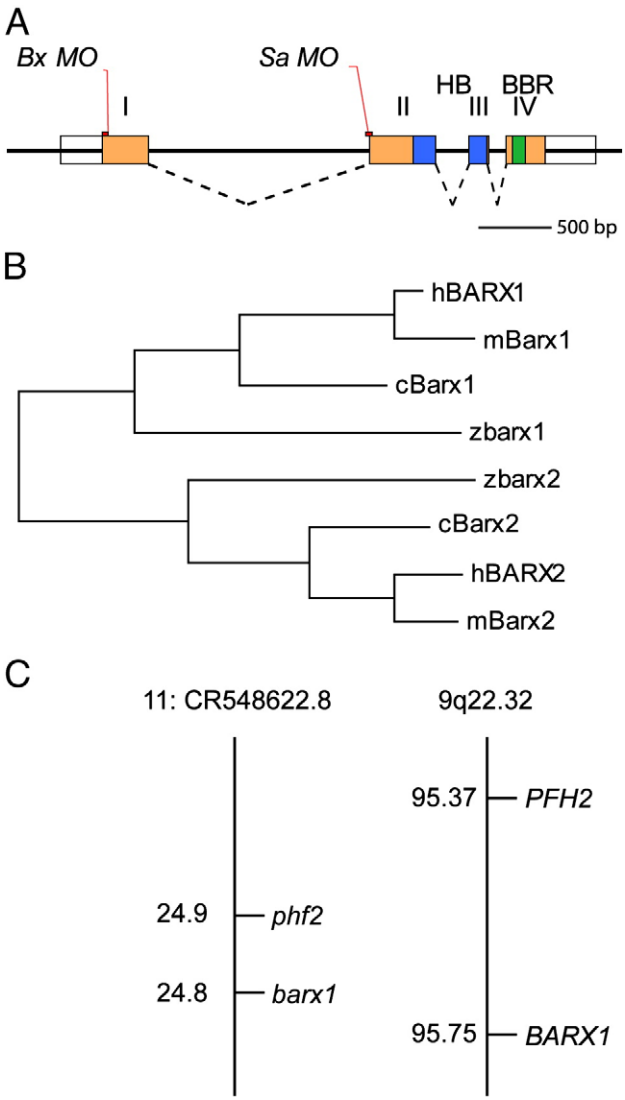

Fig. 1. Molecular analysis of zebrafish *barx1*. (A) The coding region of *barx1* consists of four exons (boxes, numbered with Roman numerals) containing the homeobox (HB, blue) and the Bar Basic Region (BBR, green); UTR's are indicated by outlined boxes. Target sites of the *barx1* ATG morpholino (*Bx MO*) and the splice-acceptor site morpholino (*Sa MO*) are indicated. (B) Phylogenetic tree comparing zebrafish (z) Barx1 with the human (h), mouse (m), and chicken (c) Barx amino acid sequences, as examined using ClustalW. GenBank accession nos.: (h1) NM021570, (m1) NM007526, (c1) NM204193, (z1) NM001024949, (h2) NM003658, (m2) NM013800, (c2) NM204896, (z2) XM001342008 respectively. (C) Syntenic relationship between zebrafish chromosome 11 fragment contig: CR548622.8 (Ensembl release 49, March 2008) and the human chromosome 9q22.32 (loci distances indicated in Mb). The *barx1* and *phf2*, PHD finger protein 2, are linked in zebrafish and humans.