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Pax2 and Pea3 synergize to activate a novel regulatory enhancer for spalt4 in the developing ear☆

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ABSTRACT

The transcription factor *spalt4* is a key early-response gene in otic placode induction. Here, we characterize the *cis*-regulatory regions of *spalt4* responsible for activation of its expression in the developing otic placode and report the isolation of a novel core enhancer. Identification and mutational analysis of putative transcription factor binding sites reveal that *Pea3*, a downstream effector of FGF signaling, and *Pax2* directly activate *spalt4* during ear development. Morpholino-mediated knock-down of each factor reduces or eliminates reporter expression. In contrast, combined over-expression of *Pea3* and *Pax2* drives ectopic reporter expression, suggesting that they function synergistically. These studies expand the gene regulatory network underlying early otic development by identifying direct inputs that mediate *spalt4* expression.

Introduction

Ectodermal placodes are transient regions of thickened ectoderm of the head that contribute to the ear, nose, lens and cranial ganglia (Baker and Bronner-Fraser, 2001). Of these, the best studied is the otic placode, which is induced adjacent to the hindbrain by signals emanating from flanking tissues (Dominguez-Frutos et al., 2009; Kil et al., 2005; Kwon and Riley, 2009; Ladher et al., 2005; Park and Saint-Jeannet, 2008). Signals such as FGFs (Leger and Brand, 2002; Maroon et al., 2002; Vendrell et al., 2000; Wright and Mansour, 2003), Wnts (Freter et al., 2008; Ohyama et al., 2006) and BMPs (Kwon and Riley, 2009) are involved in otic placode induction (Martin and Groves, 2006) and activate specific patterns of gene expression (Litsiou et al., 2005). For example, over-expression of FGFs induces ectopic otic-like structures (Alvarez et al., 2003; Kil et al., 2005; Vendrell et al., 2000), while mutations in FGFs lead to defects in ear development (Alvarez et al., 2003; Ladher et al., 2005). Following induction, the columnar placode invaginates to form the otic vesicle, which subsequently differentiates into the complex inner ear, including the cochlea, vestibular system and endolymphatic sac.

The transcription factor *spalt4*, homolog of human *SALL4* (Sweetman and Munsterberg, 2006), displays the correct localization pattern to be a key early-response gene in placode induction in the chick (Barembaum and Bronner-Fraser, 2007). It is initially expressed uniformly throughout the head ectoderm, overlapping with *Six-Eya-Dach* in the preplacodal domain. It then resolves to the presumptive otic and olfactory placode regions by stage 10, as non-placodal ectoderm loses competence to form otic placode (Baker and Bronner-Fraser, 2001; Groves and Bronner-Fraser, 2000). Expression of *spalt4* in non-placodal ectoderm is sufficient to induce invagination or ingression and expression of a number of otic genes (Barembaum and Bronner-Fraser, 2007). Interestingly, the effects of its gain- and loss-of-function resemble those of FGF over-expression and mutations, respectively. This raises the intriguing possibility that *spalt4* may be downstream of FGF and other inductive signals.

Numerous transcription factors in addition to *spalt4*, including *Pax2* (Mackereth et al., 2005), *Dlx3* (Esterberg and Fritz, 2009) and *Dlx5* (Brown et al., 2005; Robledo and Lufkin, 2006), have been found to play important roles in otic development (Baker and Bronner-Fraser, 2001). Although the position and function of a few of these regulators are documented in otic development, their order, interrelationship and direct or indirect nature of their interactions are not yet known and currently under investigation (Esterberg and Fritz, 2009; Hans et al., 2007).

To better understand important gene regulatory interactions underlying ear development, we set out to characterize the *cis*-regulatory regions of *spalt4* based on its key position in otic placode induction. We report the isolation and dissection of a novel *spalt4* regulatory module, responsible for activation of *spalt4* expression in the developing otic placode. We further interrogate this regulatory region to identify putative transcription factor binding sites and upstream regulators controlling its expression. The results reveal that *Pea3*, a downstream effector of FGF signaling, and *Pax2* directly

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activate *spalt4* during ear development. These studies expand our knowledge of known transcription factors and their direct interactions during development of the ear.

Materials and methods

Cloning

Regions of non-coding genomic DNA in the vicinity of the *spalt4* (*Sall4*) coding region were compared between chick and other vertebrates. Conserved regions were amplified from the BAC clone CH261-71D2 (BPRC, Oakland Research Institute, Oakland, CA) and cloned into the pTK vector in front of a minimal TK promoter driving GFP expression (Uchikawa et al., 2004). Mutations were made by fusion PCR (Heckman and Pease, 2007). For over-expression experiments, the protein coding regions of chicken Pea3 and Pax2 were amplified by RT-PCR and cloned into the pCIG vector. Cerulean fluorescent protein was cloned into the pCAGS expression vector.

Electroporation

Stage 4–6 embryos were collected on Whatman filter rings and placed ventral side up in an electoporation chamber, with negative electrode at the bottom. Plasmid DNA, either at 1 mg/ml for enhancer constructs or 2 mg/ml for over-expression constructs, was injected through the blastoderm into the space between the embryo and the vitelline membrane. The positive electrode was placed above the embryo and an electric current was applied of four pulses of 7 V, 50 ms in duration with a 100 ms pause in between pulses. After electroporation, embryos were transferred ventral side up to a 35 mm dish with a thin layer of egg albumin at the bottom and incubated in a humidified incubator at 37 °C for 24 h (Sauka-Spengler and Barembaum, 2008). Those embryos with high levels of fluorescence, indicating efficient eletroporation, were fixed in 4% formaldehyde overnight. Morpholino oligos were obtained from Gene-Tools (Philomath, OR) and dissolved in water at 1 or 3 mM concentrations. Plasmid DNA (pUC19) was added at a final concentration of 100 ng/μl prior to injecting into embryos. In order to electroporate the morpholino at 3 mM, the embryos were first electroporated with the enhancer construct then followed by the morpholino. The fluoresceinated morpholino oligos were made with the following sequence:

Pea3: 5′-CTG CTG GTC CAC GTA CCC CTT CAT C-3′
Pax2: 5′-GTC TGC CTT GCA GTG CAT ATC CAT G-3′
Control: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′

Implantation of beads

Stage 4 embryos were collected on Whatman filter paper rings and turned ventral side up in Ringer's solution and electroporated as described above. A small slit was made in the area opaca next to the area pellucida (Litsiou et al., 2005). A bead soaked in 50 μg/ml Fgf8 (R&D Systems, Minneapolis MN) or BSA (bovine serum albumin) was inserted into the slit and incubated in modified New culture (Chapman et al., 2001) for 5–7 h and then collected and fixed overnight in 4% paraformaldehyde.

Analysis of embryos

Embryos were collected in Ringer's solution and fixed in 4% paraformaldehyde overnight. Embryos were washed in PBT and embedded in gelatin for histochemical analysis or dehydrated in methanol for in situ hybridization. In situ hybridization was performed as described previously (Wilkinson, 1992). Antibodies to GFP (Abcam), Pax2 (Zymed), and fluorescein (Roche) were obtained commercially. Primary antibodies were visualized with Alexa Fluor 488-conjugated donkey anti-goat or Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibodies (Molecular Probes). Cerulean fluorescent protein expression was distinguished from fluorescein signal using a Zeiss 510 META 2 inverted microscope.

Results

Dissection of putative spalt4 regulatory region

Spalt4 is initially expressed throughout the preplacodal domain but resolves to the otic placode by stages 9–10 (Fig. 1A) (Barembaum and Bronner-Fraser, 2007). By stage 14, it is robustly expressed in the otic pit and maintained during formation of the otic vesicle (Fig. 1B). We set out to identify enhancer elements capable of driving reporter expression that recapitulated this spatiotemporal expression pattern.

To identify the *cis*-regulatory elements responsible for *spalt4* expression in developing ear, we probed the genomic region surrounding the *spalt4* gene using comparative genomic analysis to isolate highly conserved genomic regions with putative regulatory activity. For our study, non-coding genomic regions upstream of the *spalt4* coding region and the first intron were compared *in silico* using the UCSC gene browser (Fig. 2A) (Karolchik et al., 2008). Fragments 1 to 5 kb in size containing the putative regulatory regions with the highest homology were amplified from a chicken BAC clone and cloned into an EGFP reporter vector containing a thymidine kinase basal promoter (Uchikawa et al., 2004).

The *spalt4* gene is located on chicken chromosome 20 between genes *ZFP64* and *ATP9A*. Because the May 2006 version of the chicken genome did not contain the sequence of the first exon, we cloned the DNA corresponding to a gap in the sequence from a BAC using GC rich PCR. This region contained the first exon of *spalt4*. At the 5′ end there is over 100 kb of sequence between *spalt4* and *ZFP64*. We cloned several conserved regions upstream of the coding region, as well as within the first intron, which induced GFP

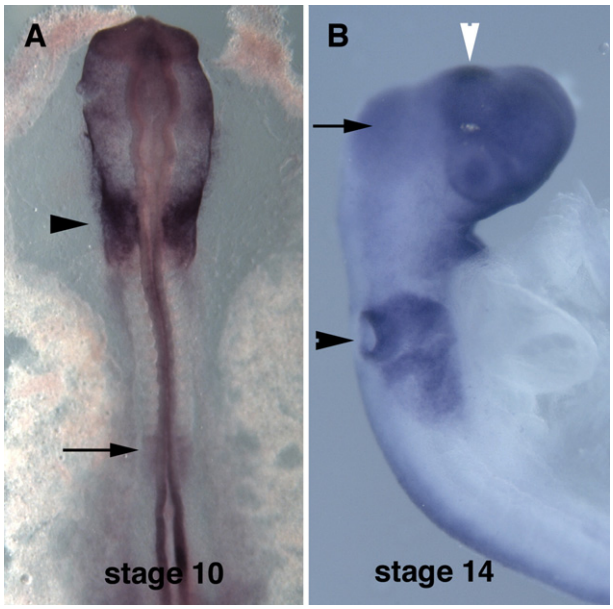

Fig. 1. In situ hybridization of chicken embryos with *spalt4* RNA probe. (A) Stage 10 embryo showing signal in the otic placode (arrowhead) and presomitic mesoderm (arrow). (B) Stage 14 embryo showing signal in the otic pits (black arrowhead) and midbrain (arrow). The region that will give rise to the epiphysis also expresses *spalt4* (white arrowhead). Other forebrain staining is background.