Endothelin-A receptor-dependent and -independent signaling pathways in establishing mandibular identity

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Summary

The lower jaw skeleton is derived from cephalic neural crest (CNC) cells that reside in the mandibular region of the first pharyngeal arch. Endothelin-A receptor (Ednra) signaling in crest cells is crucial for their development, as Ednra–/– mice are born with severe craniofacial defects resulting in neonatal lethality. In this study, we undertook a more detailed analysis of mandibular arch development in Ednra–/– embryos to better understand the cellular and molecular basis for these defects. We show that most lower jaw structures in Ednra–/– embryos undergo a homeotic transformation into maxillary-like structures similar to those observed in Dlx5/Dlx6–/– embryos, though lower incisors are still present in both mutant embryos. These structural changes are preceded by aberrant expansion of proximal first arch gene expression into the distal arch, in addition to the previously described loss of a Dlx6/Hand2 expression network. However, a small distal Hand2 expression domain remains. Although this distal expression is not dependent on either Ednra or Dlx5/Dlx6 function, it may require one or more GATA factors. Using fate analysis, we show that these distal Hand2-positive cells probably contribute to lower incisor formation. Together, our results suggest that the establishment of a ‘mandibular identity’ during lower jaw development requires both Ednra-dependent and -independent signaling pathways.

Key words: Mouse, Bone, Mandible, Patterning, Neural crest cell, Homeobox gene

Introduction

Craniofacial morphogenesis is orchestrated through a specific array of transcription factors, expressed in both spatially and temporally restricted manners, that directs formation of bone and cartilage (Cobourne and Sharpe, 2003; Francis-West et al., 1998; Graham and Smith, 2001). Many of these structures arise from cephalic neural crest (CNC) cells that emigrate to the pharyngeal arches (Le Douarin, 1982; Noden, 1983; Noden, 1988). CNC cells that contribute to the lower jaw skeleton arise from the posterior mesencephalon and hindbrain rhombomeres 1 (r1) and r2 (Coulby et al., 1993; Couly et al., 1996; Kontges and Lumsden, 1996; Lumsden et al., 1991).

Although the development of more caudal NC cell populations is partially regulated by Hox genes (Hunt et al., 1991; Prince and Lumsden, 1994), CNC cells within the first mandibular arch do not express Hox genes, a crucial aspect for proper first arch patterning (Couly et al., 1998; Creuzet et al., 2002). Mandibular arch CNC cells were initially believed to carry programming information with them from the midbrain/hindbrain region (Noden, 1983). However, it currently appears that environment signals provide patterning information to CNC cells. These signals may begin very early in development, as foregut endoderm in the chick is crucial for proper patterning of mandibular arch derivatives, including both size and polarity of structures along the embryonic axis (Couly et al., 2002). Similarly, ventral cartilage development (including Meckel’s cartilage) is disrupted in the zebrfish cas mutant, which contain very little endoderm (David et al., 2002). One potential mediator of endoderm signaling appears to be fgf3, though numerous other molecules are probably involved. In addition to this early requirement, various other secreted molecules from the surrounding ectoderm, core paraxial mesoderm and pharyngeal pouch endoderm can influence CNC cell development once the CNC cells arrive in the mandibular arch (Cobourne and Sharpe, 2003; Graham and Smith, 2001; Jernvall and Thesleff, 2000; Schilling and Kimmel, 1997; Trainor and Krumlauf, 2000).

One factor involved in CNC cell development is endothelin 1 (Edn1), a 21 amino acid peptide secreted by pharyngeal arch ectoderm, core paraxial mesoderm and pharyngeal pouch endoderm (Clouthier et al., 1998; Maemura et al., 1996; Yanagisawa et al., 1998b). Edn1 binds to the endothelin A receptor (Ednra) found on cephalic and cardiac NC cells. Targeted inactivation of Edn1 (Kurihara et al., 1994), endothelin converting enzyme 1 (Ece1; the enzyme that cleaves Edn1 from an inactive to active peptide) (Yanagisawa et al., 1998a) or Ednra (Clouthier et al., 1998) in the mouse results in severe craniofacial and cardiovascular defects. This is due in part to aberrant expression of genes involved in post-
migratory NC cell development (Clouthier et al., 1998; Clouthier et al., 2000; Ivey et al., 2003; Thomas et al., 1998). Ednra signaling during CNC cell development appears conserved among vertebrates, as pharmacological antagonism of Ednra in the rat (Spence et al., 1999) or chick (Kempf et al., 1998) results in similar craniofacial defects as those observed in Ednra−/− mice. Similarly, an edn1 mutation in zebrafish, termed suck or suc1t1, results in disruption of most cartilages of the ventral (dental) jaw (Kimmel et al., 2003; Miller and Kimmel, 2001; Miller et al., 2000).

The distal-less homeobox gene family member Dlx6 is a downstream effector of Ednra signaling in the mouse (Charité et al., 2001), which in turn induces expression of the bHLH transcription factor dHAND/Hand2 (Charité et al., 2001; Yanagisawa et al., 2003). Not surprisingly, Hand2 is one of several mandibular arch genes whose expression is disrupted in Dlx5/Dlx6−/− mouse embryos (Beverdam et al., 2002; Depew et al., 2002). In addition, maxillary first arch gene expression expands into the mandibular arch. In term Dlx5/Dlx6−/− embryos, most mandibular arch-derived bone and cartilage are missing, instead replaced with structures that appear to be mirror image duplications of maxillary structures. These findings suggest that Dlx5 and Dlx6 provide a ‘mandibular identity’ to the mandibular arch NCs.

As Dlx6 is a downstream effector of Ednra signaling, we have re-examined the development of the lower jaw in Ednra−/− embryos and followed the fate of specific populations of mandibular mesenchymal cells during this developmental process. We find that most structures of the lower jaw undergo a homeotic transformation into maxillary-like structures, with these changes reflected in earlier disruption of mandibular arch gene expression. However, normal gene expression is partially maintained in a distal mandibular arch domain that appears to be later involved in lower incisor development. This suggests that although Ednra signaling is crucial for patterning most of the CNC-derived mesenchyme and surrounding epithelium of the mandibular arch by initiating a Dlx5/Hand2 gene expression pathway, a region of the distal arch appears to be patterned by Ednra-independent mechanisms.

### Materials and methods

#### Mouse lines and genotyping

Generation and genotyping of Ednra−/− (ETα−/−) (Clouthier et al., 1998), Gata3−/− (Lim et al., 2000), Dlx5/Dlx6−/− (Beverdam et al., 2002), R26R (Soriano, 1999) and Hand2-Cre [referred to as dHAND-Cre (Ruest et al., 2003)] lines have been previously described.

#### Skeletal analysis

To analyze bone and cartilage development, E18.5 embryos were stained as previously described (McLeod, 1980). Briefly, E18.5 embryos were collected, skinned and eviscerated. The skeletons were then fixed in 95% ethanol for 3 days followed by 100% acetone for 2 days. Embryos were then stained for 5 days in 0.1% Alcian Blue (stock solution: 0.3% in 70% ethanol) and 0.005% Alizarin Red (stock solution: 0.1% in 95% ethanol) in 70% ethanol/5% glacial acetic acid at 37°C with periodic rotation. After staining, embryos were dehydrated by soft tissue resembling the soft tissue of the snout, complete with mystacial vibrissae (see black arrows in Fig. 1B inset). Within the oral cavity, palatal rugae were present in Ednra−/− embryos (Fig. 1A).

β-Galactosidase staining

To examine β-gal staining in whole embryos, E10.5 and E16.5 Ednra+/−:R26R;Hand2-Cre and Ednra−/−:R26R;Hand2-Cre embryos were collected and fixed for 1 hour in 4% paraformaldehyde. Embryo staining and photography was performed as previously described (Ruest et al., 2003). Stained E16.5 embryos were cleared for 1.5 to 2 hours in benzyl benzoate/benzyl alcohol (1:2) with rotational mixing and then photographed.

To analyze β-gal staining in embryo sections, E15.5 Ednra+/−:R26R;Hand2-Cre and Ednra−/−:R26R;Hand2-Cre embryos were collected, snap-frozen in OCT freezing media in a dry ice/ethanol bath, sectioned and stained as previously described (Ruest et al., 2003). Sections were counterstained with nuclear Fast Red and coverslipped in DPX mounting media (BDH).

#### In situ hybridization analysis

Gene expression in whole mount was analyzed using digoxigenin-labeled RNA riboprobes against Bmp4 (Furuta and Hogan, 1998), Dlx1 (McGuinness et al., 1995), Dlx2 (Robinson andMahon, 1994), Dlx5 (Liu et al., 1997), Dlx6 (Charité et al., 2001), Hand2 (Srivastava et al., 1997), Gata3 (George et al., 1994), Mxcl (Thomas et al., 1998), Twist (Chen and Behringer, 1995) and Wnt5a (Yamaguchi et al., 2000) as previously described (Clouthier et al., 1998). For sectional in situ hybridization analysis, E16.5 Ednra+/− and Ednra−/− embryos were embedded in OCT, sectioned at 14 µm onto plus-coated slides and hybridized at 65°C with a digoxigenin-labeled RNA riboprobe against Hand2. After color development, slides were dehydrated, coverslipped and photographed. For all in situ hybridization analyses, a minimum of three embryos of each genotype were examined per probe.

### Results

#### Loss of Ednra signaling results in homeotic transformation of the lower jaw

We have previously shown that targeted disruption of Ednra in mice results in neonatal lethality owing to mechanical asphyxia brought about by abnormal fusion of the lesser horns of the hyoid to the pterygoid bones, effectively closing the upper trachea (Clouthier et al., 1998). We undertook a detailed analysis of craniofacial bone development in Ednra−/− embryos. Compared with E18.5 wild-type embryos (Fig. 1A), Ednra−/− embryos had a shortened lower jaw that was covered by soft tissue resembling the soft tissue of the snout, complete with mystacial vibrissae (see black arrows in Fig. 1B inset). Within the oral cavity, palatal rugae were present in Ednra−/− embryos on both the palate and floor of the mouth (Fig. 5G,H).

Analysis of stained skeleton preparations illustrated that bone in the lower jaw of Ednra−/− embryos resided proximally (Fig. 1D). This bone, described below, was flattened and aberrantly attached to the jugal bone (j) by a bone that appeared to be a mirror image duplication of the jugal bone (Fig. 1D,F,H). This pseudo-jugal bone (j*) formed a joint with the jugal bone, although both were smaller in size than the jugal bone of wild-type embryos (Fig. 1C,E,G).

On ventral view, the bone in the lower jaw in Ednra−/− embryos appeared to be a mirror image duplication of the maxilla [Fig. 1F,H; termed pseudo-maxilla (mx*)], although it was significantly smaller than the real maxilla (Fig. 1E,G). Similar to the pseudo-maxilla observed in Dlx5/Dlx6−/− embryos (Beverdam et al., 2002; Depew et al., 2002), this pseudo-maxilla contained foramina and a second set of palatine bones (pt*; Fig. 1F,H,J). These bones projected towards each
other and elevated as though forming palatal shelves (Fig. 1J), similar to those observed in wild-type embryos (Fig. 1I), though some variation in the extent of apposition was observed among mutant embryos. As observed for other bones, both the original and pseudo-palatines bones in Ednra−/− embryos were smaller than the palatine bones of wild-type embryos, unlike relatively normal sized structures and pseudo-structures found in Dlx5/Dlx6−/− embryos. These pseudo-palatine bones were fused with other aberrant membranous bones that appeared to be duplications of the pterygoid bones (data not shown). Depew et al. (Depew et al., 2002) have hypothesized that these ectopic bones arise from mesenchyme that normally forms the tympanic and gonial bones, structures also absent in Ednra−/− embryos (Fig. 1D), though we will limit our further analysis to more distal facial structures. Likewise, although we will not discuss defects in second arch-derived elements (such as the hyoid), these structures appear to undergo changes in shape and/or size rather than to undergo any homeotic change (Fig. 1D and data not shown)

In the distal mandible, incisors were present in most mutant embryos examined but were set only in a small amount of alveolar bone and residual cartilage (Fig. 1D,H,J; insets in Fig. 1D,F). The body of Meckel’s cartilage was absent, unlike the apparent transformation of Meckel’s cartilage into a pseudo-lamina obturans observed in Dlx5/Dlx6−/− embryos (Beverdam et al., 2002; Depew et al., 2002). Furthermore, the rostral process of Meckel’s cartilage was hypoplastic at E14.5 (data not shown) and E18.5 (Fig. 1D,H,J; insets in Fig. 1D,F), in contrast to more extensive cartilage found in Dlx5/Dlx6−/− embryos, suggesting a loss of precursor cells in the absence of Ednra signaling.

Duplication of the alisphenoid bones was also observed in Ednra−/− embryos. The ala temporalis region of the alisphenoid, consisting of two cartilage processes that normally fuse to the lamina obturans (Fig. 1K, lower structure), was composed of four processes in Ednra−/− embryos (Fig. 1K, upper structure). The extra processes attached to a structure whose shape suggested it was a duplicated lamina obturans (lo*) (Fig. 1L, upper structure), though this structure was smaller than the normal lamina obturans. Taken together, it appears that most structures derived from the mandibular (distal) arch appeared to have undergone a transformation into maxillary (proximal) structures.

Gene expression boundaries in the mandibular arch

Ednra signaling is crucial for proper expression of transcription factors involved in mandibular arch development (Clouthier et al., 1998; Clouthier et al., 2000; Park et al., 2004; Thomas et al., 1998). Similar changes are observed in suc1/tel zebrafish mutants, along with expansion of more dorsal (proximal) first arch gene expression into the ventral (distal) arch, suggesting a loss of boundary identity (Miller et al., 2003). As similar

Fig. 1. Analysis of mandible structure in Ednra−/− embryos. E18.5 wild-type (Ednra+/+; A,C,E,G,I) and Ednra−/− (B,D,F,H,J) embryos. (A,B) Unlike wild-type embryos (A), the lower jaw of an Ednra−/− embryo is shortened and is covered by vibrissae (asterisks in inset in B denote follicles; arrows denote actual vibrissae) (B). (C-J) Alizarin Red and Alcian Blue staining to visualize bone and cartilage structures, respectively. (C,D) In a lateral view, the mandible in Ednra−/− embryos appears shortened and flattened (D) compared with that of the wild-type embryo (C). This bone, the pseudo-maxilla (mx*) is aberrantly connected to the jugal bone (j) through a bone resembling a duplicated jugal (j*). Incisors (i) of Ednra−/− embryos are present (inset in D) but are set primarily in mesenchyme. (E,F) A ventral view shows bilateral foramina in the pseudo-maxilla of Ednra−/− embryos (F) as well as the presence of incisors (insets). (G,H) Removal of other structures emphasizes the mirror image appearance of the pseudo-maxilla and pseudo-jugal bones in Ednra−/− embryos (H). (I,J) A frontal view of the pseudo-maxilla shown in H shows pseudo-palatine bones (pl*) attached to the pseudo-maxilla in Ednra−/− embryos (J). (K,L) Two views of the alisphenoid bone in wild-type (Ednra+/+; bottom) and Ednra−/− (top) embryos. Both the ala temporalis (at) and lamina obturans (lo) regions of the alisphenoid appear to be duplicated in Ednra−/− embryos. at*, duplicated ala temporalis; bs, basisphenoid; lo*, duplicated lamina obturans; h, hyoid.
boundary changes are observed in Dlx5/Dlx6<sup>−/−</sup> mouse embryos (Beverdam et al., 2002; Depew et al., 2002), we examined the expression of multiple genes expressed in different regions of the first pharyngeal arch in both wild-type and Ednra<sup>−/−</sup> embryos.

Wnt5a expression, observed in the proximal portion of the mandibular arch of E10.5 wild-type embryos (Fig. 2A), spread over the caudal half of the mandibular arch in Ednra<sup>−/−</sup> embryos (arrows in Fig. 2B). Similarly, Dlx1 expression in Ednra<sup>−/−</sup> embryos extended further distally along the rostral half of the mandibular arch compared with wild-type embryos (compare yellow lines in Fig. 2C,D). Expression changes were also observed for Msx1 and Twist, two transcription factors involved in multiple aspects of lower jaw development (Chen and Behringer, 1995; Han et al., 2003; Satokata and Maas, 1994; Soo et al., 2002). In E10.5 wild-type mouse embryos, Msx1 expression covered the distal half of the mandibular arch, while in Ednra<sup>−/−</sup> embryos, expression appeared to slightly expand more proximally (Fig. 2E,F). Likewise, Twist expression expanded distally in Ednra<sup>−/−</sup> embryos compared with the pattern observed in wild-type embryos (broken yellow lines in Fig. 2G,H).

Changes in epithelial gene expression were also observed in Ednra<sup>−/−</sup> embryos. In E10.5 wild-type embryos, Dlx2 is expressed within the proximal mandibular arch mesenchyme and distal mandibular arch epithelium (Fig. 2I), with little overlap between the two domains (Thomas et al., 2000). However, epithelial Dlx2 expression was lost in Ednra<sup>−/−</sup> embryos, while mesenchymal expression expanded distally (Fig. 2J). As epithelial Dlx2 expression in the mandibular arch appears to be partially regulated by Bmp4 (Thomas et al., 2000), we also examined Bmp4 expression. In contrast to wild-type embryos, in which Bmp4 expression was observed along the distal half of the rostral epithelium (arrow in Fig. 2K and inset), Bmp4 expression was absent along the epithelium of Ednra<sup>−/−</sup> embryos (arrow in Fig. 2L and inset). Taken together, our in situ hybridization analysis suggests that gene expression boundaries between the proximal and distal regions of the first arch are disrupted in the absence of Ednra signaling.

**Hand2 expression in Ednra<sup>−/−</sup> embryos**

As Hand2 expression is downregulated in both Ednra<sup>−/−</sup> and Dlx5/Dlx6<sup>−/−</sup> embryos (Beverdam et al., 2002; Clouthier et al., 2000; Depew et al., 2002) and both embryos show homeotic changes in lower jaw structures, we closely examined expression of Hand2, Dlx5 and Dlx6 in the developing mandible of Ednra<sup>−/−</sup> embryos. In contrast to the arch expression in wild-type embryos (Fig. 2M,O), Dlx5 and Dlx6 expression in Ednra<sup>−/−</sup> embryos was downregulated throughout the mandibular arch mesenchyme (Fig. 2N,P). As we have previously shown (Clouthier et al., 2000), Hand2 expression was also absent in the mandibular arch of Ednra<sup>−/−</sup> embryos (Fig. 2R), although a small Hand2 expression domain could be detected within the distocaudal arch (yellow arrows in Fig. 2R). This domain, also present in Edn1<sup>−/−</sup> embryos (Thomas et al., 1998), correlated with the domain in which neither Dlx5 nor Dlx6 is expressed (Fig. 2M,O). Sections through this distal region in wild-type embryos illustrated that Hand2 expression was confined to the mesenchyme, whereas in Ednra<sup>−/−</sup> embryos, Hand2 expression was observed in both the mesenchyme and overlying epithelium (data not shown).
To further examine this distal Hand2 domain, we took advantage of a two-component genetic system that allows us to examine both active and fated expression of Hand2 in the mandibular arch and its derivatives (Ruest et al., 2003). This system consists of a pharyngeal arch-specific Hand2 enhancer fused to a Cre cDNA. When Hand2-Cre mice (previously referred to as dHAND-Cre) are crossed with R26R mice (Soriano, 1999), β-galactosidase (β-gal) activity observed in all cells in which Hand2 is or was expressed. In E10.5 Ednra+/-;R26R;Hand2-Cre embryos, β-gal staining was observed throughout most of the mandibular arch (Fig. 3A,C). Analysis of both sagittal (Fig. 3E) and frontal (Fig. 3G) sections through the mandibular arch confirmed that labeled cells were confined to the mesenchyme. In Ednra-/-;R26R;Hand2-Cre embryos, β-gal stained cells were only observed in the distocaudal arch (Fig. 3D). In sections through this region, scattered labeled cells were observed in the arch mesenchyme, with a higher contribution in the epithelium (Fig. 3F,H).

As Dlx5 and Dlx6 appear to regulate Hand2 expression, it is possible that the distal Hand2 expression observed in Ednra-/- embryos could be the result of aberrant expression of either Dlx5 or Dlx6. We therefore examined Hand2 expression in the distal mandibular arch of Dlx5/6-/- embryos. In accordance with previous studies (Beverdam et al., 2002; Depew et al., 2002), we found that Hand2 expression was downregulated in most of the mandibular arch in E10.5 Dlx5/6-/- embryos (Fig. 3J,L).

The Hand2 enhancer driving Cre expression in Hand2-Cre transgenic mice is the only mandibular arch-specific cis-regulatory element thus far identified for Hand2 (Charité et al., 2001; McFadden et al., 2000). Although we have previously shown that this enhancer is regulated in part by Dlx6 (Charité et al., 2001), our current findings indicate that other factors may function in combination with Dlx6 to direct distal Hand2 expression. We therefore examined the sequence of the arch enhancer using MatInspector, a transcription factor binding site analysis program developed by Genomatix (www.genomatix.de). One site identified within the enhancer was the consensus-binding site for GA TA3 with an overall similarity of 0.831 (actual sequence: aggaGATCaagaa, with the underlined base pairs showing the highest conservation in mathematical models) (data not shown). GA TA3 is a member of the GA TA family of zinc-finger transcription factors shown). GA TA3 is a member of the GA TA family of zinc-finger transcription factors (George et al., 1994; Massari and Murre, 2000). Targeted inactivation of mouse Gata3 results in embryonic lethality by E11.0 in part because of noradrenaline deficiency, although this lethality can be rescued by feeding pregnant female mice a high catechol diet (Lim et al., 2000) (K.-C. Lim, unpublished). At E16.5, rescued mutant embryos show hypoplasia of the mandible, tongue and tooth primordia (Lim et al., 2000), suggesting a function for GATA3 in distal mandibular arch development. We therefore

**Fig. 3. Hand2 expression in Ednra-/- and Dlx5/6-/- embryos.**

(A-H) Ednra+/-;R26R;Hand2-Cre and Ednra-/-;R26R;Hand2-Cre embryos stained in whole mount for β-gal activity and shown in both lateral (A,B) and ventral views (C,D). For ventral views, the heart and outflow tract have been removed. Embryos (not necessarily those shown in A-D) were then sectioned along either sagittal (E,F) or frontal (G,H) planes and counterstained with nuclear Fast Red. (A,C) In E10.5 Ednra+/-;R26R;Hand2-Cre embryos, β-gal-labeled cells are observed throughout pharyngeal arches 1 and 2. (B,D) In Ednra-/-;R26R;Hand2-Cre embryos, labeled cells are observed only along the distocaudal aspect of mandibular arch. (E,G) In sagittal sections through the arches of Ednra-/-;R26R;Hand2-Cre embryos, labeled cells are confined to the neural crest-derived mesenchyme. (F,H) In sagittal sections through the arches of Ednra+/-;R26R;Hand2-Cre embryos, scattered labeled cells were observed in the distal mesenchyme, with more intense labeling observed in cells in the surrounding arch epithelium. (I-L) Analysis of Hand2 expression in Dlx5/6-/- embryos. Normal Hand2 expression (I,K) is absent in the pharyngeal arches of Dlx5/6-/- embryos (J,L), although residual expression is still observed in the distal mandibular arch (yellow arrow in inset). A small expression domain is present in the second arch (black arrows in L).
examined Hand2 expression in diet-rescued E10.5 Gata3+/− embryos. Although these embryos were found to have hypoplastic pharyngeal arches, suggesting cell death or decreased proliferation, Hand2 expression was still observed in the mandibular and second arches. However, this expression was confined to the rostral half of each arch (Fig. 4B,D), but the extent of confinement was variable (data not shown). Although this could imply a direct function for GATA3 in Hand2 expression, we also examined Dlx5 expression in Gata3+/− embryos, as GATA3 could indirectly regulate Hand2 expression through Dlx5. Similar to Hand2 expression, Dlx5 expression was also downregulated in the caudal half of the mandibular arch of Gata3−/− embryos examined (Fig. 4F,H).

If GATA3 plays a crucial role in distal mandibular arch morphogenesis, including contributing to the Ednra-independent expression of Hand2, our hypothesis dictates that Gata3 expression would continue in the absence of Ednra signaling. In support of this, mesenchymal expression of Gata3 expression was present Ednra−/− embryos (Fig. 4J,L), with expression levels at least equal to that observed in wild-type embryos (Fig. 4I,K).

**Fate of Hand2 daughter cells in the mandibular arch cells in Ednra−/− embryos**

Hand2 expression in the distal mandibular arch in the absence of Ednra signaling suggests that Hand2 may have an Ednra-independent role in lower jaw development. To investigate this aspect, we again took advantage of the R26R;Hand2-Cre mice to follow the fate of these distal cells. In E16.5 Ednra+/+;R26R;Hand2-Cre embryos, the entire lower jaw was composed of labeled cells (Fig. 5A,C,E). By contrast, labeled cells within the lower jaw of Ednra−/−;R26R;Hand2-Cre embryos were primarily observed in the cleft between the two poorly fused halves (Fig. 5B,D), with labeled cells also observed in the hypoplastic tongue and lower incisors (Fig. 5F).

To better examine the spatial distribution of cells, frozen sections of littermate embryos were stained for β-gal activity. In Ednra+/+;R26R;Hand2-Cre embryos, stained cells were observed throughout the lower jaw, including in the mandible, Meckel’s cartilage and surrounding connective tissue (Fig. 5G; data not shown). Labeled cells were also present in the tongue and lower incisor dental pulp but were not observed in the dental lamina (asterisk in inset, Fig. 5G). In Ednra−/−;R26R;Hand2-Cre embryos, labeled cells were devoid from most of the lower jaw, although presence was in the small amount of bone and cartilage that remained under the incisors (Fig. 5H). Labeled cells were also observed in the area of odontoblast formation (black arrows in inset), relatively evenly spaced with groups of unlabeled cells. Labeled cells were scattered in the remainder of the dental pulp, with the overall contribution lower than that observed in wild-type embryos. The dental lamina epithelium also contained scattered labeled cells (asterisk in inset, Fig. 5H), suggesting that the Hand2-Cre transgene was expressed at some point in oral epithelium of Ednra−/− embryos.

Compared with β-gal staining, endogenous Hand2 expression in E16.5 wild-type embryos was only observed in the odontoblast region and the mandibular bone. Hand2 expression in Ednra−/− embryos was most prominent in the odontoblast region and shaft of the vibrissae (Fig. 5I). Expression was also present in the residual bone and cartilage (data not shown). Expression was not observed in the dental lamina of either embryo, indicating that the β-gal cells observed in Ednra−/−;R26R;Hand2-Cre embryos was due to earlier mis-regulation of the endogenous gene and/or transgene. Furthermore, we did not observe either β-gal staining or endogenous Hand2 expression in the upper incisors in either embryo. These findings illustrate that normal mandibular arch gene expression is partially maintained in the lower incisor region of Ednra−/− embryos.

**Discussion**

**Ednra signaling and the establishment of a mandibular identity**

Targeted inactivation of Dlx5 and Dlx6 results in loss of
shown that the lower jaw defects observed in Ednra maximum and mandibular arches (Depew et al., 2002). We have hypothesized that molecules from regional ‘signaling centers’ between the expression, which in turn disrupts the expression of secreted hypothesized to be due in part to loss of nested Dlx genes et al., 2002; Depew et al., 2002). This transformation is homeotic transformation into maxillary structures (Beverdam mandibular identity, with mandibular structures undergoing a homeotic transformation into maxillary structures (Beverdam et al., 2002; Depew et al., 2002). This transformation is hypothesized to be due in part to loss of nested Dlx genes expression, which in turn disrupts the expression of secreted molecules from regional ‘signaling centers’ between the maxillary and mandibular arches (Depew et al., 2002). We have shown that the lower jaw defects observed in Ednra+− embryos result from a similar homeotic transformation of mandible structures into maxilla-like structures. Ednra signaling is thus the earliest identified signal involved in establishing a ‘mandibular identity’ in both CNC cells and the overlying epithelium of mandibular arch, with downstream molecules such as Dlx5, Dlx6 and Hand2 acting as effectors of this signaling (Fig. 6A). In this process, Ednra signaling may function by establishing boundaries between proximal and distal regions of the arches, thus allowing a distal arch program to be initiated and/or achieved. Zebrafish suc/et1 mutant, which contain a strong loss-of-function mutation in edn1, have hypoplastic ventral first arch cartilages that show changes in polarity, loss of jaw joints and a probable homeotic transformation of the brachioseptal ray (a ventral second arch cartilage) into an opercle (a dorsal second arch cartilage) (Kimmel et al., 2003; Miller and Kimmel, 2001; Miller et al., 2000). Polarity changes are also observed in endoderm grafting experiments in the chick, in which the relative anteroposterior direction of foregut endoderm grafts influenced polarity of lower jaw structures (Couly et al., 2002). Although the tissue from which Edn1 acts has not been elucidated, Ednra signaling is required between E8.5 and E9.25 for mandibular arch patterning (L.-B.R. and D.E.C., unpublished). It is thus plausible that Edn1 plays a key directed role in establishing positional identity of CNC cells in the mandibular arch in gnathostomes.

Although much of the lower jaw of Ednra+− embryos appears to lose its mandibular identity, limited Hand2 expression still occurs in the incisor region. As Hand2 expression is confined to the lower jaw of developing embryos, (Ruest et al., 2003) and appears crucial for normal incisor development (Abe et al., 2002), this area may reflect one in which mandibular identity is not completely lost in the absence of Ednra signaling. Furthermore, an Ednra-independent signaling pathway leading to limited Hand2 expression in the distal arch could partially explain the presence of lower incisors in the jaw of Dlx5/Dlx6−− embryos.

Ednra signaling may be additionally required for proliferation or survival of CNC cells, as most duplicated structures appear smaller than their maxillary counterparts and are smaller than those observed in Dlx5/Dlx6−− embryos (Beverdam et al., 2002; Depew et al., 2002). This could reflect a loss of precursor cells, as we have previously shown that loss
of Ednra signaling causes both a decrease in proliferation and a fourfold increase in apoptosis of mandibular arch ectomesenchyme (Clouthier et al., 2000). However, it is also possible that Ednra signaling is required for later osteogenic events, because in a mouse model of osteoblastic bone metastasis using breast cancer cells lines, antagonism of Ednra receptors decreased osteoblastic metastases (Yin et al., 2003). Analysis of Ednra function during bone development, potentially using a conditional knockout of the Ednra gene (Kedzierski et al., 2003), will be required to address this issue.

**Aberrant gene expression and lower jaw transformation**

Why loss of Ednra signaling leads to a homeotic transformation is not known. However, our analysis of gene expression suggests that loss of Bmp4 may be crucial for changes in arch development. Ectopic Bmp4 can induce epithelial Dlx2 expression in mandibular explant cultures, while introduction of ectopic noggin inhibits this expression (Thomas et al., 2000). Loss of Bmp4 in Ednra–/– embryos could thus explain why Dlx2 epithelial expression is lost. Furthermore, distal expansion of the Dlx2 mesenchymal domain could also aberrantly affect arch development, as Dlx2 can form heterodimers with Msx1 (Zhang et al., 1997). Because Msx1 appears to promote proliferation of CNC cells (Han et al., 2003), increased Dlx2 in the distal arch could increase heterodimer formation, resulting in decreased CNC cell proliferation and aberrant differentiation. It is not clear why Dlx2 expression decreases in cultured mandibular arches treated with the non-peptidic dual Ednra/Ednrb antagonist bosentan (Park et al., 2004). Although Ednrb+/– mice do not have facial defects at birth (Hosoda et al., 1994), perhaps maintenance of mesenchymal Dlx2 expression requires Edn1-mediated signaling from either Ednra or Ednrb, with the blockage of both disrupting expression.

Continued Msx1 expression in the remainder of the mandibular arch is somewhat surprising, considering that Msx1 expression is lost in Hand2–/– embryos (Thomas et al., 1998). This finding led to the hypothesis that Msx1 was downstream of an Edn1/Hand2 pathway (discussed below). However, more recent studies have illustrated that Msx1 expression is normal in Edn1+/– embryos (Ivey et al., 2003). Our results here also indicate that Msx1 expression is not dependent on Ednra signaling. It is possible that the absence of Msx1 observed in Hand2–/– embryos could be due to apoptosis of specific Msx1-expressing mesenchymal cells, as cell death is widely observed in the first arch of Hand2–/– embryos (Thomas et al., 1998). Alternatively, normal Msx1 expression may only require distal Hand2 expression, hence explaining Msx1 expression in Edn1+/– and Ednra+/– embryos. Proof of this awaits analysis of gene expression in Hand2 chimeric or conditional knockout mice.

**Hand2 as a prominent effector of Ednra signaling in the pharyngeal arches**

Comparative analysis of developmental signaling pathways in multiple species can point to common crucial mediators. One gene that lies downstream of Edn1/Ednra signaling in the pharyngeal arches in both mouse and zebrafish is the bHLH molecule Hand2. In the hand2 zebrafish mutant hands off (han), most ventral (distal) arch cartilage is missing (Miller et al., 2003). Loss of hand2 disrupts ventral gene expression, though more narrowly than observed in suc/et1 mutants. Furthermore, Hand2 appears to cooperate with Edn1 in establishing domains in the first arch that demarcate both the ventral arch and the joint region separating the upper and lower
jaws. Hand2+/− mouse embryos die from vascular failure by E10.5, preventing analysis of craniofacial bone/cartilage formation (Srivastava et al., 1997; Thomas et al., 1998; Yamagishi et al., 2000). However, misexpression of Hand2 in the chick limb bud results in digit duplication and polydactyly (Charité et al., 2000; Fernandez-Teran et al., 2000; McFadden et al., 2002), suggesting that the level of Hand2 (and the types of bHLH dimers they form) may be crucial for specifying the identity of cell populations or establishing gene expression boundaries within tissues (Firulli, 2003). Perhaps the aberrant ectodermal Hand2 expression observed in Ednra+/− embryos is another example of loss of expression boundaries within the arch. It is intriguing that the expression domain of the bHLH molecule Twist expands into the distal arch of Ednra+/− embryos, as Twist can form heterodimers with Hand2 (Firulli et al., 2003) and is required for expression of multiple transcription factors involved in mandibular arch development (Soo et al., 2002); (see also Fig. 6A). Determining how Hand2 might establish expression boundaries and the identity of its prospective partners in this process will require a better understanding of the biochemistry of Hand2 dimer formation in the pharyngeal arches.

Ednra independent regulation of Hand2 expression

Our results demonstrate that multiple mechanisms, potentially including GATA3 (see below), regulate distal Hand2 expression in the absence of Ednra signaling (Fig. 6B). However, the limited number of Hand2 daughter cells in the mandibular arch of Ednra+/− embryos suggests that Ednra-dependent and -independent mechanisms probably collaborate to fully induce Hand2 expression in wild-type embryos. Although Hand2 expression is also lost in the pharyngeal arches of sucet1 zebrafish, a cluster of Hand2-positive cells remains in proximal arch one, roughly corresponding to distocaudal arch one in the mouse (Miller et al., 2003). This indicates that Edn1/Ednra-dependent and -independent mechanisms probably collaborate to distal arch one, roughly corresponding to distocaudal arch one in the mouse (Miller et al., 2003). This indicates that Edn1/Ednra-dependent and -independent mechanisms regulating Hand2 expression in the distal arch may be conserved between mouse and zebrafish. In addition, Hand2 expression is observed in a small domain in the second arch of Dlx5+/− embryos, resembling a domain observed in sucet1 zebrafish (Miller et al., 2000). The absence of a second Hand2 domain in Ednra+/− embryos could indicate that these cells are lost in the absence of Ednra signaling or that regulation of Hand2 expression has become more complex with evolution.

One potential regulator of distal Hand2 expression may be GATA3. We have shown that loss of GATA3 partially disrupts Hand2 expression in the caudal arch. Regulation of Hand2 function through GATA factors has been previously described (McFadden et al., 2000), suggesting this may be a common mechanism for regulating Hand2 function. However, understanding regulation of gene expression based solely on expression patterns can be difficult. Even though expression of both Hand2 and Gata3 overlaps in the distal arch, Hand2 expression is lost only along the caudal half of the mandibular arch in Gata3+/− embryos. In addition, the loss occurs in both distal and proximal regions of the Hand2 domain, even though Gata3 expression is confined to the distal domain. It is clear that multiple factors are involved in regulating these genes, with our results simply providing an entry point into understanding these hierarchical pathways. Defining the exact role of GATA3 in Ednra-dependent and independent Hand2-mediated developmental processes, including odontogenesis, will require a more thorough understanding of both the molecular and cellular changes within the mandibular arch of Gata3 mutant embryos and the relationship between GATA3 and Ednra (Lim et al., 2000). Furthermore, it will be important to determine if other GATA factors are involved in regulating Hand2 or Dlx5 expression in the rostral arch, as GATA regulation of Hand2 is observed in other developmental paradigms (McFadden et al., 2000). GATA2 can also bind to a core GATC consensus sequence (as found in the Hand2 enhancer) (Ko and Engel, 1993), suggesting it as a potential candidate.

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Note added in proof

While this manuscript was in review, Ozeki et al. (Ozeki et al., 2004) reported similar homeotic changes in mandibular arch structures in Edn1+/− embryos.

References


Ednra signaling in mandibular arch development


