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Identification of novel *Hoxa1* downstream targets regulating hindbrain, neural crest and inner ear development

Nadja Makki, Mario R. Capecchi *

Howard Hughes Medical Institute and Department of Human Genetics, UT, USA

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ABSTRACT

Hox genes play a crucial role during embryonic patterning and organogenesis. Of the 39 *Hox* genes, *Hoxa1* is the first to be expressed during embryogenesis and the only anterior *Hox* gene linked to a human syndrome. *Hoxa1* is necessary for the proper development of the brainstem, inner ear and heart in humans and mice; however, almost nothing is known about the molecular downstream targets through which it exerts its function. To gain insight into the transcriptional network regulated by this protein, we performed microarray analysis on tissue microdissected from the prospective rhombomere 3–5 region of *Hoxa1* null and wild type embryos. Due to the very early and transient expression of this gene, dissections were performed on early somite stage embryos during an eight-hour time window of development. Our array yielded a list of around 300 genes differentially expressed between the two samples. Many of the identified genes play a role in a specific developmental or cellular process. Some of the validated targets regulate early neural crest induction and specification. Interestingly, three of these genes, *Zic1*, *Hnf1b* and *Foxd3*, were down-regulated in the posterior hindbrain, where cardiac neural crest cells arise, which pattern the outflow tract of the heart. Other targets are necessary for early inner ear development, e.g. *Pax8* and *Fgfr3* or are expressed in specific hindbrain neurons regulating respiration, e.g. *Lhx5*. These findings allow us to propose a model where *Hoxa1* acts in a genetic cascade upstream of genes controlling specific aspects of embryonic development, thereby providing insight into possible mechanisms underlying the human *HoxA1*-syndrome.

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Introduction

Hox proteins constitute a family of transcription factors which control gene expression networks that regulate biological processes such as neurogenesis, patterning, organogenesis and cancer (Alexander et al., 2009; Capecchi, 1997). Mouse knockout studies revealed that *Hox* genes execute their role in a specific segment or domain of the embryo, often affecting several tissues at a given axial level (Mallo et al., 2010). Although many gain- and loss-of-function experiments have been carried out, little is known about the molecular targets and the developmental pathways regulated by *Hox* genes (Hueber and Lohmann, 2008). In this study, we set out to identify the downstream targets of a specific *Hox* gene, *Hoxa1*. This gene affects the development of a diverse array of tissues in the anterior domain of the embryo including the brainstem, inner ear and heart.

Hoxa1 is strongly expressed in the neuroectoderm and mesoderm at the level of the presumptive hindbrain (precursor of the brainstem) from mouse embryonic day (E) 7.75 to 8.5 (Murphy and Hill, 1991). *Hoxa1* knockout mice die at or shortly after birth from breathing defects, which are thought to result from mispatterning of the hindbrain (Chisaka et al., 1992; Lufkin et al., 1991). During development, the hindbrain is subdivided into eight transient territories termed rhombomeres (r) (Lumsden and Krumlauf, 1996) and *Hoxa1*$^{-/-}$ embryos exhibit abnormalities in r3–r5. Additionally, the otic vesicle (embryonic progenitor of the inner ear) forms but fails to differentiate and cranial ganglia, condensations of sensory neurons in the head, are smaller and do not connect properly with the brain (Mark et al., 1993). Cranial ganglia develop in part from cranial neural crest cells, which migrate from the dorsal hindbrain (Barlow, 2002), where *Hoxa1* is expressed. So far it is unclear through which mechanisms *Hoxa1* regulates the development of neural crest cells or the inner ear. *Hoxa1* lineage analysis suggests that *Hoxa1* might play a direct role in early patterning of the otic placode (precursor of the otic vesicle) and specification of neural crest cell precursors, while they reside in the neural tube (Makki and Capecchi, 2010).

More recently, humans with homozygous truncating mutations in *HOXA1* have been identified (Athabascan Brainstem Dysgenesis Syndrome and Bosley–Salih–Alorainy Syndrome). These patients suffer from hypoventilation (requiring mechanical ventilation), deafness, facial weakness, vocal cord paralysis and swallowing dysfunction (Holve et al., 2003; Tischfield et al., 2005). In addition, patients display defects in the outflow tract of the heart, which have not been described in mice so far. Notably, the development of the cardiac outflow tract depends on the influx of neural crest cells, which

* Corresponding author at: Howard Hughes Medical Institute, University of Utah, 15 North 2030 East, Salt Lake City, UT 84112-5331, USA. Fax: +1 801 585 3425.
E-mail address: mario.capecchi@genetics.utah.edu (M.R. Capecchi).

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originate in the posterior hindbrain at the level of r6–r8 (Brown and Baldwin, 2006), where *Hoxa1* is expressed.

Despite of what we know about the importance of *Hoxa1* in proper development of several embryonic tissues in humans and mice, almost nothing is known about the transcriptional network that is regulated by this protein. In this study, we carried out a genome-wide microarray analysis to identify genes that are differentially expressed between control and *Hoxa1* null embryos. For genomic profiling, tissue was microdissected from the prospective rhombomere 3–5 region of $Hoxa1^{\Delta/\Delta}$ and wild type embryos at the 1–6 somite stage (ss). Our analysis identified novel targets of *Hoxa1* that play a role in neural crest specification, otic placode patterning, and reticulospinal neuron development.

Materials and methods

Gene targeting and genotyping

A 7.9 kb genomic DNA fragment containing the *Hoxa1* locus was subcloned into a conventional plasmid and an artificial AscI site was placed 36 bp downstream of the stop codon as described previously (Tvrdik and Capecchi, 2006). To generate the *Hoxa1* conditional allele ($Hoxa1^{c}$), one loxP site together with an EcoRI site were inserted 200 bp upstream of the *Hoxa1* transcription initiation site into a SwaI site. The downstream loxP site along with an EcoRI site and a PolII-frt-Neo-frt selection cassette were inserted into the artificial AscI site 3′ of the *Hoxa1* stop codon. Positive clones were identified by digesting genomic DNA with EcoRI, Southern blotting and hybridization with a 5′ external probe. Selected clones were further analyzed by digestion with KpnI and hybridization with an exon1 and a Neo probe. Positive ES cell clones were injected into C57BL/6 blastocysts and chimeric males were crossed to C57BL/6 females. The neomycin resistance gene was removed by crossing the mice to anFlpe-deleter line (Rodriguez et al., 2000). The *Hoxa1*-deletion allele ($Hoxa1^{\Delta}$) was generated by crossing *Hoxa1* conditional mice to an *Hprt-Cre* deleter mouse (Tang et al., 2002). Recombination was verified by Southern analysis and PCR. Genotyping was performed using multiplex PCR. The following primers were used: wild type forward NM228 5′-TGAGGCTACTC-CAGCCCAACTC-3′, deletion forward NM230 5′-CTCTCACCTCTTGC-CAGTTCAGC-3′, reverse NM229 5′-CAATTGATGTGGACACCCGATG-3′, generating a 220 bp wild type, a 326 bp conditional and a 520 bp deletion band.

Mouse breeding and tissue dissection

$Hoxa1^{\Delta/+}$ mice were maintained on a C57BL/6 background. Timed matings were set up between $Hoxa1^{\Delta/+}$ mice and embryos were harvested at E8.25. Deciduas were isolated in cold PBS and transferred into HEPES-buffered DMEM with 5% FBS on ice. Each embryo was isolated in a separate dish in PBS, extraembryonic tissues were removed and the number of somites counted. Using fine tungsten knives, the bulge region (rhombomere 3–5), including neuroectoderm, mesoderm and otic ectoderm, was isolated and the tissue trimmed by a horizontal cut at the level of the floorplate. The tissue was then transferred into 40 µl of RLT buffer (Qiagen Micro-RNA Easy kit), vortexed immediately for 1 min and stored on ice until all embryos were processed. The yolk sac was collected for DNA isolation and genotyping. Finally, the tissue was homogenized by vortexing for 5 min followed by snap freezing in liquid nitrogen and storage at −80 °C. A total of 221 embryos were collected and sorted according to genotype (verified at least twice) and somite stage. Twenty-four wild type and 24 $Hoxa1^{\Delta/\Delta}$ embryos at the 1–6 somite stage were chosen for analysis and pooled into four wild type and four mutant samples, containing one embryo of each somite stage.

RNA isolation, array hybridization and statistical analysis

RNA was isolated from the eight samples using the RNAqueous-Micro Kit (Ambion) with an on-column DNase treatment (Qiagen). The concentration and quality of the RNA was determined at the University of Utah Microarray Core Facility using a Nanodrop and Bioanalyzer (Agilent). The RNA Integrity Number (RIN) deduced from this analysis was 9.9–10 for all samples, which denotes an excellent RNA quality with no degradation (Schroeder et al., 2006). The final concentrations of total RNA varied from 15 to 20 ng/µl and 150 ng of RNA from each pool was subjected to a single linear amplification labeling reaction with Cy3. RNA was hybridized to Agilent mouse whole genome 44 K microarray slides (Agilent), using the Agilent one-color gene expression hybridization protocol. Slides were scanned (Agilent G2505B) at 5 µm resolution using an extended dynamic range protocol, and images were processed with Agilent Feature Extraction software 10.5.1.1. Within-array normalization was performed using the "Background detrending" software (Agilent). The nonuniform outlier features (spots) were removed and the intensity values were transformed to a log base 2 scale. Signal density blots showed uniform ranges and distributions of intensity values from each array and no between-array normalization was necessary. All eight array files were then compiled into a working directory and imported into the statistical analysis program "R" (Dudoit et al., 2003). Genes significantly differentially expressed were identified using the Rank Products algorithm with the default setting of 100 permutations (Breitling et al., 2004). Rank Products analysis was chosen because of its biologically meaningful emphasis on the fold change of gene expression and the reproducibility in samples with small numbers of replicates. GO analysis was performed using DAVID (Dennis et al., 2003; Huang da et al., 2009) on significantly differentially expressed genes. In case of overlapping and similar GO terms, one representative is listed, and terms that are too general were not included. Data was hierarchically clustered with Spotfire (TIBCO) and heat maps for selected genes were generated. The microarray data can be found in the Gene Expression Omnibus (GEO) of NCBI through accession number GSE25868.

Quantitative real-time PCR (qPCR)

60 ng of total RNA was linearly amplified using the qScript cDNA SuperMix (Quanta Biosciences). Reverse transcription and PCR conditions were essentially as described (Schmittgen and Livak, 2008) using the SYBR Green detection method. Primer pairs (Table S2) were obtained from the PrimerBank database (Wang and Seed, 2003). Reactions were run on a 7900HT thermal cycler (Applied Biosystems) in the Genomics Core Facility at the University of Utah. For the final experiment, three wild type and three $Hoxa1^{\Delta/\Delta}$ cDNA samples (biological replicates) were analyzed individually in three replicates of each reaction (technical replicates) and the mean threshold cycle (C_T) for each gene was derived. Relative expression levels were calculated by the ΔC_T method (Schmittgen and Livak, 2008), normalizing to the housekeeping gene β-*actin*, and data expressed as mean fold change relative to wild type. Unpaired, two-tailed Student's *t*-test was used to calculate P values between the *Hoxa1* null and control samples.

Inner ear paint-filling and RNA in situ hybridization

For inner ear paint-filling, E15.5 embryos were harvested and fixed overnight in Bodian's fixative. Embryos were washed in PBS, dehydrated in ethanol and cleared in methyl salicylate. Heads were hemisected and inner ears injected with 2% white latex paint in methyl salicylate using a micropipette (Morsli et al., 1998). For RNA in situ hybridization, Digoxigenin-labeled antisense cRNA probes were generated from plasmids carrying cDNA fragments. The following cloned mouse cDNAs were obtained, sequenced and used to prepare

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