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Alcama mediates Edn1 signaling during zebrafish cartilage morphogenesis

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ABSTRACT

The zebrafish pharyngeal cartilage is derived from the pharyngeal apparatus, a vertebrate-specific structure derived from all three germ layers. Developmental aberrations of the pharyngeal apparatus lead to birth defects such as Treacher-Collins and DiGeorge syndromes. While interactions between endoderm and neural crest (NC) are known to be important for cartilage formation, the full complement of molecular players involved and their roles remain to be elucidated. Activated leukocyte cell adhesion molecule a (*alcama*), a member of the immunoglobulin (Ig) superfamily, is among the prominent markers of pharyngeal pouch endoderm, but to date no role has been assigned to this adhesion molecule in the development of the pharyngeal apparatus. Here we show that *alcama* plays a crucial, non-autonomous role in pharyngeal endoderm during zebrafish cartilage morphogenesis. *alcama* knockdown leads to defects in NC differentiation, without affecting NC specification or migration. These defects are reminiscent of the phenotypes observed when Endothelin 1 (Edn1) signaling, a key regulator of cartilage development is disrupted. Using gene expression analysis and rescue experiments we show that Alcama functions downstream of Edn1 signaling to regulate NC differentiation and cartilage morphogenesis. In addition, we also identify a role for *neural adhesion molecule 1.1* (*nadl1.1*), a known interacting partner of Alcama expressed in neural crest, in NC differentiation. Our data shows that *nadl1.1* is required for *alcama* rescue of NC differentiation in *edn1*$^{-/-}$ mutants and that Alcama interacts with Nadl1.1 during chondrogenesis. Collectively our results support a model by which Alcama on the endoderm interacts with Nadl1.1 on NC to mediate Edn1 signaling and NC differentiation during chondrogenesis.

Introduction

Formation of the pharyngeal apparatus is a crucial part of vertebrate development because it gives rise to the cartilage, connective tissue, sensory neurons, thyroid, parathyroid and thymus. Defects in this process results in human birth defects such as DiGeorge and Treacher-Collins syndromes. Generation of this tissue is highly complex, involving extensive cell migration and signaling between cells derived from all three germ layers. NC cells migrate from the dorsal neural tube in three distinct streams into a series of pharyngeal arches and eventually give rise to cartilages and bones of the head. Each pharyngeal arch is composed of a cylinder of NC surrounding a mesodermal core. The NC is covered by ectoderm on the outside and endoderm on the inside. Between the arches, endoderm meets ectoderm forming the pharyngeal pouches, which later develop into gill clefts and the epithelial lining of the pharynx, thyroid, parathyroid and thymus (Graham, 2003).

Though NC cells carry intrinsic cues for patterning (Noden, 1983), they receive extrinsic cues from the surrounding cells and extracellular matrix as they migrate. Recently, the endoderm has been found to contribute significantly to NC development. Ablation and extirpation experiments in chicken have revealed that the endoderm carries patterning information for the NC in segments along the antero-posterior and medio-lateral axis (Couly et al., 2002; Ruhin et al., 2003). In addition, genetic mutants in zebrafish have also revealed the requirement of endoderm in formation of cartilage. The *sox32*-deficient *casanova* mutant lacks endodermal pouches and cartilages that are rescued by wild-type endodermal transplants (David et al., 2002). Likewise, the *tbx1*-deficient *van gogh* mutant fails to form segmented endodermal pouches resulting in fusion of the pharyngeal cartilages (Piotrowski and Nusslein-Volhard, 2000). Similarly, the zebrafish mutant for *integrinα5* lacks the first endodermal pouch and the anterior part of the hyoid cartilage (Crump et al., 2004). Although these data demonstrate that endoderm is essential for cartilage development, the cellular and molecular interactions between the NC and endodermal cells are not fully understood.

One major signaling factor that provides an extrinsic cue regulating NC differentiation is *endothelin-1* (*edn1*). *edn1* is expressed in the mesodermal cores, ectoderm and endoderm of the pharyngeal arches, but not in NC. Edn1 signals the NC and induces ventralization

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of pharyngeal arch cartilage (Miller et al., 2000). Mutations in *edn1*, Edn1 cleaving enzymes and other genes in the Edn1 signaling cascade (*sucker, schmerle, hoover* and *sturgeon*) cause similar cartilage defects, and have been placed in the same class (Kimmel et al., 2001; Piotrowski et al., 1996). Typically, the ventral domains of the first two arches are reduced in size, changed in orientation, and fused with the dorsal domains, while the posterior arches are mostly unaffected. Hence, Edn1 is an important signaling factor that is required non-autonomously for NC differentiation into cartilage.

Other molecular players that may signal from endoderm to NC remain elusive. While Alcama is commonly used as a marker for pharyngeal endoderm in zebrafish (Crump et al., 2004; Piotrowski and Nusslein-Volhard, 2000), its role in this tissue has not been elucidated thus far. In zebrafish, Alcama has been studied primarily for its role in neurogenesis (Diekmann and Stuermer, 2009; Fashena and Westerfield, 1999). Initially identified in chicken for its role in neurite extension (Burns et al., 1991), ALCAMA has now been shown to be involved in axonal pathfinding and axonal fasciculation (Diekmann and Stuermer, 2009; Weiner et al., 2004). Its non-neuronal roles include T-cell activation (Bowen et al., 2000; Fashena and Westerfield, 1999; Ofori-Acquah and King, 2008; Zimmerman et al., 2006), metastasis (Degen et al., 1998; Ofori-Acquah and King, 2008) and cell migration (Heffron and Golden, 2000). Human ALCAM is a transmembrane glycoprotein having five Ig domains, a transmembrane domain and a short cytoplasmic tail. It mediates cell-cell clustering through homophilic (ALCAM-ALCAM) as well as heterophilic (ALCAM-NgCAM and ALCAM-CD6) interactions (DeBernardo and Chang, 1996; Degen et al., 1998). While ALCAM can activate signal transduction pathways in neighbouring cells through heterophilic interactions (Ibanez et al., 2006), a non-autonomous role of ALCAM has not been defined thus far. In this paper we demonstrate that zebrafish Alcama, expressed in the pharyngeal endoderm, is an important non-autonomous molecule for NC differentiation. In addition, we provide evidence that Alcama mediates Edn1 signaling from the endoderm to the NC by interacting with Nadl1.1 (NgCAM in chicken) on the NC cells. These data for the first time link Alcama to Edn1 signaling and identify a role for the molecular interaction between Alcama and Nadl1.1 in cartilage formation.

Materials and methods

Fish stocks and maintenance

Fish were maintained at 28.5 °C under standard conditions (Westerfield, 2000) and were staged as described (Kimmel et al., 1995). The *sucker*^{tf216b} mutant (*edn1*$^{-/-}$) line was obtained from Zebrafish International Resource Center (Miller et al., 2000). Homozygous mutants were obtained by inbreeding of heterozygous carriers. *Tg(fli1:EGFP)* fish have been previously described (Lawson and Weinstein, 2002). Alcian Blue stained larvae of *furina*$^{-/-}$ mutants at 5 days post fertilization (dpf) were a kind gift from Chuck Kimmel (Institute of Neuroscience, University of Oregon).

Identification and genotyping of edn1$^{-/-}$ *mutants*

edn1$^{-/-}$ mutants have an A-to-T missense mutation (Miller et al., 2000). The mutants were identified by Derived Cleaved Amplified Polymorphic Sequences assay (Neff et al., 1998). DNA was extracted from the tails of stained embryos and PCR was conducted using the primers 5'-AGATGCTCCTGCGCAAGTTTTCTAG-3' and 5'-CTGACTTACTCTGGTGTGTTCACCC-3'. The mismatch in the primer which introduces a *XbaI* site in the wild-type (WT) but not in the mutant is underlined. The 93 bp PCR product, when digested with *Xba*I, gives a 68 bp product in WT. The 93 and 68 bp products were resolved on a 4% Metaphor agarose gel (Lonza). All the identified WT and mutants were included in the analysis.

Cloning and RNA transcription

RNA extracted from 48 h post fertilization (hpf) Tü larvae was used for cDNA synthesis. PCR for *alcama* was performed using the forward primer 5'-ggatccgccaccATGCATTCGGTTATCTGCCTTTTCG-3' with a *BamHI* and Kozak overhang and the reverse primer 5'-ctcgagTTAGACATCTGCTTTATGATTGTTCTCCTCC-3' with a *XhoI* overhang. The overhangs are shown in lower case. The PCR product was cloned into pCMV-Script using TOPO TA kit (Invitrogen). The *edn1* cDNA clone in pBK-CMV was obtained from ZIRC.

To make sense RNA for injection, the *edn1* and *alcama* plasmids were cut with *KpnI* and in vitro transcription was driven from the T3 promoter using mMessage Machine kit (Ambion). *ednrb1* was cloned from cDNA into TOPO TA using the primers 5'-ATGCGTTTCCAAATTATTATGGAAACAAGATGCG-3' (forward) and 5'-TCAGTGCCTAATTTGAAGTATACTTGTTGGAGAC-3' (reverse) and this plasmid was used to make ISH probe.

Morpholino anti-sense oligonucleotide and RNA injections

Translation blocking (TB) and splice site blocking (SB) Morpholinos (MOs) were designed to bind 143 bp upstream of the transcription start site and at the donor site of exon 6 *alcama* mRNA, respectively. *alcama* blocking and control MO were purchased from Gene Tools, Inc. The sequences are TB MO: 5'-GTTCTCCTTTATACAGTCCGGCGAC-3'; SB MO: 5'-GCAGTCCCTCACCTTAATGTTAAAG-3'; control MO: 5'-TGATCACCTGCAGATGGACGCTGCG-3'. The optimal doses were determined to be 1.1 ng for the TB MO and 2.2 ng for the SB MO. The control MO was injected at 1.1 ng per embryo. The TB MO for *nadl1.1*: 5-'CAGGCTGACTCTGCACTGGAGGCAT-3' has been previously described (Wolman et al., 2007) and was injected at 4.4 ng per embryo. 26 pg of *alcama* or *edn1* RNA was injected per embryo. MOs and RNA were dissolved in molecular biology grade water and pressure injected into one to four cell zebrafish embryos. For suboptimal doses, the *alcama* TB MO was used at 0.5 ng and the *nadl* MO at 1.1 ng per embryo.

Treatment with proteasome inhibitor MG-132

MG-132 was dissolved in DMSO at a stock concentration of 500 mM. Embryos were dechorionated at 5 hpf and transferred to E2 with 50 μM MG-132 (Bretaud et al., 2007) or with 1% dimethyl sulfoxide (DMSO). MG-132- and DMSO-treated control larvae were fixed at 30 hpf and stained.

Tissue labeling procedures

Alcian Blue cartilage staining and dissection were performed as described (Kimmel et al., 1998). Whole mount RNA in situ hybridization (ISH) with digoxigenin was performed as described (Miller et al., 2000). The plasmids for *dlx2a*, *dlx3b*, *dlx5a* were a kind gift from Gage DeKoeyer Crump (Keck School of Medicine, University of Southern California). The plasmid for nadl1.1 was a gift from Gavin J Wright (Cell Surface Signalling Laboratory, Wellcome Trust Sanger Institute). Alcama protein was stained using Zn-5 antibody from ZIRC at 1:500 dilution. A goat anti-mouse secondary antibody conjugated with Alexa 555 (Invitrogen) was used for fluorescence quantification purposes. DAPI staining was used for counting the number of cells in the pouches.

Imaging and quantification

Skeletons and ISH embryos were photographed on a Nikon Y-IDP microscope at 20× zoom using Spot software. Confocal images of antibody stained embryos were taken on Olympus FV1000 microscope. Images of all larvae from the same experiment were taken