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 (Couly 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 zebrafish *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 *sucker* or *suc/et1*, results in disruption of most cartilages of the ventral (distal) 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 NCCs.

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 suggest that although Ednra signaling is crucial for patterning most of the CNC-derived mesenchyme and surrounding epithelium of the mandibular arch by initiating a *Dlx*/*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*$^{-/-}$ (*ET*$_A^{-/-}$) (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 0.015% 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 cleared in 1% potassium hydroxide and successive immersions of 1% potassium hydroxide in 20%, 50% and 80% glycerol. Skeletons were photographed with an Olympus DP11 digital camera mounted on an Olympus SZX12 stereomicroscope.

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, E16.5 *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 and Mahon, 1994), *Dlx5* (Liu et al., 1997), *Dlx6* (Charité et al., 2001), *Hand2* (Srivastava et al., 1997), *Gata3* (George et al., 1994), *Msx1* (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 riboprobes 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 *suc/et1* 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

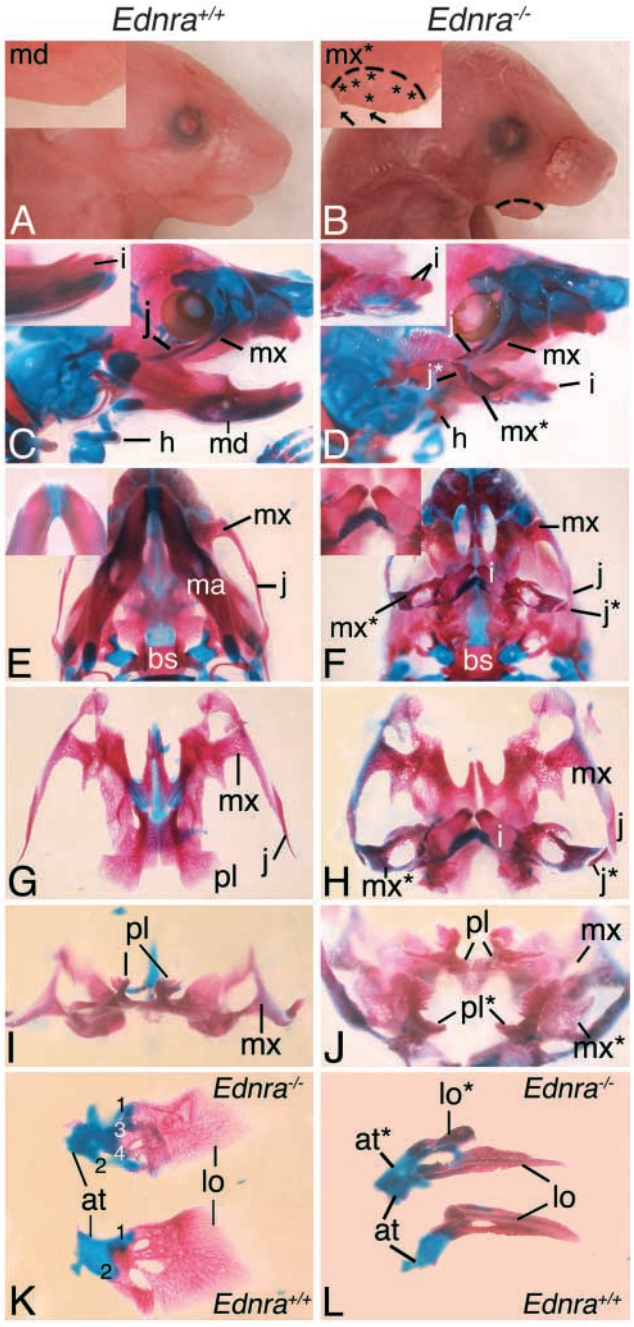

Fig. 1. Analysis of mandible structure in *Ednra*$^{-/-}$ embryos. E18.5 wild-type (*Ednra*$^{+/+}$; A,C,E,G,I) and *Ednra*$^{-/-}$ (B,D,F,H,J) embryos. (A,B) Unlike wild-type embryos (A), the lower jaw of an *Ednra*$^{-/-}$ embryo is shortened and is covered by vibrissae (asterisks in inset in B denote 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ embryos (arrows in Fig. 2B). Similarly, *Dlx1* expression in *Ednra*$^{-/-}$ 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*$^{-/-}$ embryos, expression appeared to slightly expand more proximally (Fig. 2E,F). Likewise, *Twist* expression expanded distally in *Ednra*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ embryos

As *Hand2* expression is downregulated in both *Ednra*$^{-/-}$ and *Dlx5/Dlx6*$^{-/-}$ 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*$^{-/-}$ embryos. In contrast to the arch expression in wild-type embryos (Fig. 2M,O), *Dlx5* and *Dlx6* expression in *Ednra*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ embryos, *Hand2* expression was observed in both the mesenchyme and overlying epithelium (data not shown).

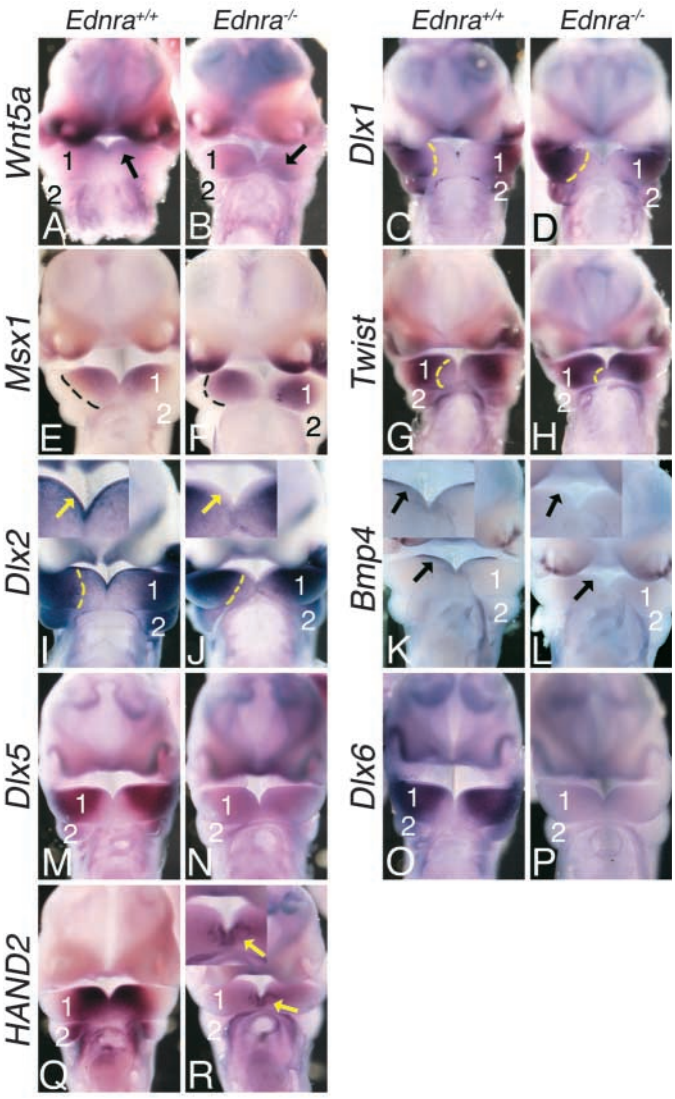

Fig. 2. Gene expression changes in *Ednra*$^{-/-}$ embryos. Whole-mount in situ hybridization analysis of gene expression in E10.5 wild-type (*Ednra*$^{+/+}$) and *Ednra*$^{-/-}$ embryos using digoxigenin-labeled cRNA riboprobes. Embryos are shown in ventral view, with the heart and outflow tract removed to aid in visualization. (A,B) *Wnt5a* expression spreads into the distal half of the mandibular arch in *Ednra*$^{-/-}$ embryos (black arrow in B). (C,D) *Dlx1* expression spreads into the distal mandibular arch in *Ednra*$^{-/-}$ embryos (C; compare broken yellow lines in C and D). (E,F) *Msx1* expression appears to spread proximally in *Ednra*$^{-/-}$ embryos (F; compare broken black lines in E and F). (G,H) *Twist* expression expands distally in *Ednra*$^{-/-}$ embryos (H; compare broken yellow lines in G and H). (I,J) *Dlx2* expression also spreads more distally in *Ednra*$^{-/-}$ embryos (J; compare broken yellow line in I and J). This expansion is accompanied by loss of epithelial expression (compare arrows in insets). (K,L) *Bmp4* expression is also downregulated on the rostral epithelium of *Ednra*$^{-/-}$ embryos (L; compare arrows in insets in K and L). (M,N) *Dlx5* expression is almost completely lost in the mandibular arch of *Ednra*$^{-/-}$ embryos (N). (O,P) *Dlx6* expression is also absent in the mandibular arch of *Ednra*$^{-/-}$ embryos (P). (Q,R) *Hand2* expression, which is observed in the distal two-thirds of wild-type embryos (Q), is almost completely absent in *Ednra*$^{-/-}$ embryos, though some expression remains in the distal arch (R; arrow in inset). 1, mandibular region of first pharyngeal arch; 2, second pharyngeal arch.

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 is 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/Dlx6*$^{-/-}$ 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/Dlx6*$^{-/-}$ embryos (Fig. 3J,L). However, limited *Hand2* expression remained in the distal arch, though the expression level was generally less than that observed in *Ednra*$^{-/-}$ embryos. This suggests that at least some of the distal *Hand2* expression in *Ednra*$^{-/-}$ embryos occurs in a Dlx5/Dlx6-independent manner. Expression was also observed in a small domain in the second arch (black arrows in Fig. 2L), resembling that observed in *suc*/*et1*-mutant zebrafish (Miller et al., 2000).

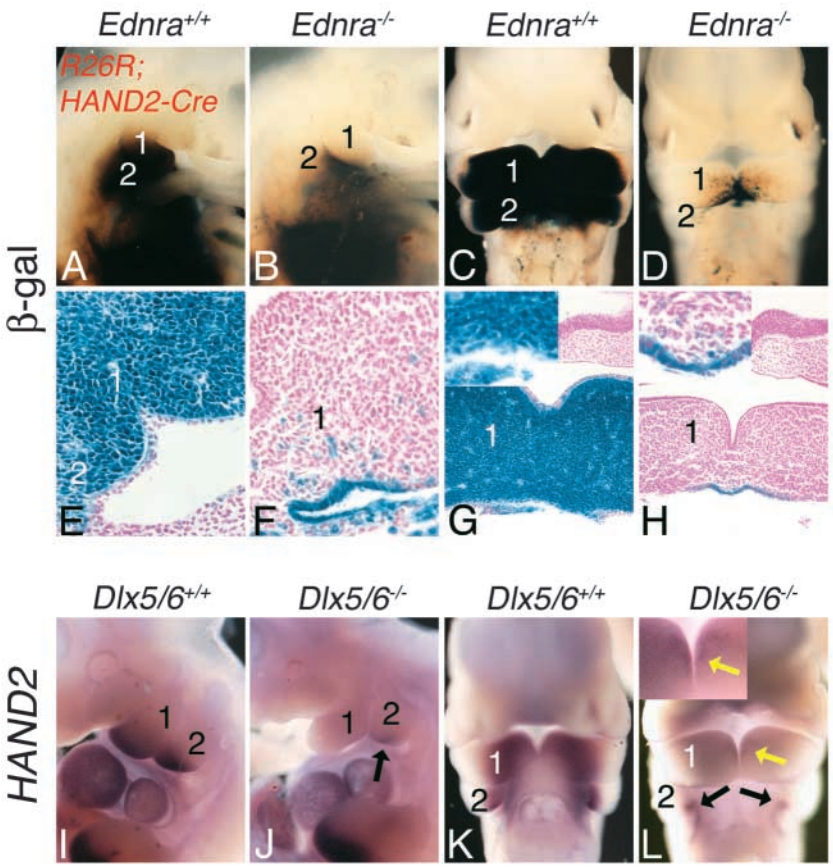

Fig. 3. *Hand2* expression in *Ednra*$^{-/-}$ and *Dlx5/6*$^{-/-}$ embryos. (A-H) *Ednra*$^{+/+}$*;R26R;Hand2-Cre* and *Ednra*$^{-/-}$*;R26R;Hand2-Cre* embryos stained in whole mount for β-gal activity and shown in both lateral (A,B) and ventral views (C,D). For ventral views, the heart and outflow tract 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 are 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/Dlx6*$^{-/-}$ embryos. Normal *Hand2* expression (I,K) is absent in the pharyngeal arches of *Dlx5/Dlx6*$^{-/-}$ 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).

GATA3 and distal *Dlx5/Hand2* expression

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 GATA3 (nngaGATAanann), with an overall similarity of 0.831 (actual sequence: aggaGATCagaga, with the underlined base pairs showing the highest conservation in mathematical models) (data not shown). GATA3 is a member of the GATA 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 noradrenalin 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

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).

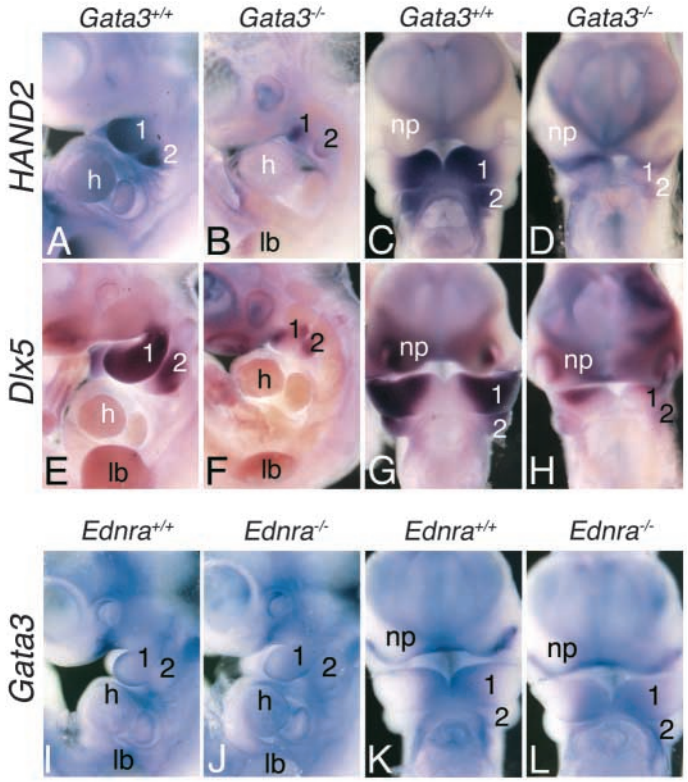

Fig. 4. Gata3 in mandibular arch development. E10.5 wild-type (*Gata3*$^{+/+}$, A,C,E,G; *Ednra*$^{+/+}$, I,K), *Gata3*$^{-/-}$ (B,D,F,H) and *Ednra*$^{-/-}$ (J,L) embryos following whole-mount in situ hybridization analysis using DIG-labeled riboprobes against *Hand2* (A-D), *Dlx5* (E-H) and *Gata3* (I-L). Embryos are shown in both lateral (A,B,E,F,I,J) and ventral (C,D,G,H,K,L) views. (A-D) Unlike wild-type embryos (A,C), *Hand2* expression in *Gata3*$^{-/-}$ embryos is observed only in the rostral mandibular arch (B,D); expression is also decreased in the second arch. There is an overall decrease in arch size in *Gata3*$^{-/-}$ embryos. (E-H) *Dlx5* expression is observed only along the rostral half of the mandibular arch of *Gata3*$^{-/-}$ embryos (F,H). (I-L) Mandibular arch expression of *Gata3*, observed in the distal arch mesenchyme of wild-type embryos (I,K), appears unchanged in *Ednra*$^{-/-}$ embryos (J,L). 1, mandibular region of the first pharyngeal arch; 2, second pharyngeal arch; h, heart; lb, limb bud; np, nasal prominence.

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 were present 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

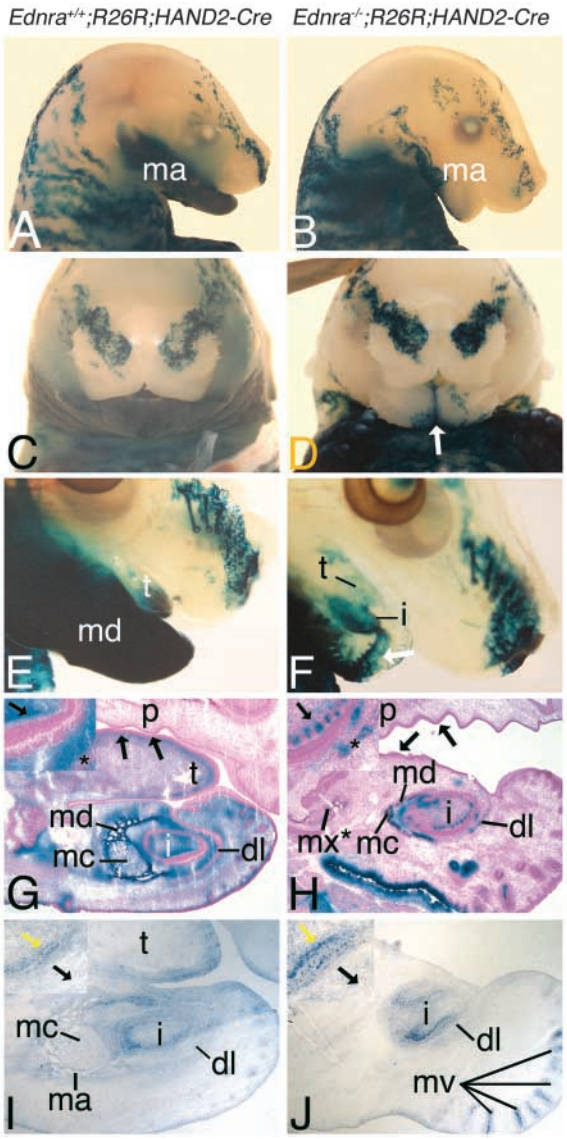

Fig. 5. Fate of Hand2 daughter cells in *Ednra*$^{-/-}$*;R26R;Hand2-Cre* embryos. E16.5 *Ednra*$^{+/+}$*;R26R;Hand2-Cre* (A,C,E,G,I) and *Ednra*$^{-/-}$*;R26R;Hand2-Cre* (B,D,F,H,J) embryos stained for β-gal activity in whole-mount (A-F) or sections (G,H), or analyzed for *Hand2* expression using sectional in situ hybridization (I,J). (A,B) In a lateral view, β-gal-labeled cells are observed throughout the lower jaw of *Ednra*$^{+/+}$*;R26R;Hand2-Cre* embryos (A). In *Ednra*$^{-/-}$*;R26R;Hand2-Cre* embryos, staining is confined to ventral surface of the mandible (B). (C,D) In a ventral view, few labeled cells are present in the lower jaw in *Ednra*$^{-/-}$ embryos (D), although the labeling is very prominent in the cleft between the two mandibular halves (arrow in D). (E,F) After clearing in benzyl benzoate:benzyl alcohol, little structural detail is apparent in the lower jaw of wild-type embryos because of the extensive labeling (E). By contrast, labeled cells in *Ednra*$^{-/-}$ embryos are present in the epithelial seam between the two arch halves (arrow in F), hypoplastic tongue (t) and lower incisors (i). (G, H) Stained sagittal cryosections illustrate labeling in most lower jaw structures and surrounding soft tissue of wild-type embryos, including the mandible (md), Meckel's cartilage (mc) and dental pulp of the incisors (arrow in inset) (G). Labeling is not observed in the dental lamina (dl; * in inset). In *Ednra*$^{-/-}$ embryos, labeled cells are present in the dental pulp of the lower incisors (arrow in inset), but are more scattered compared with wild-type embryos. A few labeled cells are also present in the small residual bone and cartilage underneath the incisors and in the dental lamina (* in inset) (H). Rugae are obvious along the both the top and bottom of the oral cavity (arrows). (I,J) *Hand2* expression is observed in the dental pulp of both wild-type and mutant embryos, with highest expression in the odontoblast layer (yellow arrow in insets). Expression is not observed in either embryo within the dental lamina (black arrow in insets). p, secondary palate tissue.

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 brachiostegal 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 Edn1 is one of the key secreted factors that establish 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.

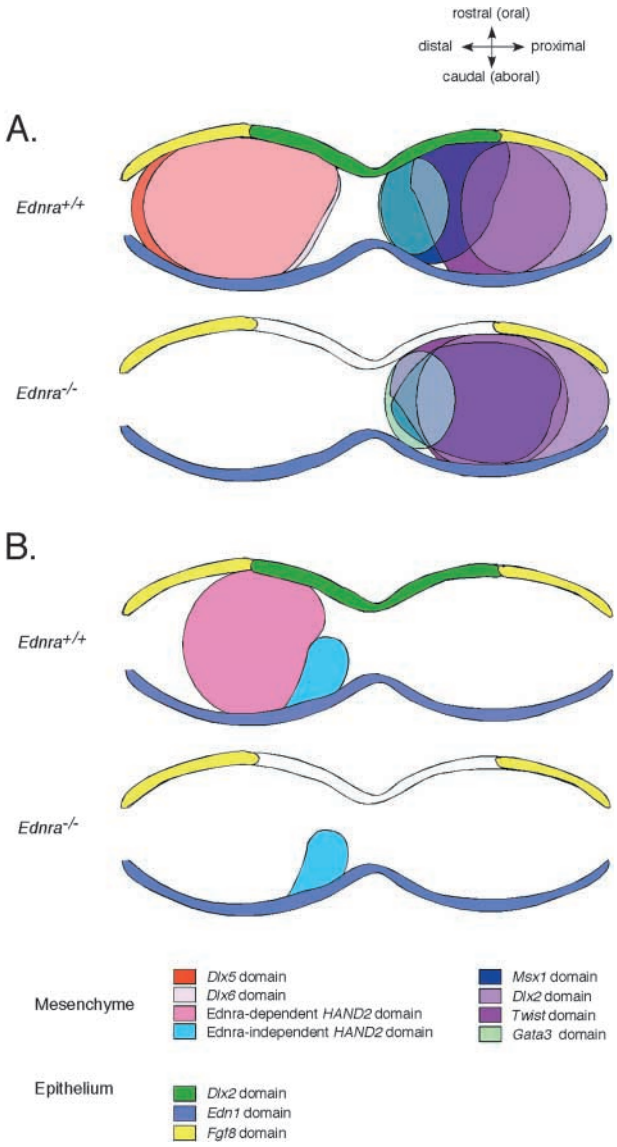

Fig. 6. Gene expression domains in E10.5 *Ednra*$^{+/+}$ (top) and *Ednra*$^{-/-}$ (bottom) embryos. The sketches illustrate a ventral view of the mandibular arch, with the orientation of each arch half depicted above. Color-coded keys define both mesenchymal and epithelial gene expression domains. (A) In wild-type (*Ednra*$^{+/+}$) embryos, the overlapping expression domains of *Dlx5* and *Dlx6* are shown on the left-hand side, while the right side depicts *Msx1*, *Dlx2*, *Twist* and *Gata3* expression domains. Epithelial expression of *Fgf8*, *Dlx2* and *Edn1* are also shown on both sides. In *Ednra*$^{-/-}$ embryos, expression of both *Dlx5* and *Dlx6* is disrupted in the arch mesenchyme, as is *Dlx2* expression in the rostral arch epithelium. By contrast, mesenchymal expression domains of *Msx1*, *Dlx2* and *Twist* expand either proximally or distally, creating a large region of overlap. The *Gata3* expression domain appears unchanged. (B) *Hand2* expression is shown in two domains: one that requires Ednra signaling and one that does not require Ednra signaling.

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 arch mesenchyme 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 *suc*/*et1* 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 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/Dlx6*$^{-/-}$ embryos, resembling a domain observed in *suc/et1* zebrafish (Miller et al., 2000). The absence of a second arch *Hand2* domain in *Ednra*$^{-/-}$ embryos could indicate either 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.

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