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Hoxa1 lineage tracing indicates a direct role for *Hoxa1* in the development of the inner ear, the heart, and the third rhombomere

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ABSTRACT

Loss of *Hoxa1* function results in severe defects of the brainstem, inner ear, and cranial ganglia in humans and mice as well as cardiovascular abnormalities in humans. Because *Hoxa1* is expressed very transiently during an early embryonic stage, it has been difficult to determine whether *Hoxa1* plays a direct role in the precursors of the affected organs or if all defects result from indirect effects due to mispatterning of the hindbrain. In this study we use a *Hoxa1-IRES-Cre* mouse to genetically label the early *Hoxa1*-expressing cells and determine their contribution to each of the affected organs, allowing us to conclude in which precursor tissue *Hoxa1* is expressed. We found *Hoxa1* lineage-labeled cells in all tissues expected to be derived from the *Hoxa1* domain, such as the facial and abducens nuclei and nerves as well as r4 neural crest cells. In addition, we detected the lineage in derivatives that were not thought to have expressed *Hoxa1* during development. In the brainstem, the anterior border of the lineage was found to be in r3, which is more anterior than previously reported. We also observed an interesting pattern of the lineage in the inner ear, namely a strong contribution to the otic epithelium with the exception of sensory patches. Moreover, lineage-labeled cells were detected in the atria and outflow tract of the developing heart. In conclusion, *Hoxa1* lineage tracing uncovered new domains of *Hoxa1* expression in rhombomere 3, the otic epithelium, and cardiac precursors, suggesting a more direct role for *Hoxa1* in development of these tissues than previously believed.

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Introduction

Homeobox (*Hox*) genes encode a family of transcription factors that regulate embryonic patterning and organogenesis (Alexander et al., 2009; Capecchi, 1997; Iimura and Pourquie, 2007). *Hoxa1* is one of the earliest and most anteriorly expressed *Hox* genes (Murphy and Hill, 1991). Mice with a targeted disruption of *Hoxa1* die shortly after birth from breathing defects, which are thought to result from mispatterning of the hindbrain (Chisaka et al., 1992; Lufkin et al., 1991). In addition, the inner ear fails to differentiate and cranial ganglia are smaller. Patients with homozygous truncating mutations in *HOXA1* (Bosley–Salih–Alorainy syndrome (BSAS) or Athabascan brainstem dysgenesis syndrome (ABDS)) suffer from hypoventilation, deafness, facial weakness, vocal cord paralysis, swallowing dysfunction, carotid artery abnormalities, and conotruncal heart defects (Bosley et al., 2007, 2008; Holve et al., 2003; Tischfield et al., 2005).

Hoxa1 expression is first detected at embryonic day E7.5 in the primitive streak, in newly formed mesoderm, and overlying neuroectoderm (Murphy and Hill, 1991). At E7.75, *Hoxa1* expression reaches its anterior domain in the presumptive hindbrain, the embryonic precursor of the brainstem, and at E8.5, *Hoxa1* has retreated from this region (Murphy and Hill, 1991). Thus, *Hoxa1* is only expressed for about 12 hours in its most anterior domain.

The hindbrain or rhombencephalon is subdivided into eight transient swellings called rhombomeres, abbreviated r1–r8 (Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996). Loss of *Hoxa1* function results in the absence of r5 and size reduction of r4. This leads to loss of the abducens (6N) and strong reduction of the facial nuclei (7N), which is most likely the reason for horizontal gaze abnormalities and facial weakness in human patients. In addition to the defects in r4- and r5-derivatives, $Hoxa1^{-/-}$ mice also exhibit abnormal neurogenesis in r3, namely presence of cell patches with an r2 molecular identity, premature neuronal differentiation, and abnormal navigation of motor axons (Helmbacher et al., 1998). Previous studies have described the anterior border of *Hoxa1* expression to be in the hindbrain below the preotic sulcus at the future r3/r4 boundary (Barrow et al., 2000; Murphy and Hill, 1991). Therefore, the development of r3 was proposed to be nonautonomous and dependent on interactions with *Hoxa1*-expressing cells in r4 (Helmbacher et al., 1998).

Besides the hindbrain, another severely affected organ in $Hoxa1^{-/-}$ mice is the inner ear (Chisaka et al., 1992; Lufkin et al., 1991). The otic vesicle forms but fails to differentiate. Similarly, humans with mutations in *HoxA1* have undifferentiated inner ears and are deaf.

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Development of the inner ear commences with an ectodermal thickening called the otic placode, which invaginates to form the otic cup and subsequently the otic vesicle. All components of the adult inner ear are derived from the otic ectoderm, including patches of sensory cells within the epithelium and sensory neurons in the spiral and vestibular ganglia, which innervate these patches. Because no *Hoxa1* expression has been detected in the precursor of the inner ear, the abnormalities in *Hoxa1*$^{-/-}$ embryos were attributed to the disruption of hindbrain signals necessary for inner ear patterning (Mark et al., 1993). To date, little is known about how *Hoxa1* performs its function during inner ear development and what signals are regulated by this gene.

Hoxa1$^{-/-}$ embryos also show defects in cranial ganglia and the stapes bone of the ear (Chisaka et al., 1992), structures that develop in part from neural crest cells (NCCs), which delaminate from rhombomere 4 after *Hoxa1* expression has retracted from this region. This delamination takes place in two waves. The first wave migrates into BA2, where it forms cartilage, which differentiates into bone and connective tissue. The second wave of r4-NCC condenses lateral to the neural tube and gives rise to glia of the facio-acoustic ganglion complex (7/8G) (Baker et al., 1997; Kontges and Lumsden, 1996). All glia cells in this ganglion are derived from NCCs, whereas almost all neurons originate from ectodermal placodes (Barlow, 2002). The defects in NCC derivatives in *Hoxa1*$^{-/-}$ mice lead to the hypothesis that *Hoxa1* might specify the developmental program of cranial neural crest cells (Lufkin et al., 1991).

An even more dramatic phenotype is observed in embryos lacking both *Hoxa1* and its paralog *Hoxb1*, which, in addition to neural crest defects, almost completely lack the second branchial arch (BA2) and its mesodermal derivatives (Gavalas et al., 1998; Rossel and Capecchi, 1999). During development, cells from the cranial paraxial mesoderm surrounding r3–r6 migrate into the core of BA2 and give rise to the muscles of facial expression as well as the muscles of the jaw and upper neck. It has so far been controversial whether loss of mesodermal derivatives is secondary due to the absence of r4 NCCs in the double knockout or if *Hoxa1* (in redundancy with *Hoxb1*) plays a direct role in mesoderm development (Morrison, 1998).

A study in humans demonstrated that *HOXA1* has a previously unrecognized role in development of the cardiovascular system (Tischfield et al., 2005). Humans with homozygous mutations in *HOXA1* exhibit outflow tract (OFT) and internal carotid artery (ICA) abnormalities (Tischfield et al., 2005). The OFT develops from mesodermally derived myocardial cells and is later infiltrated and remodeled by cardiac neural crest cells, originating in the hindbrain at the level of r6–r8 (Brown and Baldwin, 2006; Kirby and Waldo, 1995; Snider et al., 2007). *Hoxa1* is expressed in the neural tube at the level from which cardiac NCCs arise (Murphy and Hill, 1991) in addition to the foregut and mesoderm adjacent to the cardiac field (Godwin et al., 1998; Ryckebusch et al., 2008). However, no *Hoxa1* expression has been detected in myocardial precursors within the cardiac field (Godwin et al., 1998; Ryckebusch et al., 2008). Since the cardiovascular defects have not been analyzed in mice, it is unknown at which step of development and in what tissue *Hoxa1* function is required.

In this study, we present new insight into the role of *Hoxa1* during embryogenesis by genetically labeling early *Hoxa1*-expressing cells, using the Cre/loxP system (Branda and Dymecki, 2004), and following their fate into later stages of development (*Hoxa1* lineage tracing). Our analysis demonstrates that *Hoxa1* lineage does not exhibit a sharp anterior border at the r3/r4 boundary but extends into r3. We also find that *Hoxa1* lineage gives rise to all neural crest cells, which populate the second branchial arch and contribute to cranial ganglia. In contrast, no *Hoxa1* lineage is detected in mesodermal derivatives of BA2. Interestingly, *Hoxa1* lineage is seen in a restricted pattern in derivatives of the otic placode and myocardium, both structures that were not thought to express *Hoxa1*.

Materials and methods

Gene targeting and genotyping

To generate the *Hoxa1-IRES-Cre* allele, a 7.9-kb *Cla*I fragment containing the *Hoxa1* locus from 129/SV genomic DNA was subcloned. An *Asc*I site placed 36 bp downstream of the stop codon was used to insert an IRES-Cre-frt-MC1-Neo-frt cassette (Arenkiel et al., 2003). The targeting vector was electroporated into R1 ES cells, which were cultured under positive selection using G418. Correctly targeted ES cell clones were identified by Southern hybridization (Fig. 1B) and used to generate chimeras, which were crossed to C57BL6 mice. The neomycin resistance gene was removed by crossing the mice to a FLPe deleter line (Rodriguez et al., 2000). *Hoxa1-IRES-Cre* homozygous mice are viable and fertile.

Genotyping was performed using multiplex PCR with the following primers: wt 5′ (AGCGATGAGAAAACGGAAG), wt 3′ (GGG ACG AGA AAG GAA GAG AG), Cre 5′ (CAA TAC CGG AGA TCA TGC AAG), generating a 220 bp wt and 382 bp engineered band. Lineage analysis was carried out using the previously described *R26R-EYFP*, *R26R-lacZ*, and *nLacZ* lines (Haldar et al., 2008; Soriano, 1999; Srinivas et al., 2001). All mouse use complied with protocols approved by the University of Utah Institutional Animal Care and Use Committee.

β-Galactosidase staining and RNA in situ hybridization

For β-gal staining, tissues were dissected in PBS, pH 7.4 with 2 mM $MgCl_2$, fixed for 15 min to 2 hours depending on tissue size in 1% formaldehyde, 0.2% glutaraldehyde, 25 mM EGTA, 2 mM $MgCl_2$, 0.02% NP-40 in PBS, washed in PBS with 2 mM $MgCl_2$, and moved into X-gal staining solution (0.8 mg/ml X-gal, 25 mM $K_3Fe(CN)_6$, 25 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP-40 in PBS). Facial nerve staining was carried out after removal of the skin and surrounding tissues. Brains of adult mice were isolated after perfusion with 2% formaldehyde, cryosectioned, stained with X-gal, and mounted in Celvol. All stainings were carried out overnight at room temperature.

Whole-mount in situ hybridization with a digoxigenin-labeled antisense probe generated from a plasmid containing a 216 bp *Hoxa1* exon1 fragment was carried out as described (Henrique et al., 1995).

Immunostaining and analysis

Tissues were fixed at 4 °C for 1–2 hours in 4% formaldehyde, rinsed in PBS, equilibrated to 30% sucrose, and embedded in OCT. Cryosections were cut at 10 μm, washed in PBS and preincubated in blocking solution (2% BSA, 10% NGS, 0.1% Triton in PBS, pH 7.2). Primary antibodies were applied overnight at 4 °C in a humid chamber, followed by secondary detection using Alexa Fluor-conjugated (Molecular Probes) or DyLight-conjugated (Jackson ImmunoResearch) secondary antibodies. Immunodetection was carried out using an SP5 confocal system (Leica) or an inverted microscope (Axiovert 200M; Zeiss) equipped with a SensiCam camera (The Cooke Corporation). Data were acquired using the LAS AF or SlideBook™ software and processed using Adobe Photoshop. For hindbrain flat mounts, embryonic hindbrains were isolated, and the neural tube was cut along the roof plate. Brains were fixed, stained, and visualized as above.

The following primary antibodies were used in this study: mouse anti-AP2 (DSHB; 1:75), chick anti-GFP (Aves Labs; 1:500), rabbit anti-GFP (Abcam; 1:4000 or Molecular Probes; 1:2000), mouse anti-GFP (Molecular Probes; 1:250), rabbit anti-Hox-B1 (Covance; 1:250), mouse anti-Islet1 (DSHB; 1:30), rat anti-MBP (Chemicon; 1:75), mouse anti-Myogenin (DSHB; 1:25), rabbit anti-p75 (Chemicon; 1:100), mouse anti-Pax7 (DSHB; 1:15), rabbit anti-Phox2b (kind gift from C. Goridis and J. P. Brunet; 1:1000), rabbit anti-Sox2 (Chemicon;

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