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ScienceDirect
Developmental Biology 310 (2007) 154–168
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Hand transcription factors cooperatively regulate development of the distal midline mesenchyme

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Received for publication 15 March 2007; revised 23 July 2007; accepted 26 July 2007
Available online 3 August 2007

Abstract

Hand proteins are evolutionally conserved basic helix–loop–helix (bHLH) transcription factors implicated in development of neural crest-derived tissues, heart and limb. *Hand1* is expressed in the distal (ventral) zone of the branchial arches, whereas the *Hand2* expression domain extends ventrolaterally to occupy two-thirds of the mandibular arch. To circumvent the early embryonic lethality of *Hand1* or *Hand2*-null embryos and to examine their roles in neural crest development, we generated mice with neural crest-specific deletion of *Hand1* and various combinations of mutant alleles of *Hand2*. Ablation of *Hand1* alone in neural crest cells did not affect embryonic development, however, further removing one *Hand2* allele or deleting the ventrolateral branchial arch expression of *Hand2* led to a novel phenotype presumably due to impaired growth of the distal midline mesenchyme. Although we failed to detect changes in proliferation or apoptosis between the distal mandibular arch of wild-type and *Hand1/Hand2* compound mutants at embryonic day (E)10.5, dysregulation of *Pax9*, *Msx2* and *Prx2* was observed in the distal mesenchyme at E12.5. In addition, the inter-dental mesenchyme and distal symphysis of Meckel's cartilage became hypoplastic, resulting in the formation of a single fused lower incisor within the hypoplastic fused mandible. These findings demonstrate the importance of Hand transcription factors in the transcriptional circuitry of craniofacial and tooth development.

Keyword: Cranofacial; Neural crest; Incisor; Mandible

Introduction

Defects in migration, expansion, and differentiation of neural crest cells result in a variety of birth defects affecting craniofacial and cardiovascular structures. The neural crest is a transient and pluripotent population of cells that is formed in the dorsal lip of the neural tube as a result of inductive interactions between the neural plate and the surface ectoderm (Selleck et al., 1998). During craniofacial development, neural crest cells migrate ventrolaterally and populate the branchial arches, where they proliferate and form the frontonasal process and discrete swellings that demarcate each branchial arch. Lineage trace studies utilizing a *Wnt1-Cre* transgene and the ROSA26 conditional reporter (R26R) mouse line have confirmed *in vivo* that cranial neural crest cells eventually differentiate into bone, cartilage, teeth, cranial ganglia, and connective tissue of the face and neck (Chai et al., 2000; Chai and Maxson, 2006).

Early in embryonic development, the ectomesenchyme of the mandibular arch can be divided along a horizontal axis into two equal parts that have distinct developmental capabilities. The rostral (oral) ectomesenchyme participates in tooth formation via interactions with the overlying oral ectoderm, while the caudal (aboral) mandibular ectomesenchyme gives rise to Meckel's cartilage (reviewed in Miletich and Sharpe, 2004). Meckel's cartilage is unique among cartilages in that it exists provisionally prior to permanent mandibular bone formation in the first branchial arch (Sohal et al., 1999).

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.07.036

The mandible first appears as a mesenchymal condensation at E11.5 in mice. At around E13.5, bone begins to form by means of intramembranous ossification, which involves differentiation of mesenchymal precursors to active osteoblasts along the Meckel's cartilage in a proximo-distal direction (Ramaesh and Bard, 2003). As intramembranous ossification proceeds, BrdU-positive proliferating cells diminish from the body of the mandible except at the proximal and distal symphysis of Meckel's cartilage where proliferating cells contribute to the lengthening mandible (Ramaesh and Bard, 2003). By E16.5, the body of the mandibular bone is surrounded by a periosteum and the distal region of the mandible encloses the Meckel's cartilage followed by gradual disintegration of Meckel's cartilage (Ramaesh and Bard, 2003). In contrast to the posterior (proximal) end of Meckel's cartilage, which undergoes endochondral ossification to form middle ear ossicles (Kronenberg, 2003), the anterior (distal) and intermediate portions of Meckel's cartilage degenerate, undergo apoptosis, and are resorbed and replaced by bone (Ishizeki et al., 1999; Trichilis and Wroblewski, 1997). Recently, it was reported that the development and growth of incisors may contribute to osteoclast differentiation and activation, as well as initiation of resorption of Meckel's cartilage (Sakakura et al., 2005).

Tooth development is regulated by inductive tissue interactions between the oral epithelium and the subjacent mesenchyme of the first branchial arch. Numerous signaling pathways have been implicated in each stage of tooth development (reviewed in Matalova et al., 2004). In particular, Fibroblast growth factor-8 (Fgf8), bone morphogenetic protein-4 (Bmp4), sonic hedgehog (Shh), and Wnt7b are epithelial-derived growth factors that induce an auto-regulatory positive feedback loop as well as mutual inhibitory signals to form incisor-molar boundaries within the epithelium (Hardcastle et al., 1998; Jeong et al., 2004; Sasaki et al., 2005; Stottmann et al., 2001). Tooth initiation becomes morphologically distinguishable between embryonic day (E) 11.5 and E12 when the oral ectoderm thickens in prospective tooth-forming regions of the mandibular and maxillary arches (reviewed in Tucker and Sharpe, 2004). This thickened ectoderm, known as the dental lamina, proliferates and starts to invaginate into the underlying neural crest-derived mesenchyme. Around E13.5 at the bud stage of tooth development, the mesenchyme proliferates and condenses around the developing epithelial bud. During this period, the mesenchyme induces the epithelial enamel knot, which becomes the signaling center of the developing tooth. By E14.5, the tooth bud takes the shape of a cap, and neural crest-derived ectomesenchymal cells are concentrated at the dental papilla. Cytodifferentiation of the tooth occurs after E16.5 at the bell stage, in which the ectoderm gives rise to the enamel-secreting ameloblasts, and the ectomesenchyme to dentine-secreting odontoblasts, pulp, and alveolar bone.

In the present work, we investigated the function of *Hand* genes in the development of mandibular arch derivatives. The class II bHLH proteins Hand1 (eHand, Thing1, and Hxt) and Hand2 (dHand, Thing2, and Hed) are expressed in partially overlapping domains in postmigratory neural crest cells in the branchial arches. *Hand1* expression is confined to the ventral one-third of the mandibular arch and arch two, whereas *Hand2* is expressed in the ventral two-thirds of the mandibular arch and arch two (Clouthier et al., 2000; Cserjesi et al., 1995; Srivastava et al., 1995; Srivastava et al., 1997). *Hand1*$^{-/-}$ embryos die by E8.5 due to yolk sac deficiency and *Hand2*$^{-/-}$ embryos die around E10.5 because of cardiac insufficiency (Firulli et al., 1998; Srivastava et al., 1997). Although *Hand2*$^{-/-}$ embryos show retarded branchial arch development and increased apoptosis, it is not clear whether the phenotype is a primary effect of the absence of *Hand2* in neural crest cells or secondary to heart abnormalities.

Despite early embryonic lethality of homozygous embryos, other genetically manipulated mice offer some insights into the role of *Hand* genes in the development of branchial arch-derived tissues. Mice lacking the endothelin-1-dependent *Hand2* branchial arch enhancer die perinatally and exhibit a spectrum of craniofacial defects, including cleft palate, mandibular hypoplasia and cartilage malformations (Yanagisawa et al., 2003). Lineage analysis using *Hand2*-Cre:R26R embryos showed *Hand2*-progeny cells in both mandibular and molar mesenchyme at around E11.5, and later in dental papilla as well as in chondrogenic and osteogenic structures including Meckel's cartilage (Ruest et al., 2003). In transgenic embryos expressing β-galactosidase under the same 7.4 kb upstream region of the *Hand2* gene, transgene expression was observed in the broad mesenchyme of the incisor region. It was also reported that treatments of tooth germ explants with *Hand2* anti-sense oligodeoxynucleotide resulted in impaired differentiation of ameloblasts and odontoblasts, suggesting a potential role of Hand2 in early tooth formation (Abe et al., 2002).

To further explore roles of *Hand* genes during craniofacial development, we deleted the *Hand1* gene in the branchial arch ectomesenchyme using *Cre* recombinase controlled by the neural crest cell-specific *Wnt1* promoter. We also manipulated the expression domain and the level of both *Hand* genes in the branchial arches during embryogenesis by generating compound mutants carrying *Hand1* conditional alleles and mutated *Hand2* alleles. We found that development of the distal midline mesenchyme of the mandibular arch was sensitive to the total *Hand* gene dosage.

Materials and methods

Mouse strains

The *Hand1*-null allele, referred to as *Hand1*^{lacZ} or *Hand1*^{KO}, and *Hand1* conditional allele, referred to as *Hand1*^{loxP}, have been described previously (Firulli et al., 1998; McFadden et al., 2005). The *Hand2*-null allele and *Hand2* mutant allele lacking the ventrolateral branchial arch enhancer, referred to as *Hand2*^{BA}, have been described elsewhere (Srivastava et al., 1997; Yanagisawa et al., 2003). A transgenic mouse line expressing Cre recombinase under control of the neural crest specific promoter *Wnt1* (*Wnt1*::*Cre*) has been previously characterized (Jiang et al., 2000). Animals were kept on a 12 h/12 h light/dark cycle under specific, pathogen-free conditions. Wild-type or heterozygous littermates were used as controls. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees.

RT-PCR

Mandibular and second branchial arches were collected from wild-type (*Hand1*$^{loxP/loxP}$; $n=13$), mutant (*Wnt1*::*Cre*; *Hand1*$^{lacZ/loxP}$; $n=9$, *Wnt1*::*Cre*; *Hand1*$^{loxP/loxP}$; $n=8$) and heterozygous (*Hand1*$^{lacZ/loxP}$; $n=14$) embryos at E10.5 and pooled for RNA preparation. Total RNA was isolated using Trizol