

Regionalisation of early head ectoderm is regulated by endoderm and prepatterns the orofacial epithelium

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Summary

The oral epithelium becomes regionalised proximodistally early in development, and this is reflected by the spatial expression of signalling molecules such as *Fgf8* and *Bmp4*. This regionalisation is responsible for regulating the spatial expression of genes in the underlying mesenchyme. These genes are required for the spatial patterning of bone, cartilage orofacial development and, in mammals, teeth. The mechanism and timing of this important regionalisation during head epithelium development are not known. Using lipophilic dyes to fate map the oral epithelium in chick embryos, we show that the cells that will occupy the epithelium of the distal and the proximal

mandible primordium already occupy different spatial locations in the developing head ectoderm prior to the formation of the first pharyngeal arch and neural crest migration. Moreover, the ectoderm cells fated to become proximal oral epithelium express *Fgf8* and this expression requires the presence of endoderm. Thus, the first fundamental patterning process in jaw morphogenesis is controlled by the early separation of specific areas of ectoderm that are regulated by ectoderm-endoderm interactions, and does not involve neural crest cells.

Key words: Oral epithelium, Ectoderm, Fate map, DiI, DiO, Chick

Introduction

In both mice and Aves, the gene expression profile of both the oral ectoderm and the underlying mesenchyme is markedly different in the proximal and distal domains (Barlow et al., 1999; Sharpe, 1995). Bead experiments and epithelium removal experiments support the idea of Mina et al. that the mandible primordium is separated into discrete functional units (Mina et al., 2002). In the proximal domain, morphogenesis appears to be dependent upon the FGF signalling pathway (Trump et al., 1999; Mina et al., 2002). *Fgf8* is expressed in the proximal oral epithelium and regulates the expression of several transcription factors in the underlying mesenchyme, including the homeobox transcription factor *Barx1* (Barlow et al., 1999; Ferguson et al., 2000). In the medial/distal region, *Bmp4* is believed to be one of the major signals that controls and regulates the expression of transcription factors in the underlying mesenchyme. *Bmp4* activates the expression of *Msx1* and *Msx2* in the distal mesenchyme, and represses the expression of *Barx1* (Tucker et al., 1998a; Ferguson et al., 2000).

It has been established that the expression of transcription factors (and consequently the establishment of the proximodistal domains in the ectomesenchyme of the developing mandible) is controlled by the spatially restricted expression of signalling factors in the overlying oral epithelium. This suggests that the oral epithelium contains a crude proximodistal pre-pattern that is transferred and refined into overlapping proximodistal domains of homeobox genes in the ectomesenchyme. This proximodistal regionalisation of the

oral epithelium is fundamental to patterning of the hard tissue of the jaws and, in heterodontic mammals, also of teeth.

Although the origins of the neural crest cells in the first pharyngeal arch have been mapped (Couly et al., 1996; Lumsden et al., 1991) (reviewed by Graham, 2001), relatively little attention has been paid to the origins of the surface epithelium of the mandibular primordium. Because this epithelium appears to contain the first patterning information in the developing lower jaw, it is important to understand how this early pattern is produced. One possibility is that ectodermal cells in the mandibular primordium having a proximal fate have a different developmental origin and history to the cells that have a distal fate. In order to investigate the lineage origins of the oral epithelium, DiI/DiO-lineage tracing was used to follow the fate of early cranial surface ectoderm cells. Developmental stages after the head-process stage were chosen for this study because previous work by Streit indicates that at earlier stages otic, neural crest, epibranchial placode and epidermis cell precursors were all intermingled (Streit, 2002). We thus chose this stage as our starting point to help map the process through which proximal and distal ectoderm territories become defined.

Avian embryos were chosen because of the ease with which lineage tracing can be performed. Expression of epithelial signalling molecules and mesenchymal transcription factors in the early developing first pharyngeal arch is largely conserved between chick and many other vertebrates, including mouse, and one might expect that the mechanism of proximodistal patterning of the mandibular primordium is also similarly conserved (Chen et al., 2000).

Materials and methods

Ectoderm labelling using micro-injection

Leghorn chicken eggs were incubated at 38.5°C for approximately 30–36 hours in a humidified incubator until the embryos had between 4 and 10 somites, i.e. Hamburger Hamilton (HH) stage 8 to 10 (Hamburger and Hamilton, 1952). To allow the embryos to be easily visualised, Fount India ink (Pelikan), diluted 1:10 in PBS, was injected into the subgerminal cavity of the egg. In order to label the dorsal side of the chick embryo, a small hole was made in the vitelline membrane using a fine needle. The embryo was then aligned relative to *x* and *y* co-ordinates with an eye piece graticule. The anterior neuropore and the first somite were used as *y*-axis landmarks, and the developing neural tube and the lateral edges of the embryo were used as landmarks for the *x*-axis (Fig. 1A). Cell tracker-1,1-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI, Molecular Probes, USA), or 3,3'-dioctadecyl-5,5'-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine (DiO, Molecular Probes, USA), diluted in ethanol at 2 mg/ml, was backfilled into fine glass micro-capillary needles and injected into cells on the embryonic surface ectoderm. Only the right side of the embryo was labelled, the left side was used as an untreated control. Micromanipulators were used to position the needles and the DiI forced out of the needle onto the embryo surface by N₂ gas pressure. The eggs were then sealed and incubated for a further 48 hours, or until the embryos had reached between HH stages 18 to 22.

In order to label the ventral side of the embryo, the vitelline membrane covering the entire rostral half of the embryo had to be carefully removed. The head process was folded back and held in place using a fine needle and the embryo lined up on the graticule using the lateral edges, the sub-blastodermic fold and the anterior neuropore as landmarks (Fig. 1B). The CM-DiI was then administered and the embryos incubated as described previously.

Ectoderm labelling with DiO crystals

DiO crystals were produced and added to the embryo surface ectoderm as described by Clarke (Clarke, 1999). In addition to the *x* and *y* co-ordinates, the position of the label was recorded by digital photography.

Dissection and fixation

Embryos were dissected from the eggs and the extra-embryonic membranes removed. The embryos were fixed at 4°C in 4% paraformaldehyde (PFA) overnight and then transferred to 1% PFA for storage.

Microscopy and photography

Using epifluorescent microscopy, cells labelled with either CM-DiI or DiO were identified. DiI-labelled cells were observed using a rhodamine, 590 nm barrier filter (Leica), and DiO-labelled cells with a GFP2, 510 nm low-pass barrier filter (Leica). Photographs of the embryos were taken under both visible and epifluorescent light and the images merged using PhotoShop (Adobe, USA).

Vibratome sectioning

Following whole-mount photography, embryos were embedded in 20% gelatin diluted into PBS and the blocks submerged in 4% PFA for 48 hours. Vibratome sections (40 µm thick) were cut and mounted under a glass coverslip in Vectashield mounting solution (Vector Laboratories, UK).

In situ hybridisation

In situ hybridisation was performed on PFA-fixed embryos as described by Mootoosamy and Dietrich (Mootoosamy and Dietrich, 2002). Chick *Fgf8* and *Bmp4* cDNA probes were kindly provided by Ivor Mason and Anthony Graham, respectively.

Isolation of coronal tissue explant containing the presumptive mandibular region

Eggs were incubated as described above, or until they had reached stage 9, and the whole embryo dissected from the surrounding membranes in Dulbecco's Modified Eagle's medium (Sigma) supplemented with 10% foetal calf serum (Gibco-BRL). The stage 9 embryos were positioned with the ventral side facing upwards. Using fine tungsten needles a coronal cut was made approximately two-fifths of the distance between the anterior neuropore and the sub-germinal fold. The most rostral segment containing the neuropore was discarded. The remaining head tissue rostral to the sub-germinal fold was then isolated by a second incision just above the sub-germinal fold. The caudal head and trunk tissue was discarded. The remaining coronal head segment contained the presumptive mandibular region as predicted from the fate map.

Isolation of ventral tissue explant

Coronal segments were isolated as described above and placed so that the neural tube and foregut endoderm were visible. Using fine tungsten needles incisions were made on the lateral edges cutting through the surface ectoderm, mesoderm and foregut endoderm (e.g. Fig. 8A). At the midline, the ventral and dorsal foregut endoderm are closely apposed, so at this point the ventral tissue fragments had to be carefully teased away from the more dorsal tissue. The dorsal tissue fragment containing the neural tube was discarded and the ventral tissue explant cultured.

Ventral-lateral explants, which correspond to tissues fated to occupy the most proximal part of the mandible, and ventral-medial explants, which correspond to the tissues fated to occupy the most distal part of the mandible were isolated in a similar fashion (Fig. 8C).

Unilateral endoderm removal

Using fine tungsten needles a ventral-medial incision was made in the isolated coronal head segment (isolated as described above). The ventral head tissue was opened out and fine tungsten wire (0.1 mm; Goodfellow) was inserted between the apposing ectoderm and endodermal tissues on the right side of the head segment. It is notable that at stage 9, the neural crest cells have not yet completed their migration, so between the ectoderm and the endoderm there is a large amount of extracellular space. Therefore, using the tungsten wire, it is possible to dissect and remove the endoderm tissue from the ectoderm.

Culture of chick explants

Tissue explants were cultured under the conditions described by Tucker et al. (Tucker et al., 1998a; Tucker et al., 1998b). Briefly, explants were placed on nitrocellulose membrane filters (Millipore), supported by stainless steel mesh (Goodfellow), in organ-welled dishes, and partly submerged in MEM supplemented by 10% FBS. Explants were cultured at 37°C in a humidified incubator containing 5% CO₂ for 2–3 days. Following incubation, growth was stopped by adding ice cold 100% methanol and fixing in 4% PFA, as described previously.

Results

Fate map of the first pharyngeal arch epithelium

Because the oral epithelium of the lower jaw is derived from the ectoderm of first pharyngeal arch, the first step was to ascertain the axial level from which the cells giving rise to the first pharyngeal arch ectoderm are derived. Initially, relatively large labels were placed on the surface ectoderm of HH stage 8 to 10 embryos by the addition of DiO crystals. More focal labellings were produced by pressure injection of CM-DiI or DiO. Initially dye applications were made predominantly along

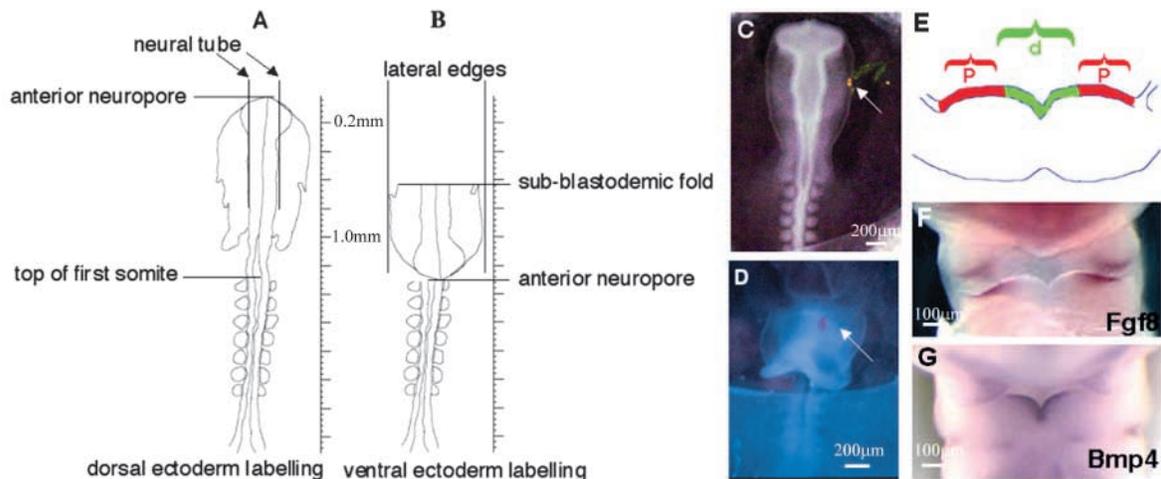


Fig. 1. Examples of DiI and DiO labelling of chick embryos, and the locations of the proximal and distal domains of the oral mandibular primordium. (A) Dorsal view of a stage 9 embryo. (B) Stage 9 embryo viewed from the dorsal side with the head folded back at the sub-blastodermic fold to expose the ventral surface ectoderm. The sub-blastodermic fold, anterior neuropore and the lateral edges of the embryo were used as landmarks, and the DiI/DiO position was recorded relative to these structures. (C) Labelling of the lateral edge of the dorsal side of a stage 10– chick embryo with a DiO crystal (arrow). (D) DiI labelling of the ventral side of stage 8+ head ectoderm. (E) Representation of the chick mandibular primordium at stages 18 to 20. The proximal (p) oral ectoderm where *Fgf8* is expressed is shown in red and the distal (d) oral ectoderm where *Bmp4* is expressed is shown in green. (F,G) Whole mount in situ hybridisation of *Fgf8* (F) and *Bmp4* (G). Drawings are not to scale. d, distal ectoderm; p, proximal ectoderm.

the lateral edge of the embryo on the dorsal side. Following application of the dye, the axial level at which the DiI/DiO had been administered was visualised using a binocular microscope, and the position along the lateral edge measured relative to the y-axis on the eye-piece graticule, using the anterior neuropore and the first somite as vertical landmarks (Fig. 1A,C). The anterior neuropore was aligned to the zero point on the y-axis of the eye piece graticule and the top of the first somite with the 100 mark. If the DiO/DiI was not placed on the lateral edge, the distance from the edge was measured as a percentage of the embryo width using the x-axis of the graticule.

Out of 325 embryos labelled, 98 survived incubation, reaching stages of between 18 and 22. Embryos having craniofacial malformations or morphological differences between the labelled side and the unlabelled control side were discarded. The remaining embryos emitting fluorescence were vibratome sectioned to confirm that the label was indeed on the surface epithelium and not in the underlying mesenchymal cells. In total, 59 embryos containing epithelial DiI/DiO label were analysed further, and the final location of DiI/DiO following incubation recorded. Particular note was taken of the presence or absence of fluorescence in the maxilla and the location on the mandible. The proximal epithelium of the mandibular primordium was characterised as both the oral region, where *Fgf8* (Fig. 1E,F) is expressed, and the part of the mandible away from the oral aspect. Regions more distal to this were characterised as distal epithelium (Fig. 1E,G). As fluorescence of DiI and DiO is lost following in situ hybridisation, the characterisation of whether the label was proximal or distal was predicted by comparison of the position of the label in the experimental embryo with a stage-matched embryo on which an *Fgf8* in situ hybridisation had been performed.

Labelling from the dorsal aspect of stage 8–10 embryos did

not result in any ectodermal fluorescence in the oral region at stages 18–22, except when the lateral edges were labelled. These data indicate that relative to the dorsal side of the embryo, only cells on the very lateral edge are able to give rise to the cells on the epithelium of the mandibular and maxillary primordia. The locations along the lateral edge of labels that gave rise to the ectoderm rostral to the maxillary primordia, maxilla, oral and aboral mandible were found to be distinct, even as early as stage 8 (Figs 2, 3). The relative distance along the vertical axis of the embryonic head, and the region where the mandibular primordium was labelled, was comparable between stages 8 to 10 (Fig. 2). When cells on the most dorsolateral edge were labelled, at a distance approximately one third of the distance between the anterior neuropore and the first somite at stage 9– (7 somites), fluorescence was observed in the oral maxillary primordium and in the most proximal region of the oral epithelium of the mandibular primordium (Fig. 3A–D). When the lateral edge was labelled from the dorsal aspect at the same stage but more caudally, approximately halfway between the anterior neuropore and the first somite, the aboral surface of the first branchial arch was fluorescent (Fig. 3E,F). Labels were also identified in the maxillary primordia of the first pharyngeal arch (Fig. 3G), and in more rostral regions (Fig. 3H). Thus, the epithelium of the different jaw primordia is demarcated early in the development of the head.

Origins of the proximodistal axis of the oral epithelium

By administering DiI/DiO to the lateral edge surface ectoderm from the dorsal side of stage 8 to 10 embryos, it was possible to label the most proximal part of the primordia of both the mandibular and maxillary oral epithelium. However, no fluorescence was ever observed in the most distal part of the oral epithelium of the mandibular primordium. It therefore

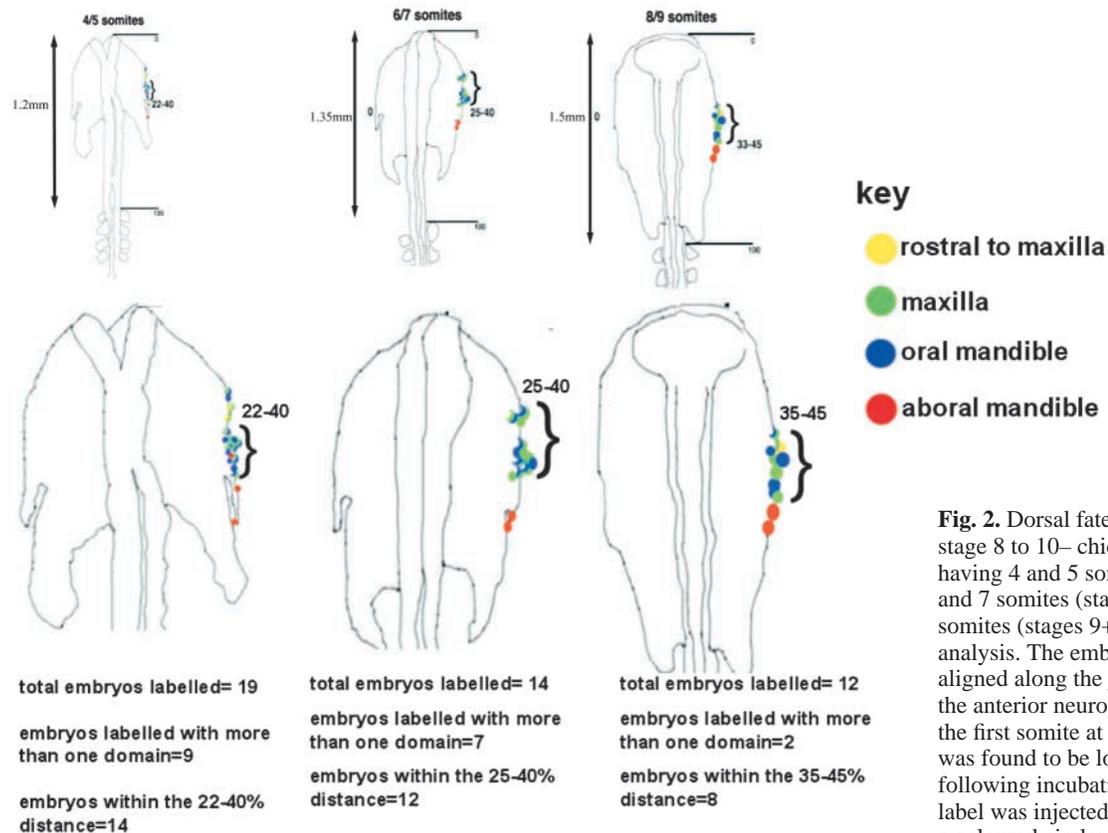


Fig. 2. Dorsal fate map of the lateral edges of stage 8 to 10- chick embryos. Embryos having 4 and 5 somites (stages 8 and 8+), 6 and 7 somites (stages 9- and 9), and 8 and 9 somites (stages 9+ and 10-) were pooled for analysis. The embryos, when labelled, were aligned along the y-axis of the graticule with the anterior neuropore at the zero position and the first somite at position 100. Where label was found to be located in a single area following incubation, the position at which the label was injected along the y-axis is shown by a coloured circle; the different coloured circles represent different fates. Where two structures

were labelled following incubation, half circles of each of different colour are placed at the point of labelling. The range of percentage distances along the y-axis between the anterior neuropore and the first somite, where the labels were fated predominantly to the axial level of the maxilla and the mandible, are shown. Also shown is the number of embryos used for each age category and the number of embryos that had more than one structure labelled from an individual DiI/DiO injection.

appears likely that cells having a more distal location had their origins on the ventral head surface ectoderm. Gaining access to the ventral surface ectoderm involved removing the vitelline membrane covering the most rostral part of the embryo, folding the head process back and holding it in place with a fine needle while the DiI/DiO was administered (Fig. 1). Out of 386 embryos labelled, 119 survived. Out of the surviving 119 embryos, 55 were useful and were photographed and vibratome sectioned to confirm the location of the cells on the surface epithelium.

When cells close to the ventral lateral edge at the level predicted to contribute to the primordia of the mandible and maxilla were labelled, they populated the same areas of the facial epithelium as those from the dorsal most lateral edge (Fig. 4A-C). This suggested that the same group of cells were being labelled in both experimental sets. Labelling closer to the ventral midline resulted in cells populating the distal oral epithelium. Where cells adjacent to the ventral midline were labelled, the most distal part of the mandibular primordium contained fluorescence (Fig. 4D-F).

These results show that at stage 8 to 10, the cells that normally give rise to the proximal and the distal oral ectoderm of the mandibular primordium are already separated into different populations occupying different spatial locations (Fig. 5). Thus not only is the ectoderm that will form the jaw

primordial fated very early, but it appears that the future proximodistal regions of the oral epithelium are fated prior to, and consequently independently of, neural crest cell migration. It is also of interest that in embryos where the maxillary primordium was labelled, the proximal oral epithelium of the mandibular primordium was also frequently labelled. Given that both are derived from the first pharyngeal arch, it is unclear from these data whether the epithelium of the maxillary and proximal mandible primordia comes from the same population of cells, or whether the resolution of the labelling technique used is not fine enough to detect subtle differences in cellular locations.

***Fgf8* expression identifies the ectodermal precursors of the proximal epithelium of the mandibular primordium**

Shigetani et al. identified an *Fgf8* expression domain on the lateral and ventral head ectoderm at an axial level just rostral to the midbrain-hindbrain junction in stage 11 chick embryos (Shigetani et al., 2000). Their data suggested that this domain corresponds to the presumptive maxilla-mandibular regions. Whole-mount in situ hybridisation of *Fgf8* was used to map expression at stages 11, 12+ and 14-. *Fgf8* expression could clearly be detected on the lateral edge and the ventral surface ectoderm at stage -11 (Fig. 6A,B). Expression of *Fgf8* in the

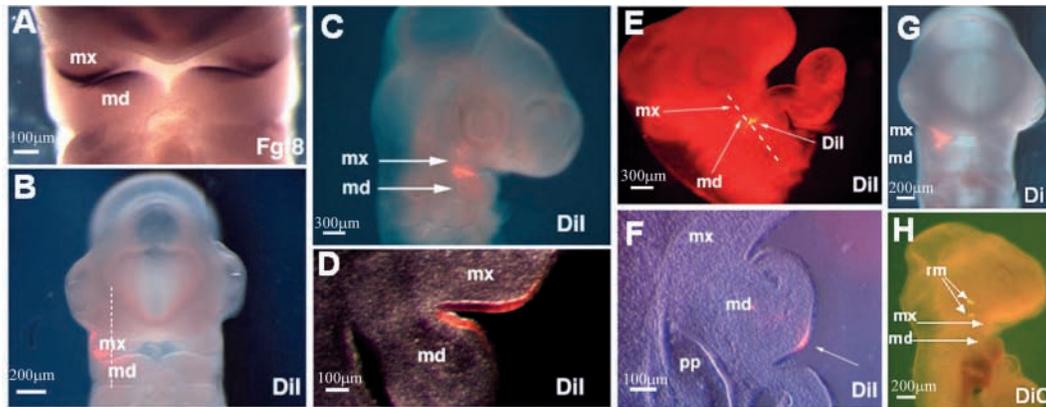


Fig. 3. (A) In situ hybridisation showing maxilla and proximal mandibular primordial expression of *Fgf8*. (B-D) A DiI labelled embryo at stage 9, from the dorsal side. DiI was injected on the lateral edge, one-third the distance between the anterior neuropore and the first somite; the embryo was then incubated for an additional 48 hours (until approximately stages 18-20). (B) Frontal view of embryo following 48 hours of incubation. Cells containing DiI are located in the maxilla and the proximal oral mandible primordia. (C) Side view of embryo. (D) Vibratome section (40 μ m) showing DiI-labelled cells located on the oral side of maxilla and mandible primordia. The plane of section is shown in B by the white vertical line. (E,F) Chick embryo labelled at stage 9 with DiI from the dorsal side and incubated for 48 hours. DiI was placed on the lateral edge of the embryo halfway between the anterior neuropore and the first somite. (E) Side view of whole embryo, fluorescent cells are located on the aboral side of the first pharyngeal arch. The white line represents the plane of vibratome sectioning. (F) Vibratome section (40 μ m) showing that DiI is in the aboral epithelium. (G) Example of DiI labelling of the maxillary primordium of the first pharyngeal arch. (H) Example of DiO labelling of ectoderm rostral to the maxillary primordium of the first arch. mx, maxilla; md, mandible; pp, pharyngeal pouch.

region of presumptive first pharyngeal arch ectoderm appeared to be restricted to the ectoderm (x, Fig. 6G,H), whereas expression in the more caudal pharyngeal arches was observed in both ectoderm and endoderm (y, Fig. 6G,I). Expression of *Fgf8* became stronger as development proceeded, and by stage -14, *Fgf8* could be seen to be demarcating the proximal epithelium of the oral mandibular primordium (Fig. 6B-F). To determine whether this early ectodermal *Fgf8* domain demarcates the future mandibular region, DiI and DiO labelling was performed on stage 11- to 12+ embryos. Initially experiments were performed to check whether the *Fgf8* domain

could be easily targeted at stages 11 to 12+. DiI or DiO was injected onto the lateral ventral surface ectoderm at an axial level just rostral to the midbrain-hindbrain junction, and the embryos harvested, fixed and photo-converted using DAB as described by Streit (Streit, 2002). Once satisfied that the *Fgf8* domain could be accurately labelled (data not shown), further embryos were labelled and development allowed to proceed for approximately 24 hours. Fluorescence was observed on the proximal oral epithelium of the mandibular primordium (Fig. 7). This data suggests that at stages 11 to 12+ the *Fgf8* expression domain represents expression in the future

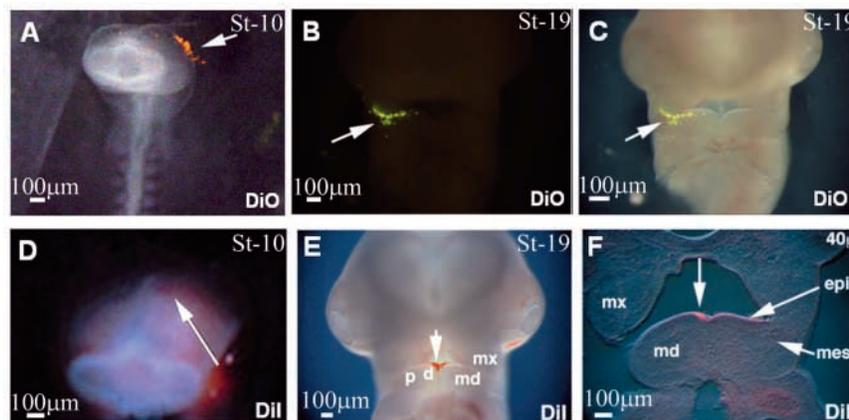


Fig. 4. DiO and DiI labelling of stage 10 chick embryos, followed by 48 hours of incubation (until approximately stages 18-20). (A) The site at which the DiO crystal was placed, the lateral edge and the proximal ventral ectoderm. (B) Image of embryo taken after 48 hours of incubation using epifluorescence and a GFP2 filter; DiO is located on the proximal mandibular primordium. (C) Image shown in B merged with a visible light image using Photoshop 6 (Adobe, USA). (D) DiI injected into cells on the ventral head ectoderm close to the midline. (E) Image of DiI fluorescence of embryo shown in D after 48 hours. Image taken using an epifluorescent microscope with a rhodamine filter and merged with visible image using Photoshop. Fluorescent cells are located in the distal oral mandibular primordium. (F) Vibratome section (40 μ m) illustrating the epithelial location of the DiI. d, distal mandible; epi, epithelium; md, mandible; mes, mesenchyme; mx, maxilla; p, proximal. The location of the DiI is highlighted by the position of the white arrows.

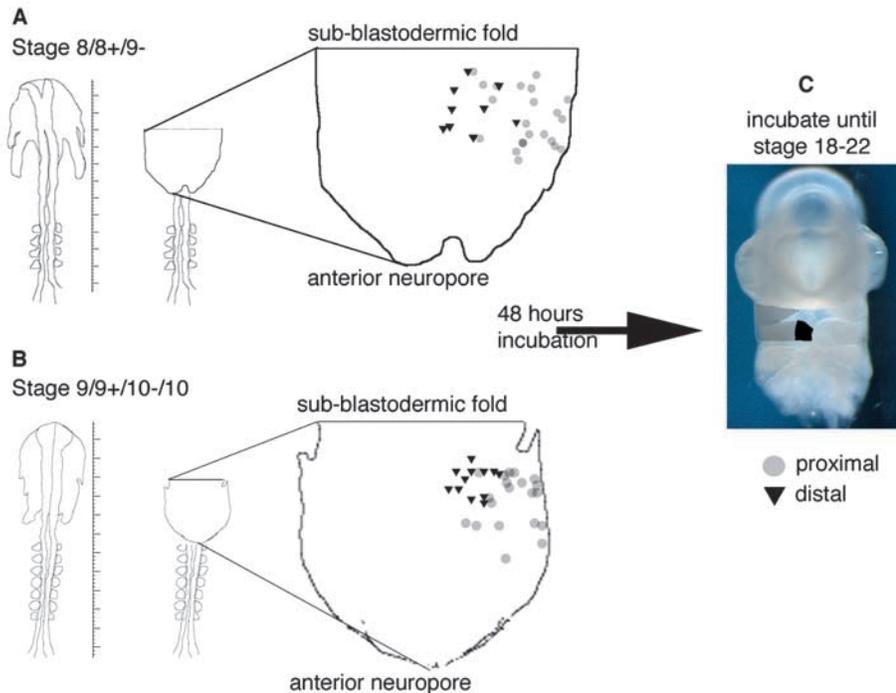


Fig. 5. Fate of DiI/DiO-labelled cells on the ventral surface ectoderm, with diagrammatic representation of the labelling of stage 8-10 embryos. The fate maps of stage 8, 8+ and 9- embryos were pooled (A), as were those of stage 9, 9+, 10- and 10 embryos (B). The position of labelled cells is shown as either grey circles or black triangles. Grey circles represent cells fated to occupy the proximal mandible epithelium and black triangles the distal mandible epithelium in stage 18-22 chick embryos, as shown by the shading of the chick mandible in C.

developing mandible, and that thereafter the cells of the proximal mandible can be traced by their expression of *Fgf8*.

Regulation of *Fgf8* expression

The localised expression of *Fgf8* in the early ectoderm precursors of the proximal oral epithelium could conceivably be induced either from lateral or dorsoventral adjacent tissues. In order to investigate this, proximal *Fgf8*-positive, and distal *Fgf8*-negative, regions of stage 9 embryos were dissected and cultured in vitro to determine whether separation of *Fgf8*-expressing ectoderm from adjacent lateral tissue affected expression. At stage 9, *Fgf8* is not yet expressed in the presumptive mandibular ectoderm. After 2.5 days in culture, *Fgf8* expression was observed in proximal ectoderm, indicating that lateral tissues are not required to maintain expression (Fig. 8).

The close proximity of endoderm to the *Fgf8*-positive ectoderm suggested that endoderm might have a role in regulating ectodermal *Fgf8* expression. This was tested by physically ablating the endoderm from one side of the dissected tissue explants of stage 9 embryos, adjacent to the *Fgf8*-positive ectoderm (Fig. 9A,D). Ablation was performed at stage 9, prior to the onset of *Fgf8* expression in the presumptive mandibular region. After 2.5 days in culture, expression was not present in the ectoderm from the ablated side but was present in the control side of the explants (Fig. 9D,E). Dorsoventral signalling from the endoderm is thus required for the early ectodermal expression of *Fgf8* that marks the precursors of the proximal oral epithelium. This data supports the work of Couly et al. (Couly et al., 2002), who ablated whole stripes of endoderm in ovo at stage 8+ to 9-. The area that they categorised as stripe 2 corresponds to the endoderm underlying the presumptive maxilla and mandible ectoderm. Following 5 days of incubation, they observed underdevelopment of both the maxillary and mandibular

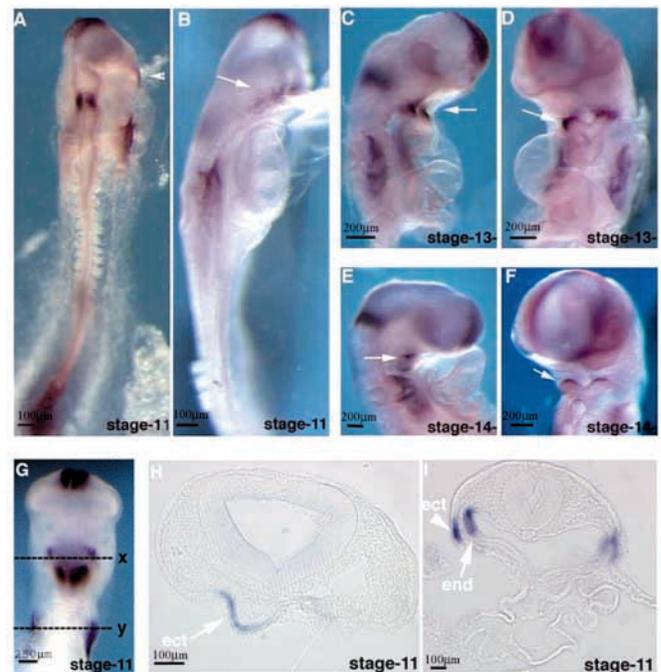


Fig. 6. Whole-mount in situ hybridisation of chick embryos using a probe for *Fgf8*. (A,B) Dorsal (A) and side (B) views of a stage 11 embryo. The white arrow and arrowhead indicates the *Fgf8* domain believed to demarcate the proximal pharyngeal arch ectoderm. (C,D) Side and ventral views of a stage 13- embryo. The white arrows indicate the *Fgf8* domain on the developing first pharyngeal arch. (E,F) Stage 14- embryos with the mandible primordium clearly visible, white arrow pointing to the *Fgf8* domain. (G) Ventral aspect of a stage 11 chick embryo showing positions of vibratome sections (40 μ m) in H and I, cut at axial levels x and y, respectively. ect, ectoderm; end, endoderm.

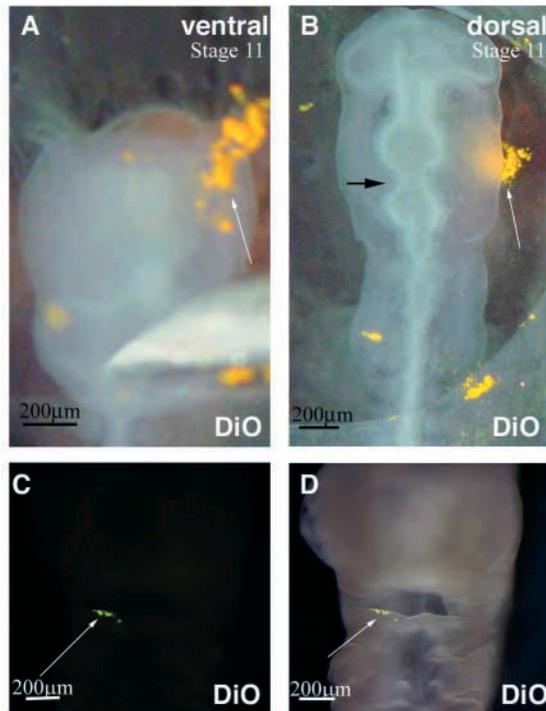


Fig. 7. DiO labelling of a stage 11 chick embryo. (A) The head of the embryo is bent back along the sub-blastodermic fold to show the position of the DiO crystal on the ventral surface ectoderm. (B) View of the same embryo from the dorsal aspect. The black arrow shows the position of the midbrain-hindbrain junction. (C,D) The same embryo following 24 hours of incubation. (C) DiO, viewed under fluorescence and a GFP2 filter. (D) The visible and the fluorescent images merged using Photoshop 6 (Adobe, USA). Note that the DiO fluorescence is located on the proximal surface ectoderm of the mandibular primordium. White arrows indicate the DiO crystal.

primordia, and absence of Meckel's cartilage on the operated side. Additionally, they also observed partial loss of expression of the transcription factor *Pitx2* on the operated side. Interestingly, Withington et al. illustrated that the endoderm was required for the expression of *Fgf8* in the forebrain (Withington et al., 2001). Removal of definitive endoderm at stage 4+ to 5 resulted in the loss of *Fgf8* expression in the forebrain at stage 11.

Regulation of *Bmp4* expression

In the developing head ectoderm the expression of *Bmp4* is dynamic and it is not until stage 14 that its expression becomes restricted to the distal oral epithelium of the mandibular primordium. In order to determine whether the signals responsible for the expression of *Bmp4* in the developing mandibular and maxillary primordia are intrinsic to ventral tissues, the ventral head tissue was dissected (including ectoderm, mesoderm and endoderm) and cultured (Fig. 8A). Tissues were dissected at stage 9, prior to the migrating neural crest cells populating the ventral head. Expression of *Bmp4* was observed in these ventral tissue explants after 2.5-3 days of culture (Fig. 8B). This suggests that signals from the neural crest cells are not necessary for expression of *Bmp4* in the ectoderm.

In order to determine whether the ventral-medial tissues of stage 9 embryos have an intrinsic ability to express *Bmp4* independently of planar signals from the ventral-lateral tissues, ventral-medial and ventral-lateral tissues, tissue explants of stage 9 embryos were dissected (Fig. 8). These explants were cultured for 2.5-3 days and assayed for *Bmp4* expression. Both ventral-lateral and medial-lateral cultured explants express *Bmp4* (Fig. 8). The observation that the ventral-medial explants express *Bmp4* does indeed suggest that the cells of stage 9 embryos are fated to express *Bmp4* later in development. However, the fact that the ventral-lateral tissues also express *Bmp4* indicates that the situation may be more complex. One explanation for the observation that *Bmp4* is expressed in both sets of explants may lie with the dynamic expression of *Bmp4* in the developing chick head, and the fact that tissues in culture do not develop at the same rate as those in vivo. Another possible reason might be because *Bmp4* is also expressed in the developing maxillary primordia (Wall and Hogan, 1995) of the developing first pharyngeal arch and our fate mapping indicates that, like the proximal mandibular epithelium, the ectoderm of the maxillary primordia arises from the lateral surface ectoderm of the early embryonic head. At stage 14, when the distal mandibular ectoderm begins to express *Bmp4* the endoderm no longer underlies the ectoderm. Also by stage 14, migrated neural crest cells occupy tissue underlying the ectoderm in the first pharyngeal arch, hence creating a physical barrier to signalling molecules arising from the endoderm. At stage 14, it is therefore unlikely that signals from the endoderm are responsible for the highly localised and restricted expression of *Bmp4* in the distal ectoderm of the mandible primordium.

Discussion

The pharyngeal arches are composed of cell populations of different developmental origin. Cells originating from the cranial neural crest and mesoderm make up the core, while the epithelial covering is of endodermal and ectodermal origin (Francis-West et al., 1998). The cranial neural crest cells give rise to the cartilage, intramembranous bone and the connective tissues of the face. Despite many decades of research, there is still controversy concerning how and when the patterning of the cranial neural crest, and consequently the skeletal elements, occurs. Presently, it is believed that the cranial neural crest cells obtain morphogenic information from a variety of sources. There is evidence that some of the specific signals are received by the crest cells before they migrate from the neural tube, and that they respond by expressing specific combinations of Hox genes (Krumlauf et al., 1993). Neural crest cells fated to occupy the first arch are devoid of Hox gene expression. These crest cells are thought to obtain their positional information from the surrounding tissues, both during the migration itself and once they have reached their final destination (reviewed by Richman and Lee, 2003; Couly et al., 2002).

The work of Couly et al. implicates the foregut endoderm as a major source of patterning signals (Couly et al., 2002). They found that, in the early chick, the foregut endoderm is able to pattern the non-Hox expressing cranial neural crest cells that occupy the jaw. Their data indicates that, at a general level, the endoderm appears to specify the identity and polarity. However, the nature of the signals arising from the endoderm is at present unknown.

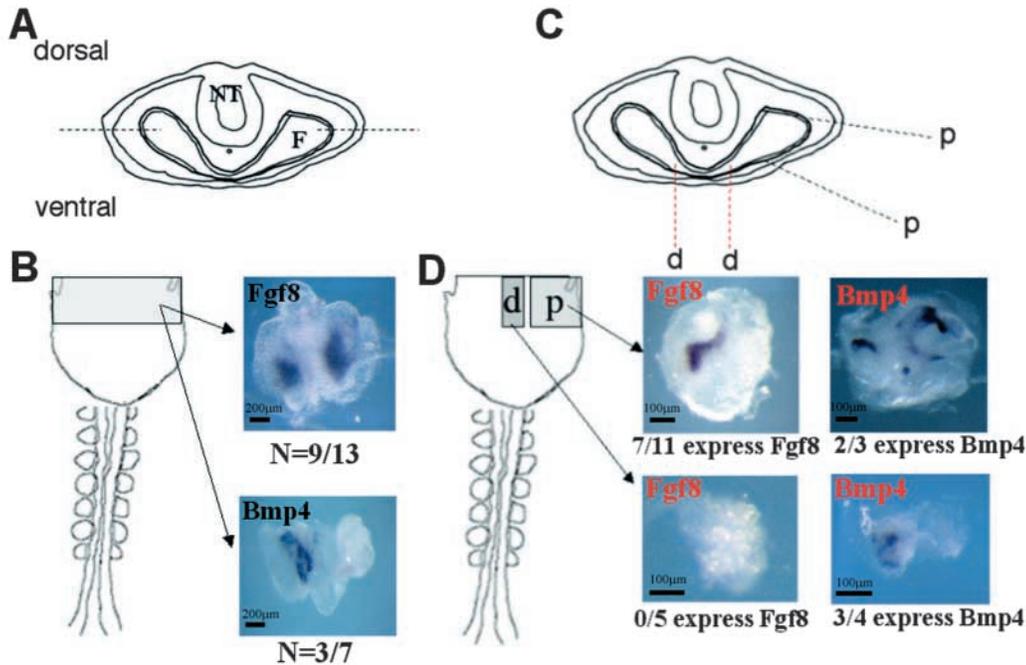


Fig. 8. *Fgf8* and *Bmp4* expression in cultured explants of stage 9 chick embryos. Stage 9 coronal segments, corresponding to approximately one-third of the length of the total head, rostral to the subgerminal fold and encompassing the presumptive mandibular region (as predicted from the fate map), were dissected as shown. (A) Isolation and separation of ventral head tissues. (B) *Fgf8* expression in explanted tissue in two lateral domains and a single *Bmp4* domain. (C) Isolation of ventral-lateral (p) tissues and ventral-medial tissues (d). (D) *Fgf8* is expressed in ventral-lateral explants (p) but not in ventral-medial explants (d). *Bmp4* is expressed in both ventral-lateral and ventral-medial explants.

Previous work in our laboratory, and in numerous others, indicates that the oral ectoderm plays an important role in the high resolution patterning of the neural crest of the first pharyngeal arch. In order for the skeletal elements of the jaw to be shaped, positioned and orientated correctly in three-dimensional space, the mesenchyme of the mandible must be patterned along three different axes. These axes can be described as rostrocaudal, proximodistal and dorsoventral. The early patterning of the mesenchyme of the mandibular primordium along these three axes is reflected in the specific graded expression of numerous transcription factors. For

example, *Lhx6* and *Lhx7* are expressed exclusively in the rostral mesenchyme, in contrast to *Gsc*, which is expressed specifically in the caudal region (Tucker et al., 1999).

Expression of *Barx1* marks the proximal mesenchyme of the mandibular primordium, whereas *Msx1* is expressed in the distal mesenchyme. The expression of these transcription factors is controlled by signalling molecules originating in the oral ectoderm. The expression of *Fgf8* proximally and *Bmp4* distally induces the expression of *Barx1* and *Msx1*, respectively (Ferguson et al., 2000; Tucker et al., 1998a; Tucker et al., 1998b). We are interested in how the skeletal structures of the

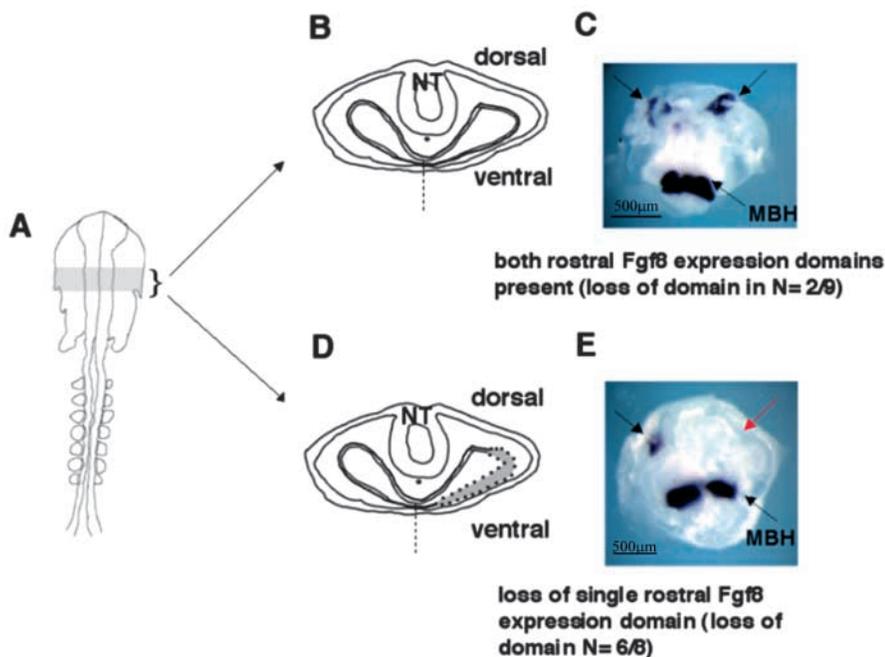


Fig. 9. *Fgf8* expression in the presumptive mandibular ectoderm is lost following removal of the underlying endoderm. (A) Shaded segment is the approximate level at which regions were dissected from stage 9 embryo. (B) Coronal segment; ventral head tissue cut at the midline to expose the foregut endoderm. (C) *Fgf8* is expressed at the midbrain-hindbrain junction (MBH) and in two more rostrally located lateral domains (black arrows), corresponding to the presumptive maxillo-mandibular domain. (D) Coronal segment; ventral head tissue cut at the midline to expose the foregut endoderm and the foregut endoderm removed unilaterally. (E) *Fgf8* expression in an explant with unilateral endoderm removal. *Fgf8* is expressed as normal in the neural tube at the midbrain-hindbrain boundary (MBH). Expression is lost in the lateral domain on which the endodermal removal had taken place (red arrow), but is present on the contralateral side (black arrow).

jaw are patterned in the proximodistal direction. Although it has been established that the proximodistal ectomesenchyme is patterned as a result of spatially restricted signals arising from the oral ectoderm, how the spatially restricted expression domains in the oral ectoderm are established and maintained, and how they interact with each other, is at present not fully understood.

Gene expression studies suggest that the proximodistal patterning of orofacial epithelium is evident in the chick embryo from stage 14, when distinct domains of *Fgf8* and *Bmp4* expression are first visible (Shigetani et al., 2000). Important questions are: when are these territories specified and does this require the presence of neural crest-derived cells? Shigetani et al. identified a domain of *Fgf8* expression in the ventral head ectoderm of stage 11 chick embryos in the presumptive maxillo-mandibular region (Shigetani et al., 2000). As development proceeds, the *Fgf8* expression domain expands and corresponds to the developing maxillary and mandibular prominences. DiI labelling of the stage 11 to 12+ chick *Fgf8* expression domain indicated that it did demarcate the cells fated to occupy the epithelium of the proximal oral mandibular primordium. The expression of *Bmp4* in the early developing chick embryo is however more dynamic. Shigetani et al. (Shigetani et al., 2000) first observed expression of *Bmp4* in the rostral head ectoderm at stage 12. Weak expression was observed ventral to the presumptive pharyngeal ectoderm. At stage 13, more distinct expression was reported; this expression was ventral to, and had a slight overlap with, the *Fgf8* expression domain. Shigetani et al. (Shigetani et al., 2000) first reported distinct expression of *Bmp4* in the distal domain of the pharyngeal arch at stage 14. It therefore seems that *Bmp4* does not demarcate the distal pharyngeal ectoderm until stage 14.

In order to understand the origin of the morphogenic signals that pattern the oral ectoderm, we have fate mapped the cells that give rise to the proximal and the distal domains of the oral ectoderm. Labelling of the ectoderm between stages 8 to 10, predicted that the progenitor cells of the mandibular primordium were located at a level between one-quarter and two-fifths of the distance between the anterior neuropore and the first somite along the anteroposterior axis. Couly and Le-Douarin (Couly and Le-Douarin, 1990) crudely segmented the surface ectoderm between the anterior neuropore and the first somite of a stage 8 (3 somite) embryo into six ventrolateral segments of equal width, and transplanted these segments between quail and chick. Transplanting a tissue strip one-sixth and one-third of the distance between the anterior neuropore and the first somite resulted in quail cells occupying the ectoderm overlying the maxillary and mandibular primordia. The results we present using DiI/DiO labelling are consistent with data of Couly and Le-Douarin (Couly and Le-Douarin, 1990). However, our more precise mapping reveals that, from at least stage 8, the cells fated to occupy the proximal and the distal oral ectoderm occupy different spatial locations in the developing embryo. The fate map thus shows that the cells that go on to express *Fgf8* in the proximal oral epithelium of stage 18 to 22 chicks are located on the ventral-lateral head ectoderm as early as stage 8. Cells in the distal oral mandibular primordium (which at stage 14 start to express *Bmp4*) occupy a more medial position in the early chick embryo. This data indicates that the cells occupying the proximal and the distal

mandibular primordia occupy different locations in the early embryo and, consequently, are likely to have a different developmental history.

As the cells are located at different positions in the head, it is possible that they are exposed during their developmental history to different morphogens, or perhaps different concentrations of morphogenic factors. These factors may be planar or may arise from the underlying mesenchyme or endoderm. Prior to neural crest migration and the cranial neural crest cells reaching the maxillo-mandibular region, the ectoderm of the mandible is in close proximity to the foregut endoderm. Couly et al. implicated the foregut endoderm as a source of morphogenic signals responsible, at least in part, for patterning the skeletal elements of the jaw (Couly et al., 2002). Removal of the endoderm that is in close proximity to the *Fgf8*-positive head ectoderm in stage 9 embryos resulted in a loss of expression of *Fgf8*. When the ventral head tissue was dissected from the dorsal tissue at stage 9 (Fig. 8), and earlier (data not shown), and cultured in vitro, *Fgf8* expression was still observed. This indicates that absent or aberrant neural crest migration is not responsible for the failure of ectoderm to express *Fgf8* in the absence of endoderm. It also suggests that, in addition to its role in patterning facial mesenchyme, the endoderm has an earlier role in patterning the orofacial ectoderm.

Although the ventral-lateral head ectoderm and the more medial head ectoderm are fated to occupy different positions on the oral ectoderm, the point at which they are specified to follow either the 'proximal' or 'distal' developmental route is unclear. *Fgf8* is not expressed in the ventral head ectoderm until stage 11, our DiI labelling of cells at these stages suggests that these are indeed the same cells that later go on to occupy the proximal epithelium at stages 18 to 22. *Bmp4* expression is much more dynamic, and it is not until stage 14 that it is expressed specifically in the distal epithelium. When and how during development the proximal and distal ectoderm is induced and programmed to express *Fgf8* and *Bmp4*, and when the proximal or distal fate is specified, is currently under investigation. However, we speculate that the proximal and the distal ectoderm are both specified and committed to their respective fates independently and at different times during embryogenesis.

Candidate molecules within the head ectoderm that may play a role in the control of *Fgf8* and *Bmp4* expression include *Pitx2* (Amand et al., 2000). *Pitx2* is a bicoid transcription factor that is expressed in the early developing embryo and has overlapping expression domains with *Fgf8*. In the chick, *Pitx2* has been shown to be expressed in the presumptive maxillo-mandible region of stage 11 embryos (Amand et al., 1998). The data of Lu et al. (Lu et al., 1999) showed that mice null for *Pitx2* have defective development of the maxillo-mandible facial prominences. Lu et al. suggested that the expression of *Fgf8* is absent in these mutants (Lu et al., 1999); however, Lin et al. reported that, in mice null for *Pitx2*, *Fgf8* was still expressed in the facial region, albeit at a reduced level (Lin et al., 1999). Lu et al. (Lu et al., 1999) reported that the expression of *Bmp4* in the *Pitx2* null mutants expanded into the whole proximodistal ectoderm mandibular primordium. This mandibular phenotype is comparable to that reported by Stottmann et al. (Stottmann et al., 2001) in *Noggin/Chordin* null mutant mice. Whether (or how) *Pitx2* expression affects

Noggin expression is not known. Whether *Pitx2* is responsible for initiating the expression of *Fgf8* in the developing ventral ectoderm of the embryonic head, or whether it is only responsible for its maintenance, is at present unclear from the literature.

In summary, we have identified cells in the chick embryo that are fated to occupy the proximal and distal oral ectoderm of the mandibular primordium. We have found that cells fated to occupy the proximal (*Fgf8*-expressing) domain and distal (*Bmp4*-expressing) domains of stage 18 to 20 chick mandible epithelium occupy different spatial locations as early as stage 8 in ventral head ectoderm, prior to the formation of neural crest. In addition, the cells on the ventral head ectoderm that express *Fgf8* at stage 11 to 12 are the same cells that are fated to occupy the proximal oral mandibular primordium and that express *Fgf8* at stage 18 to 22. The co-ordination and control of orofacial morphogenesis is thus a process that begins early in embryogenesis with the demarcation of boundaries in the cranial ectoderm that prefigure the proximodistal organisation of oral epithelium.

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