Downregulation of \textit{Dlx5} and \textit{Dlx6} expression by Hand2 is essential for initiation of tongue morphogenesis

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**SUMMARY**

Lower jaw development is a complex process in which multiple signaling cascades establish a proximal-distal organization. These cascades are regulated both spatially and temporally and are constantly refined through both induction of normal signals and inhibition of inappropriate signals. The connective tissue of the tongue arises from cranial neural crest cell-derived ectomesenchyme within the mandibular portion of the first pharyngeal arch and is likely to be impacted by this signaling. Although the developmental mechanisms behind later aspects of tongue development, including innervation and taste acquisition, have been elucidated, the early patterning signals driving ectomesenchyme into a tongue lineage are largely unknown. We show here that the basic helix-loop-helix transcription factor Hand2 plays key roles in establishing the proximal-distal patterning of the mouse lower jaw, in part through establishing a negative-feedback loop in which Hand2 represses \textit{Dlx5} and \textit{Dlx6} expression in the distal arch ectomesenchyme following \textit{Dlx5}- and \textit{Dlx6}-mediated induction of \textit{Hand2} expression in the same region. Failure to repress distal \textit{Dlx5} and \textit{Dlx6} expression results in upregulation of \textit{Runx2} expression in the mandibular arch and the subsequent formation of aberrant bone in the lower jaw along with proximal-distal duplications. In addition, there is an absence of lateral lingual swelling expansion, from which the tongue arises, resulting in aglossia. Hand2 thus appears to establish a distal mandibular arch domain that is conducive for lower jaw development, including the initiation of tongue mesenchyme morphogenesis.

**KEY WORDS:** Aglossia, bHLH, Craniofacial, Hinge and caps, Homeosis, Neural crest cell, Mouse

**INTRODUCTION**

Most of the craniofacial skeleton arises from neural crest cells (NCCs), including the bone, cartilage and connective tissue of the face, neck and tongue (Hall, 1982). Arising along the neural folds of vertebrates before migrating ventrally into the pharyngeal arches (Bronner-Fraser, 1995; Couly et al., 1996; Kontges and Lumsden, 1996; Le Douarin, 1982), NCCs are patterned by numerous environmental cues (Chai and Maxson, 2006). These signals, organized into hierarchical cascades, initiate NCC patterning and differentiation, thus determining both their identity and fate (Clouthier and Schilling, 2004; Depew and Simpson, 2006).

In contrast to our knowledge of the signals regulating bone and cartilage development in the mandibular portion of the first pharyngeal arch, the early signals initiating tongue morphogenesis are poorly understood. Development of the tongue involves NCCs both from three pharyngeal arches and from somitic myoblasts (Noden and Francis-West, 2006). Although the mouse mandibular arch is a complex structure composed of multiple tissue and gene expression domains at embryonic day (E) 10.5 (Depew and Simpson, 2006; Clouthier and Schilling, 2004), a noticeable tongue bud is not evident. However, by E12.5, a recognizable tongue is present and includes somitic myoblasts that have migrated through the hypoglossal duct and that will give rise to the tongue musculature (Noden and Francis-West, 2006). Besides transforming growth factor-beta receptor 2 (Tgfrb2) signaling (Hosokawa et al., 2010), the identity of other factors regulating this early aspect of tongue morphogenesis is not known.

One key pathway responsible for establishing the proximal-distal organization of the pharyngeal arches is initiated by signaling from the endothelin-A receptor (Ednra) located on NCCs (Clouthier et al., 1998; Yanagisawa et al., 1998). Ednra signaling is induced soon after NCCs reach the pharyngeal arches by its ligand, endothelin-1 (Edn1) (Clouthier et al., 1998; Maemura et al., 1996; Miller et al., 2000; Yanagisawa et al., 1998). This initiates one or more signaling cascades responsible for NCC identity and fate within the mandibular pharyngeal arch (Clouthier et al., 2010; Clouthier and Schilling, 2004). Two induced factors, \textit{Dlx5} and \textit{Dlx6}, contribute to the ‘Dlx code’ hypothesized to establish proximal-distal identity within the arches (Depew et al., 2002; Depew and Simpson, 2006; Depew et al., 2005; Jeong et al., 2008). Loss of \textit{Ednra}, \textit{Edn1}, or \textit{Dlx5} and \textit{Dlx6} results in homeotic transformation of mandibular arch-derived bone and soft tissue structures into more maxillary-like deriveds (Beverdam et al., 2002; Depew et al., 2002; Ozeki et al., 2004; Ruest et al., 2004). However, aglossia is not observed in any mouse mutants in the Ednra pathway (Ednra, Edn1, \textit{Ece1}, \textit{Mef2c}, \textit{Dlx5}, \textit{Dlx6}) (Clouthier et al., 2010).

One direct transcriptional target of \textit{Dlx5} and \textit{Dlx6} is the gene encoding the basic helix-loop-helix transcription (bHLH) factor Hand2 (Charité et al., 2001). Although \textit{Hand2} mutant mice die by E10.5 owing to vascular defects (Thomas et al., 1998; Yamagishi et al., 2000), fate mapping \textit{Hand2} daughter cells using the \textit{Hand2} arch-specific enhancer (Charité et al., 2001) illustrated that these cells were found throughout most of the lower jaw (Ruest et al., 2003a). In addition, ventral arch cartilage derivatives in \textit{hand2} mutant zebrafish (\textit{han}6), which survive longer than mouse...
mutants), are severely affected (Miller et al., 2003) and early gene expression is disrupted. In mice, targeted deletion of the Hand2 arch-specific enhancer (Hand2\textsuperscript{H11032}) results in only partial loss of the Hand2 expression domain in the mandibular arch and limited developmental changes (Yanagisawa et al., 2003). However, NCC-specific deletion of Hand1 (the other Hand gene expressed in the pharyngeal arches) (Clouthier et al., 2000; Cserjesi et al., 1995) on the hypomorphic Hand2\textsuperscript{H11032} background resulted in cleft palate and a small, thickened mandible (Funato et al., 2009). Based on in vitro findings, the changes in the mandible were hypothesized to result from a loss of Hand2-Runx2 interaction, leading to changes in bone ossification. However, the full function of Hand2 in facial morphogenesis is not clear, as Hand2 was not completely inactivated in these mice.

To address problems associated with global Hand2 inactivation, two groups have created Hand2 conditional knockout mice. Conditional ablation of Hand2 in NCCs has revealed a crucial role for Hand2 in the development of sympathetic ganglion neurons (Hendershot et al., 2008, 2007), the enteric nervous system (Hendershot et al., 2007), D’Autreaux et al., 2007) and the cardiac outflow tract (Holler et al., 2010). In addition, conditional inactivation of Hand2 in the developing palatal epithelium has uncovered a role for Hand2 in palatal shelf fusion (Xiong et al., 2009). However, the role of Hand2 in NCC patterning and differentiation during craniofacial development has yet to be examined. In this report, we show that inactivating Hand2 specifically in NCCs leads to changes in jaw development, with the most striking being aglossia. These changes are preceded by an earlier failure to downregulate Dlx5 and Dlx6 expression in the distal mandibular arch, indicating that Hand2-mediated repression of Dlx5 and Dlx6 expression is crucial for establishing a domain in the mandibular arch that is conducive for tongue mesenchymal morphogenesis.

MATERIALS AND METHODS

Mice

Hand\textsuperscript{fl/+;Wnt1-Cre} (Hand\textsuperscript{fl/+} (Hendershot et al., 2007; Hendershot et al., 2008), Hand\textsuperscript{cko} (Hendershot et al., 2008), Wnt1-Cre (Danilian et al., 1998) and R26R (Soriano, 1999) mice have been described previously.

Breeding and genotyping

Hand\textsuperscript{fl/+} mice were bred with Wnt1-Cre transgenic mice to generate Hand\textsuperscript{fl/+}, Wnt1-Cre animals. Hand\textsuperscript{fl/+}, Wnt1-Cre mice were bred with Hand\textsuperscript{cko} mice to generate Hand\textsuperscript{fl/+}, Wnt1-Cre embryos (referred to as Hand\textsuperscript{fl/+}). For fate-mapping experiments, Hand\textsuperscript{fl/+} mice were bred with R26R mice (Soriano, 1999) to homozygosity. These mice were then bred with Hand\textsuperscript{fl/+}, Wnt1-Cre animals to create Hand\textsuperscript{fl/+}, Wnt1-Cre;R26R\textsuperscript{+/-} embryos. Hand\textsuperscript{fl/+}, Cre and lacZ genotyping were performed as described previously (Ruest and Clouthier, 2009; Soriano, 1999; Hendershot et al., 2008).

Skeleton staining

Skeleton (Ruest and Clouthier, 2009) and cartilage (Clouthier et al., 1998) staining was performed as described previously. Stained bone and cartilage preparations were analyzed and photographed using an Olympus SZX12 stereomicroscope fitted with a DP11 digital camera.

Whole-mount \( \beta \)-galactosidase staining

\( \beta \)-galactosidase staining was performed as described previously (Ruest et al., 2003a).

Histology

For histological analysis, E18.5 embryos were fixed, embedded in paraffin, sectioned and stained as described previously (Ruest et al., 2004). For immunostaining, sections were incubated with monoclonal anti-skeletal myosin (1:400; MY-32; Sigma, St Louis, MO, USA), anti-TROMO-1 (1:25; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and/or Alexa Fluor 568 phalloidin (Invitrogen) as described previously (Clouthier et al., 1997). Trichrome staining was performed as described previously (Clouthier et al., 1997). After staining, all sections were examined and photographed with an Olympus BX50 compound microscope fitted with an Olympus DP71 digital camera.

In situ hybridization

Whole-mount in situ hybridization (ISH) analysis was performed as described previously (Clouthier et al., 1998) using digoxigenin (DIG)-labeled antisense cRNA riboprobes against Dlx1, Dlx2, Dlx3, Dlx5, Dlx6, Foxf1 (Foxf1 – Mouse Genome Informatics), goosecoid homeobox (Gsc – Mouse Genome Informatics), Hand1, Hand2, Msx1, Msx2, Pitx1, Pitx2, Runx2, sonic hedgehog and Twist1. Embryos were photographed using an Olympus SZX12 microscope as described above.

Real-time PCR

Mandibular pharyngeal arches were collected and stored in RNA Later (Ambion) until genotyped. RNA was collected from arches using the QiAShredder and RNeasy kits (Qiagen). cDNA was prepared from total RNA using the Quantitect cDNA Synthesis Kit (Qiagen). Real-time quantitative PCR was performed using 5 ng of cDNA and the Quantitect SYBR Green PCR Kit (Qiagen), including Quantitect Assay primers (Qiagen). RT-PCR and data analysis was performed using a MyiQ2 machine (BioRad).

Volume measurements for the Runx2 expression domain

Volume measurements for Runx2 expression following ISH were conducted using ImageJ software (http://rsb.info.nih.gov/ij/) (Abramoff et al., 2004). Briefly, the scale (pixels/micron) was calculated using a micrometer and the ImageJ ruler tool, then was set globally in ImageJ. A region of interest (ROI) was specified using the polygon tool. The lower jaw ROI was specified as the entire lower jaw. We included the tongue in control embryos to ensure that our area measurements between control and Hand2\textsuperscript{cko} embryos were equivalent (as Hand2\textsuperscript{cko} embryos lack a tongue). The Runx2 ROI was specified as the entire region exhibiting DIG reactivity. Once the ROIs were outlined, the total area contained in the outlined region was calculated in ImageJ using the Measure tool. The percentage of expression was calculated by dividing the Runx2 ROI area by the lower jaw ROI area. Measurements were conducted on three embryos of each genotype and values averaged.

Analysis of cellular proliferation and apoptosis

Three 7 \( \mu \)m sections through the rostral, middle and caudal aspects of the mandibular arch, each separated by 60 \( \mu \)m, were used. Analysis of cell death at E10.5 was performed using the DeadEnd Labeling Kit (Promega) as described previously (Abe et al., 2007). Detection of cell death at E11.5 was performed using the In Situ Cell Death Detection Kit (Roche). Proliferating cells at both ages were detected using 5-ethyl-2’-deoxyuridyline (EdU) incorporation and the Invitrogen Click-It EdU Kit (Promega). At E10.5, these assays were conducted on consecutive sections; at E11.5, assays were performed on the same section. After staining, slides were counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, 2.0 \( \mu \)g/ml) for 10 minutes, washed and coverslipped using ProLong Gold Antifade Reagent (Invitrogen). Images were captured using the DP71 digital camera, with reconstruction of the entire arch region accomplished using TrakEM2, a component of Fiji (http://pacific.mpi-cbg.de). To quantify cell death and proliferation in specific arch regions, a grid was manually placed over the arch image using Adobe Photoshop. Vertical grid lines were placed on the midline and the outermost regions of the left and right lateral arch halves. The midline of the left or right arch half was then calculated by dividing the distance between lateral outer edge and arch midline in half; this established the boundary between the anterior/posterior arch and the lateral arch. Horizontal grid lines were calculated by setting the upper and lower limits at the point where the arch midline reaches the ectoderm. The length between upper and lower horizontal lines was then divided in two to create the horizontal midline of the arch, separating the anterior and posterior arch. Cell counts within the left and right lateral regions and posterior and anterior regions were then
manually counted using the Cell Counter plugin for ImageJ. The final incidence of proliferation and cell death was calculated as the number of proliferating or TUNEL-positive cells as a percentage of total cells.

**Cell culture**

MC3T3 cells (ATCC, Manassas, VA, USA) were maintained in MEM alpha (Invitrogen) supplemented with 20% fetal bovine serum (Sigma), penicillin/streptomycin (Invitrogen), and fungizone (Invitrogen) at 37°C in a humidified chamber with 5% CO₂.

**Luciferase assays**

MC3T3 cells seeded on 12-well plates were transfected with 0.05 μg pRL-TK (Promega), 0.5 μg pGL3-156i-luc [generated by placing the 156i enhancer both 5’ and 3’ of the firefly luciferase cDNA in the pGL3 (Promega) vector] or pGL3-156iΔEbox-luc [created using the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene); primers 5’- CAAAATGGATGGCACTGAAGCTGGAGGCTTTGTTC-3’ and 5’- GAACAAAGCCTCCAGCTTCAGTGCCATCCATTG-3’ were used to change the putative E-box site CAGCTG to AAGCTG within the pGL2-156i-luc template vector], and 0.8 μg of each expression vector using Fugene 6 (Roche). Expression vectors included: pCS2+MT-DIx5, pCS2+MT-Hand2, pCS2+MT-Hand1 (gift from Hiromi Yanagisawa, University of Texas Southwestern Medical Center, Dallas, TX, USA) and pcDNA3.1+MT-ΔAN1.98-Hand2 (gift from Anthony Firulli, Indiana University School of Medicine, Indianapolis, IN, USA). Experiments were performed in triplicate and collected 24 hours after transfection. Lysate was prepared and firefly (experimental reporter) and renilla (normalizing reporter) luciferases were measured simultaneously using the Dual-Luciferase Reporter Assay System (Promega) and a Luminoskan Ascent 2.4 machine (Thermo Scientific). Activity of the experimental reporter was calculated by dividing the firefly luciferase value by the renilla luciferase value. Fold change was then calculated by dividing the expression vector activity by the empty expression vector (mock) activity.

**Statistical analysis**

All statistical analysis of results was performed using an unpaired two-tailed t-test with Prism software (GraphPad).

**RESULTS**

**Changes in facial development in Hand2fl/fl, Wnt1-Cre embryos**

Mice homozygous for the conditional allele (Hand2fl/fl) were viable, healthy and fertile (Hendershot et al., 2007; Hendershot et al., 2008) and showed no alterations in skull morphology (data not shown). We next crossed Hand2fl/fl mice with the Wnt1-Cre transgenic strain to target the Hand2 gene deletion to NCCs (Chai et al., 2000). Grossly, E18.5 Hand2fl/fl, Wnt1-Cre (Hand2cko) embryos had retrogrowtha and low-set pinnae (data not shown) along with mystacial vibrissae (the whisker-like sensory organs normally confined to the snout) on the lower jaw epithelium (data not shown).

Alizarin Red and Alcian Blue-stained E18.5 Hand2cko skeletons revealed a loss of both the angular processes of the mandible and the tympanic rings, significant retrogrowtha and an almost complete loss of Meckel’s cartilage (Fig. 1B,D,H,J,L). Bone structures resembling duplicated pterygoid bones were fused to the actual pterygoid bones (Fig. 1F), indicating a role for Hand2 in establishing or maintaining NCC identity. Also, in contrast to Hand2fl/fl embryos (Barbosa et al., 2007; Funato et al., 2009; Yanagisawa et al., 2003), gross cleft palate was not observed (Fig. 1F), indicating that Hand2 does not play a direct role in NCC-mediated palatal shelf elevation. In the lower jaw, the mandible was dysmorphic, resembling the mandible seen in Hand2fl/fl, Handfl/fl, Wnt1-Cre embryos (Barbosa et al., 2007; Funato et al., 2009). A second bilateral membranous bone ran medial to the mandible (asterisks in Fig. 1H,J), though its identity was not clear. Other changes included aberrantly ossified body and lesser horns of the hyoid bone, with the latter fused, either unilaterally or bilaterally, to duplicated palatine bones (Fig. 1H,J). In addition, both lesser horns also formed articulations with the malformed cartilage anlage of the mallei (Fig. 1H,J; data not shown),
whereas the greater horns articulated with the styloid process (data not shown). Finally, only a single midline incisor was observed in most embryos (Fig. 1H, J, L), though two closely abutting lower incisors were also observed in some embryos (data not shown). Because skull defects in E18.5 Hand2<sup>fl/fl</sup>; Wnt1-Cre embryos were identical to those observed in Hand2<sup>ko/ko</sup>; Wnt1-Cre embryos (data not shown), the remainder of our analysis was performed using Hand2<sup>ko/ko</sup>; Wnt1-Cre embryos. Taken together, these changes suggest that Hand2 plays crucial roles in both NCC patterning and establishment of NCC identity in the mandibular arch.

**Histological analysis of skull structures reveals aglossia**

In serial frontal sections through the head of E18.5 control and Hand2<sup>ko</sup> embryos, aberrant bone was observed running along the medial mandible (Fig. 2B). Only a single incisor was present along the midline, whereas upper and lower molar development appeared normal (Fig. 2B; data not shown). Mystacial vibrissae on the lower jaw epithelium were also observed (Fig. 2D), and rugae, raised epithelial ridges normally confined to the roof of the mouth, were duplicated on the lower oral cavity surface (Fig. 2D). That duplicated vibrissae and rugae were also observed in Edn1, Ednra and Dlx5/Dlx6 mutant embryos (Depew et al., 2002; Ozeki et al., 2004; Ruest et al., 2004) suggest that loss of Hand2 might contribute to these mandibular to maxillary transformations.

The most striking finding in Hand2<sup>ko</sup> embryos was the absence of a tongue (Fig. 2B,D,F,H,L). In frontal and sagittal sections, a cleft existed where the tongue should normally reside, though a small amount of muscle was present at the cleft base (asterisk in Fig. 2D; m in Fig. 2H,J). Although the tongue was absent, the tongue epithelium was present. In both control and Hand2<sup>ko</sup> embryos, the delineation between the non-keratinized and keratinized stratified squamous epithelium of tongue was readily apparent (red arrows in Fig. 2A,B; black arrows in Fig. 2F). Squamous cells overlying the keratinized epithelium were also present in control and Hand2<sup>ko</sup> embryos, though fewer were observed in Hand2<sup>ko</sup> embryos (Fig. 2E-H). We also identified taste buds in both control (Fig. 2I) and Hand2<sup>ko</sup> (Fig. 2J) embryos using an antibody against cytokeratin-8 (CK8, Troma-1; Krt8 – Mouse Genome Informatics), a taste bud marker (Thirumangalathu et al., 2009). Taken together, our results indicate that although a failure of NCC patterning, proliferation or differentiation leads to a near absence of tongue mesenchyme, partial tongue epithelial development occurs.

Tongue morphogenesis requires the interaction of NCC-derived cells and myoblasts (Hosokawa et al., 2010). Aglossia could, therefore, result from failed somitic myoblast migration to the future tongue domain. However, the muscle below the tongue of Hand2<sup>ko</sup> embryos was stained by phalloidin (an F-actin marker) (Fig. 2J). Similarly, anti-myosin staining was observed in the anterior lower jaw of Hand2<sup>ko</sup> embryos (white arrow in Fig. 2L), though it was far more scattered than that observed in control embryos (Fig. 2K). Thus, although we cannot rule out a partial myoblast migration defect, the aglossia does not appear to be due to a complete absence of myoblasts.

**Tongue mesenchyme morphogenesis is never initiated**

We next examined gross oral morphology in control and Hand2<sup>ko</sup> embryos between E12.5 and E13.5. As a marker, we used sonic hedgehog (Shh) expression, which is expressed by the incisor buds, palatal rugae and developing taste papilla of the tongue. At E12.5,
Hand2 function in craniofacial development

**Fig. 3. Tongue development of Hand2^cko embryos.** Shh expression in Hand2^wt (left column) and Hand2^cko (right column) mouse embryos. (A,B) Oral view of the upper jaw from E12.5 control and Hand2^cko embryos shows Shh expression in developing mystacial vibrissae (mv), incisors (in) and rugae (r). (C-F) In E12.5 control embryos, Shh expression is observed in the fungiform papilla of the tongue (t) (C,E). By E13.5, Shh expression was also observed in the anterior soft tissue and aberrant mystacial vibrissae (mv') (D). (G-H) At E13.5, Shh expression in the lower jaw of controls remains in fungiform papilla (arrow in G), whereas expression in the anterior oral cavity in Hand2^cko embryos has organized into horizontal stripes resembling rugae of the upper jaw (arrows in H). The aberrant proximal-distal seam has persisted in the lower jaw and joined two other seams in the anterior oral cavity that resemble the fusion point of the primary and secondary palates in the upper jaw (see blue lines in H'). md, mandible.

Shh expression in the upper jaw of control and Hand2^cko embryos was observed in the mystacial vibrissae, palatal rugae and incisor fields (Fig. 3A,B). In the lower jaw of control embryos, Shh expression was observed in the incisor domains and fungiform papilla on the tongue (Fig. 3C,E). By contrast, although Shh expression in E12.5 Hand2^cko embryos marked the incisor fields, expression was also observed in the anterior soft tissue and aberrant mystacial vibrissae (Fig. 3D,F), but was absent in the posterior soft tissue, consistent with absence of tongue development (asterisk in Fig. 3F). By E13.5, Shh expression in control embryos was still observed in the fungiform papilla (Fig. 3G). In E13.5 Hand2^cko embryos, Shh expression in the lower jaw had resolved into lateral lines that resembled developing palatal rugae (black arrows in Fig. 3H). Although a midline cleft was still present, it met two other clefts anteriorly that together formed the appearance of primary and secondary palates (see lines in Fig. 3H'). The primary and secondary palates are complex structures; not all aspects of these structures were duplicated in the lower jaw of Hand2^cko embryos. However, it does appear that loss of Hand2 disrupts tongue morphogenesis and leads to partial transformation of NCC fate from a mandibular to maxillary identity.

**Changes in proliferation and cell death in Hand2^cko embryos**

Aglossia could result from defects in NCC or somitic myoblast migration to the pharyngeal arches, or their subsequent proliferation and survival. Because Hand2 expression within the ectomesenchyme is first observed at ~E9.25-9.5 (Clouthier et al., 2000; Thomas et al., 1998), NCC migration defects were not expected (Abe et al., 2007; Chai et al., 2000). To confirm this, we crossed the Hand2 conditional mutant strain into the R26R Cre reporter strain (Soriano, 1999) and found that in E10.5 embryos, the distribution of β-galabeled cells was similar in the arches of both Hand2^fl/fl;R26R;Wnt1-Cre and Hand2^cko;R26R;Wnt1-Cre embryos (data not shown). This indicates that NCC migration was unaffected by loss of Hand2.

Conventional targeting of Hand2 in mice results in widespread mandibular arch mesenchyme apoptosis by E10.0 (Thomas et al., 1998). Thus, we examined changes in both cellular proliferation and apoptosis in E10.5 and E11.5 control and Hand2^cko mandibular arches using a grid pattern that would allow us to determine if and where (lateral, anterior or posterior) changes were occurring (Fig. 4A). Because the tongue arises from the posterior arch, changes in this region could provide a partial mechanism for the observed aglossia. We found that the percentage of proliferating cells was similar between control and Hand2^cko embryos at E10.5 in the lateral and posterior arch (Fig. 4B). However, in the anterior arch, EdU incorporation was slightly but significantly elevated. By E11.5, proliferation was statistically similar in control and Hand2^cko embryos throughout the mandibular arch (Fig. 4B). Examination of cell death using TUNEL revealed a significant increase in apoptotic nuclei in the lateral mandibular arch of both E10.5 and E11.5 Hand2^cko embryos compared with that observed in control embryos (Fig. 4C). Although the incidence of apoptosis trended higher in the posterior arch domain of E11.5 Hand2^cko embryos, the change was not statistically significant. These data indicate that decreased proliferation and increased cell death are not major contributors to the observed aglossia.

**Gene expression analysis of Hand2^cko embryos**

To define the molecular changes that accompany the observed cellular changes, we performed whole-mount ISH analysis on E10.5 control and Hand2^cko embryos. As expected, Hand2 expression was absent in the pharyngeal arches of Hand2^cko embryos (Fig. 5B). Surprisingly, expression of Hand1 was almost completely downregulated in the mandibular arch (Fig. 5D). This suggests that Hand1 expression in this area is under transcriptional control of another signaling pathway. Gsc, a gene involved in mouse middle ear development (Rivera-Perez et al., 1995; Yamada et al., 1995), is downregulated in the pharyngeal arches of hans6^zebrafish (Miller et al., 2003), a pattern also observed in the pharyngeal arches of Hand2^cko embryos (Fig. 5F). By contrast, the zebrafish msx genes msx1 and msx2 are upregulated in the pharyngeal arches of hans6 embryos (Miller et al., 2003). However, we found that the expression...
of Msx1 and Msx2 was unchanged in Hand2cko embryos (Fig. 5L,N), as were Pitx1 and Pitx2 (Fig. 5P,Q), both involved in lower jaw and incisor development (Gage et al., 1999; Lin et al., 1999; Liu et al., 2003; Mitsiadis and Drouin, 2008). Expression of Twist1 was also examined, as it can interact with Hand2 to influence limb development (Firulli et al., 2005; McFadden et al., 2002) and shows an expanded expression domain that parallels the decreased Hand2 expression domain in Ednra–/– embryos (Ruest et al., 2004). However, Twist1 expression in Hand2cko embryos did not appear grossly different (Fig. 5T). Expression of the distal-less gene family members Dlx2 and Dlx3 was also not noticeably changed (Fig. 5H,J). Finally, FoxF1 expression was examined, as it appears to be involved in distal lower jaw morphogenesis downstream of Shh (Jeong et al., 2004). However, FoxF1 expression was unchanged in Hand2cko embryos (Fig. 5V).
Loss of Hand2 leads to aberrant maintenance of both Dlx5 and Dlx6 expression

We also examined the expression of Dlx5 and Dlx6, two genes that induce arch expression of Hand2 (Charité et al., 2001; Depew et al., 1999; Depew et al., 2002). In E9.5 Hand2cko embryos, Hand2 expression was absent (data not shown), whereas Dlx5 and Dlx6 were both expressed throughout the mandibular arch of control and Hand2cko embryos (Fig. 6A-D). By E10.5, Hand2 expression in control embryos was observed in the disto-oral mandibular arch mesenchyme (Fig. 6E,E’), but was absent in Hand2cko embryos (Fig. 6F,F’). Dlx5 expression in E10.5 control embryos was downregulated in the distal and oral aspects of the mandibular arch but remained in the proximal arch (Fig. 6K,K’). By contrast, Dlx5 expression was present throughout the mandibular arch of E10.5 Hand2cko embryos (Fig. 6L,L’). By E11.5, Hand2 message was present on the disto-oral surface of the mandibular arch in control embryos corresponding to the lateral lingual swelling (Fig. 6G). As observed in E10.5 control embryos, this coincided with a repression of Dlx5 expression in the lateral lingual swelling (Fig. 6M). In E11.5 Hand2cko embryos, Hand2 expression was absent (Fig. 6H), with Dlx5 expression continuing in the disto-oral region (Fig. 6N). By E12.5, Hand2 expression in control embryos was confined to the tongue and distal mandibular arch (Fig. 6I), whereas Dlx5 expression was confined to the tongue and proximal jaw (Fig. 6J). In E12.5 Hand2cko embryos, Hand2 expression was absent (Fig. 6J) whereas Dlx5, owing to the absence of the tongue, was only observed in the proximal jaw (Fig. 6P).

Hand2 represses Dlx5 and Dlx6 expression through the Dlx5/Dlx6 pharyngeal arch-specific enhancer

To examine how Hand2 might repress Dlx5 and Dlx6 expression, we transfected MC3T3-E1 cells with a luciferase reporter construct under control of the arch-specific intergenic enhancer from the Dlx5/Dlx6 region. We measured luciferase activity induced by Dlx5 and Dlx6 promoters in Hand2fl/fl and Hand2cko cells. As expected, the activity in Hand2cko cells was lower than in control cells for both Dlx5 and Dlx6 promoters. These results suggest that Hand2 represses Dlx5 and Dlx6 expression through the Dlx5/Dlx6 arch-specific enhancer.
hand2 regulates mandibular arch patterning through negative regulation of Dlx5 and Dlx6

Dlx5 and Dlx6 induce Hand2 expression in the distal half of the mandibular arch (Charité et al., 2001). Here, we have shown that Hand2 then acts in a negative-feedback loop to shut down Dlx5 and Dlx6 expression in the Hand2 domain, acting through the Dlx5 and Dlx6 intergenic arch-specific enhancer (Fig. 8). Although it might be surprising that changes limited to this distal domain can cause such extensive changes in lower jaw development, we have shown through analysis of Hand2 daughter cell fate that the Hand2 domain contributes to the entire lower jaw and much of the middle ear (excluding the incus and stapes) (Ruest et al., 2003a). Further, Bronner-Fraser and colleagues have illustrated in axolotl that the distal half of the mandibular arch gives rise to the lower jaw (mandible) whereas the proximal portion of the mandibular arch gives rise to more proximal hinge structures of the jaw (the palaatoquadrate) (Cerny et al., 2004).

So why does the maintained presence of Dlx5 and Dlx6 lead to aglossia? Targeted disruption of smoothened (Smo – Mouse Genome Informatics) in the mandibular arch mesenchyme leads to loss of distal arch tissue and subsequent absence of the tongue and most of the lower jaw (Jeong et al., 2004). However, in these mutants, the expression of Fox genes, including FoxF1, was disrupted in the mandibular arch, accompanied by increased Hand2 expression and distal arch cell death. Such changes were not observed in Hand2cko embryos. Based on these findings, it does not appear that aglossia is due to absence of sufficient arch tissue. Rather, it would seem more likely that the distal arch tissue is repatterned owing to the presence of aberrant signals that include Dlx5 and Dlx6, with this disrupting normal developmental interactions with other cell types, including somitic myoblasts. That some tongue muscle is present in near-term embryos probably reflects the complex signaling environment required for proper tongue muscle morphogenesis (Noden and Francis-West, 2006).

The findings from this study support the ‘hinge and caps’ model of jaw development (for a review, see Depew and Simpson, 2006). This model proposes that the polarity and modularity of the upper and lower jaws is driven by the articulation of the first arch prominences (the hinge) and signals located on the distal aspects of the two prominences and from the lamboidal junction (where the maxillary prominence meets the olfactory placode) (the caps). Our
and colleagues proposed that Hand2 could both repress early ossification of mandibular arch tissue (Barbosa et al., 2007; Funato et al., 2009), loss of either of these functions could lead to premature ossification and thus mandibular hypoplasia. Here, we show a third potential mechanism in which Hand2 functions in a negative-feedback loop to inhibit distal Dlx5 and Dlx6 expression. As Dlx5 can induce Runx2 (Holleville et al., 2007; Miyama et al., 1999), failure to downregulate Dlx5 and Dlx6 expression in the distal arch mesenchyme would probably lead to enhanced Runx2 expression (as shown in the present study) and thus the premature establishment of signaling cascades favorable to bone formation. Enhanced Runx2 expression could also pattern neighboring cells that were competent to respond to Runx2 but do not normally see it and could explain the presence of the aberrant bone in the lower jaw of our Hand2<sup>cko</sup> embryos. It remains to be seen whether Runx2 binds Hand2 in vivo (Abe et al., 2009), though our model could allow aberrant Runx2 activity without a physical interaction.

**Hand gene dosage and bHLH dimer pools**

As findings from this and other studies emerge illustrating the importance of Hand family members in developmental processes, one aspect to consider is whether both Hand1 and Hand2 have unique or redundant functions. A neural crest-specific deletion of Hand1 on a hypomorphic Hand2 background leads to jaw defects (Barbosa et al., 2007; Funato et al., 2009), suggesting that Hand proteins act redundantly in far distal arch development (i.e. in the Hand2 domain in which Hand1 is expressed). However, it is not possible to prove this point specifically, as we showed that Hand2 regulates Hand1 expression in the arch. The converse (that loss of Hand1 in the NCC leads to loss of Hand2) is not true (Barbosa et al., 2007). One intriguing question to be addressed is whether the small amount of Hand1 remaining in the distal arch is functionally significant. Answering this will require NCC-specific deletion of both Hand1 and Hand2.

Another aspect to consider is how bHLH dimer pools change in response to loss of specific bHLHs within a cell. Although bHLHs can bind other factors through their 5' end (Bialek et al., 2004; Funato et al., 2009), they typically form dimer pairs with the ubiquitous class A bHLH molecules (such as E-proteins) or other class B bHLH proteins (Cai and Jabs, 2005; Firulli, 2003). When one dimer partner is removed, the stoichiometry of dimer pools is disrupted, allowing for potential changes in binding partners and subsequent aberrant gene regulation (Cai and Jabs, 2005). Dimer partner choice is known to influence strongly the cellular response to Twist family proteins (Firulli et al., 2003), with changes in Twist1-Hand2 dimerization resulting in Saethre-Chotzen syndrome (Firulli et al., 2005). In addition, conditional disruption of either Hand2 (this study) or Twist1 (Rinon et al., 2007) in NCCs results in defects in craniofacial bone and cartilage structures. To understand fully the role of Hand2 in facial morphogenesis, it will therefore be important to understand how loss of Hand2 affects the function of other Twist family members, including Twist1.

**Note added in proof**

While this manuscript was in review, Talbot et al. (Talbot et al., 2010) reported that the expression of several Dlx genes expands into the ventral arches of hand2 mutant zebrafish embryos.

**Fig. 8. Model of Hand2 activation and regulation of Dlx5 and Dlx6 during mandibular arch development.** At E9.5, ectodermal-derived Edn1 activates Ednra signaling in neural crest cells (NCCs), thus inducing Dlx5 and Dlx6 expression broadly within the mandibular arch. Dlx5 and Dlx6 in turn activate Hand2 expression distally and Dlx3 expression proximally. In E10.5 control animals, Dlx5 and Dlx6 expression is restricted from the distal arch by Hand2, which also activates or maintains expression of Hand1 and Gsc. In Hand2<sup>cko</sup> embryos, absence of Hand2 leads to continued Dlx5 and Dlx6 expression in the distal arch and aberrant expression of Runx2. In addition, Gsc expression is lost and Hand1 expression is greatly reduced.

findings support this model by illustrating that the loss of a cap signal (Hand2) specifically expressed in the distal mandibular arch mesenchyme disrupts jaw polarity (duplication of maxillary elements in the lower jaw). Further, our findings illustrate the importance of modal genes within the lower jaw, as improper regulation of cap signals (in this case, the failure to downregulate Dlx3 and Dlx6 expression in the distal arch) probably contributes to the observed Hand2<sup>cko</sup> phenotype in the jaw, including changes in distal jaw shape and fused incisors. It will be important to understand better the communication between Hand2 and the hinge region and to elucidate the molecules through which such communication occurs.

**Hand2 function is crucial for osteogenic fates**

Mouse embryos hypomorphic for Hand2 have premature ossification of mandibular arch tissue (Barbosa et al., 2007; Funato et al., 2009). Based on both in vitro and in vivo data, Yanagisawa and colleagues proposed that Hand2 could both repress early Runx2 expression and antagonize Runx2 activity via physical association and subsequent sequestration (Funato et al., 2009). Because Runx2 is required for both the maintenance and differentiation of preosteoblastic mesenchyme into osteoblasts and subsequent initiation of osteogenesis (Bialek et al., 2004; Flores et al., 2006; Goldring et al., 2006; Hinoi et al., 2006), loss of either of these functions could lead to premature ossification and thus mandibular hypoplasia. Here, we show a third potential mechanism in which Hand2 functions in a negative-feedback loop to inhibit distal Dlx5 and Dlx6 expression. As Dlx5 can induce Runx2 (Holleville et al., 2007; Miyama et al., 1999), failure to downregulate Dlx5 and Dlx6 expression in the distal arch mesenchyme would probably lead to enhanced Runx2 expression (as shown in the present study) and thus the premature establishment of signaling cascades favorable to bone formation. Enhanced Runx2 expression could also pattern neighboring cells that were competent to respond to Runx2 but do not normally see it and could explain the presence of the aberrant bone in the lower jaw of our Hand2<sup>cko</sup> embryos. It remains to be seen whether Runx2 binds Hand2 in vivo (Abe et al., 2009), though our model could allow aberrant Runx2 activity without a physical interaction.
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Competing interests statement
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