INTRODUCTION

Development of many vertebrate organs is controlled by interactions between epithelial and mesenchymal cells. Mammalian tooth development provides an easily manipulated system for studying such interactions. In addition, the development of different types of teeth, molars, incisors etc., enables the role of such interactions in pattern formation to be studied. Teeth develop from interactions between oral epithelium and mesenchyme cells derived from cranial neural crest cells (ectomesenchyme) on the mandibular and maxillary derivatives of the first branchial arch. Classical tissue recombination experiments between presumptive incisor (distal) and molar (proximal) epithelium and ectomesenchyme have been used to determine which cells carry the instructive information to determine tooth type. The majority of the results obtained from these experiments indicate that tooth type is determined by the epithelium does not affect spatial ectomesenchymal expression. Significantly, however, the response of ectomesenchyme cells to epithelial regulatory signals was found to be different in the mandibular and maxillary primordium. Thus, whereas both mandibular and maxillary arch epithelia could induce Dlx2 and Dlx5 expression in the mandible and Dlx2 expression in the maxilla, neither could induce Dlx5 expression in the maxilla. Reciprocal cell transplantations between mandibular and maxillary arch ectomesenchymal cells revealed intrinsic differences between these populations of cranial neural crest-derived cells. Research in odontogenesis has shown that the oral epithelium of the mandibular and maxillary primordium has unique instructive signaling properties required to direct odontogenesis, which are not found in other branchial arch epithelia. As a consequence, development of jaw-specific skeletal structures may require some prespecification of maxillary ectomesenchyme to restrict the instructive influence of the epithelial signals and allow development of maxillary structures distinct from mandibular structures.

SUMMARY

The cellular origin of the instructive information for hard tissue patterning of the jaws has been the subject of a long-standing controversy. Are the cranial neural crest cells prepatterned or does the epithelium pattern a developmentally uncommitted population of ectomesenchymal cells?

In order to understand more about how orofacial patterning is controlled we have investigated the temporal signalling interactions and responses between epithelium and mesenchymal cells in the mandibular and maxillary primordia. We show that within the mandibular arch, homeobox genes that are expressed in different proximodistal spatial domains corresponding to presumptive molar and incisor ectomesenchymal cells are induced by signals from the oral epithelium. In mouse, prior to E10, all ectomesenchyme cells in the mandibular arch are equally responsive to epithelial signals such as Fgf8, indicating that there is no pre-specification of these cells into different populations and suggesting that patterning of the hard tissues of the mandible is instructed by the epithelium. By E10.5, ectomesenchymal cell gene expression domains are still dependent on epithelial signals but have become fixed and ectopic expression cannot be induced. At E11 expression becomes independent of epithelial signals such that removal of the epithelium does not affect spatial ectomesenchymal expression. Significantly, however, the response of ectomesenchyme cells to epithelial regulatory signals was found to be different in the mandibular and maxillary primordium. Thus, whereas both mandibular and maxillary arch epithelia could induce Dlx2 and Dlx5 expression in the mandible and Dlx2 expression in the maxilla, neither could induce Dlx5 expression in the maxilla. Reciprocal cell transplantations between mandibular and maxillary arch ectomesenchymal cells revealed intrinsic differences between these populations of cranial neural crest-derived cells. Research in odontogenesis has shown that the oral epithelium of the mandibular and maxillary primordium has unique instructive signaling properties required to direct odontogenesis, which are not found in other branchial arch epithelia. As a consequence, development of jaw-specific skeletal structures may require some prespecification of maxillary ectomesenchyme to restrict the instructive influence of the epithelial signals and allow development of maxillary structures distinct from mandibular structures.

Key words: Branchial arch, Dlx genes, Maxilla, Mandible, Mouse
genes in ectomesenchyme cells determines tooth type (Sharpe, 1995; Thomas and Sharpe, 1998). Functional support for this model came from targeted gene mutations of Dlx genes, Dlx1 and Dlx2 which are specifically expressed in proximal (presumptive molar) mesenchymal cells in the mandible and maxilla (Thomas et al., 1997). Thus mouse embryos lacking functional Dlx1 and Dlx2 genes have a tooth patterning phenotype where development of maxillary molar teeth is inhibited but development of all other teeth is normal. Targeted mutations in activin βA, which is expressed in tooth germ ectomesenchyme cells, similarly resulted in abnormal tooth patterning where only maxillary molar teeth developed (Ferguson et al., 1998). These findings thus support the idea that the instructive information for tooth patterning resides in the ectomesenchyme cells. Implicit in this interpretation is that there is a predetermination of cranial neural crest cells that populate the proximal and distal regions of the mandible and maxillary processes.

In order to determine whether prespecification of cranial neural crest cells might specify tooth patterning and also to explain why experimental alterations of ectomesenchymal homeobox gene expression result in changes in tooth patterning whereas recombinations show that pattern is controlled by epithelial signals, we investigated the temporal regulation of ectomesenchymal gene expression in proximodistal domains. We have tested the ability of mandibular ectomesenchyme of different ages (E9.5-E11.5) to express specific proximal or distal marker genes both in the absence of epithelial signals and after supplying the epithelial signal FGF8, on beads, to proximal or distal domains of mandibular explants. We show that prior to E11, expression of marker genes in mandibular arch mesenchyme is dependent on localised signals from the overlying oral epithelium and that prior to E10.5 all ectomesenchymal cells are equally competent to respond to these epithelial signals, suggesting there is no apparent prepattern within the ectomesenchyme and that the epithelium is indirectly responsible for determining tooth type by regulating ectomesenchymal gene expression. This supports work where inhibition of BMP signalling in distal ectomesenchyme cells of E10 mandibular primordia explants, using Noggin resulted in a transformation of tooth type from incisor to molar (Tucker et al., 1998b). This transformation was probably mediated by loss of BMP-inducible distal ectomesenchyme gene expression such as Msx1 and ectopic expression of the proximal gene Barx1 that is repressed by Bmp4. Furthermore, we have identified specific time points during a period of 24 hours after which the competence of ectomesenchyme to respond to an epithelial signal changes, and finally is lost. These results provide a molecular explanation for the conflicting data obtained from earlier epithelial-mesenchymal recombination studies.

The fact that the gene knock-outs described above appear to have different effects on maxillary versus mandibular molars suggests that these teeth have independent modes of development. The question arises as to whether there are underlying differences in the epithelium or ectomesenchyme of the mandibular and maxillary primordia. Several epithelial signals, such as sonic hedgehog (Bitgood and McMahon, 1995; Hardcastle et al., 1998), Bmp4 (Vainio et al., 1993; Aberg et al., 1997) and Fgf8 (Neubuser et al., 1997; Kettunen and Thesleff, 1998; Tucker et al., 1998b) have been found to play an important role during tooth development. These and all other genes that have been reported to be expressed in the oral epithelium to date, are present in the oral epithelium of both primordia (http://HONEYBEE>HELSINKI.FI/toothexp/). Thus intrinsic differences between the mandibular and maxillary ectomesenchyme is a more likely explanation. Using Dlx genes that are differentially expressed in the mandibular and maxillary ectomesenchyme, and hetero- and homotypic recombinations as well as implantation of FGF8-soaked beads, we set out to establish whether the mandibular and maxillary ectomesenchymal cells respond differently to epithelial signals, and if so, why. Our data indicates that the ectomesenchymal cells of the mandibular and maxillary primordia appear to be intrinsically different in their response to epithelial signals and these differences can thus explain the differences in hard tissue patterning of the upper and lower jaws.

**MATERIALS AND METHODS**

**Explant cultures**
Mice of the CD-1 strain were used. Timed matings were set up such that noon of the day on which vaginal plugs were detected was considered as E0.5. Mandible and maxillary primordia from embryos at E9.5-E11.5 were dissected in D-MEM with glutamax-1 (Gibco BRL) as indicated in Fig. 3J. To accurately assess the age of embryos, somite pairs were counted and the stage confirmed using morphological criteria e.g. relative sizes of maxillary and mandibular primordia, extent of nasal placode invagination, and the size of limb buds. To remove epithelium, explants were incubated in Dispase (Gibco BRL) made up in calcium and magnesium free PBS at 2 units/ml for 8-10 minutes at 37°C, washed in D-MEM with 10% foetal calf serum and the tissues were mechanically separated using fine tungsten needles. The explants were cultured as previously described (Ferguson et al., 1998) on membrane filters supported by metal grids following the Trowel technique as modified by Saxén (Trowel, 1959; Saxén, 1966). After the period of culture, explants were washed in ice-cold methanol and fixed in fresh 4% paraformaldehyde for 1 hour at room temperature. Cultures were then prepared for either whole-mount digoxigenin (DIG) in situ hybridisation or radioactive section in situ hybridisation.

**Bead experiments**
FGF8 beads: heparin acrylic beads (Sigma) were washed several times in PBS, then incubated overnight at 4°C in 1 mg/ml FGF8b (R&D Systems) before use. Control beads: Affi-Gel agarose beads (BioRad) were washed several times in PBS. These were used directly, or were dried out and added to BSA at 1 μg/ml for 1 hour at 37°C before use.

**Recombination experiments**
Recombinations were carried out at E10.25. The epithelium and mesenchyme were isolated following incubation in Dispase. Maxillary epithelia were replaced on maxillary mesenchyme (homo- and heterotypic recombinations) or recombined with mandibular mesenchyme (heterotypic recombination) and vice versa. The recombinations were assembled and cultured for 24 hours as described previously (Ferguson et al., 1998).

**Transplantations**
Mandibles and maxillae were dissected from CD1 (wild type) or ROSA-26 (Zambrowicz et al., 1997) embryos at E9.5-E10.25. For preparation of donor mesenchymal cells the aboral halves of the mandibles and maxillae were cut away and the oral epithelium removed. A small group (plug) of mesenchymal cells was isolated...
from the presumptive molar region of mandibles and maxillae. Mesenchymal plugs that were isolated from wild-type embryos were labelled by soaking in a solution of DiI (Sigma; stock solution of 3 mg/ml in DMF) of 3 μl/ml in DMEM for 30 minutes at 37°C, then rinsed in medium before transplantation. Where ROSA-26 embryos were used, tissue was first checked for β-galactosidase activity. Limb tissue was fixed and prepared for X-gal staining according to Sanes et al. (1986).

For preparation of host explants the aboral halves of the mandibles and maxillae were cut away and a small plug of mesenchymal cells was removed from the presumptive molar region, taking care not to damage the overlying oral epithelium. The labelled plugs of mesenchymal cells were then inserted into the host explants from the aboral side which were subsequently cultured oral side up on membrane filters.

**In situ hybridisation**

Whole-mount DIG in situ hybridisation was carried out as described by Pownall et al. (1996). Radioactive in situ hybridisation was carried out as described by Wilkinson (1995). Antisense probes were generated from mouse cDNA clones that were gifts from several laboratories: Barx1 (Jean-Francois Brunet, Developmental Biology Institute of Marseilles, France); Dlx2 and Dlx5 (John Rubenstein, University of California, San Francisco); and Lhx7 (Vassilis Pachnis, National Institute of Medical Research, Mill Hill, London). An antisense probe for lacZ was generated from a cDNA template derived by subcloning a fragment of lacZ from a commercially available plasmid into the MCS of pBluescript II (Promega).

**RESULTS**

**Time-dependent response of oral ectomesenchyme to epithelial signals**

Analysis of early expression patterns of the homeobox genes Msx1 and Barx1, in the developing mandible has revealed that these are markers of presumptive incisor and molar mesenchyme, respectively (Tissier-Seta et al., 1995; Tucker et al., 1998a,b). Their expression patterns are established in response to signals emanating from the overlying mandibular epithelium. This has been demonstrated by the fact that expression is lost in mandible explants, after removal of the epithelium at E10.5 (Tucker et al., 1998a,b).

To investigate the nature of the response of ectomesenchymal cells to inductive signals from the epithelium, between E9.5-E11.5, the period known to involve epithelium to mesenchymal signalling, mandibles were explanted at different stages (embryonic age was carefully assessed by counting numbers of somite pairs and by other
morphological criteria – see Methods) and cultured intact or without epithelium (Fig. 1). Removal of epithelium at E9.5 and E10.5 resulted in a loss of Msx1 and Barx1 expression in the mesenchyme within 6 hours (Fig. 1A,B). However, when epithelium was removed from explants at E11.5, the mesenchyme was found to retain correct spatial expression of these genes (Fig. 1C,D).

Fgf8, which is present in the oral epithelium has been reported to induce a number of mesenchymally expressed genes, namely Pax9 (Neubuser et al., 1997), Dlx6 and Lhx7 (Grigoriou et al., 1998) and Activin βA (Ferguson et al., 1998) as well as Barx1 (Tucker et al., 1998b) and Msx1 (Kettunen and Thesleff, 1998). The competence of ectomesenchyme to respond to Fgf8 signals at different time points was investigated by implanting FGF8-soaked beads into mandible explants from which epithelium had been removed (Fig. 2). When beads were implanted six hours after removal of the epithelium, at E9.5 or E10.0, Barx1 and Msx1 expression was induced around the beads regardless of their position in the ectomesenchyme (Fig. 2A-C). Thus, Barx1 was induced ectopically in presumptive incisor ectomesenchymal cells (Fig. 2A). Likewise, Msx1 expression was induced in presumptive molar ectomesenchymal cells (Fig. 2B). Therefore, gene expression endogenous to proximal and distal mesenchymal domains can be induced in the same cells, indicating that Barx1 and Msx1 expression is not mutually exclusive. When beads were implanted six hours after removal of the epithelium at E10.5, again following loss of ectomesenchymal gene expression, Barx1 and Msx1 expression was induced only in their endogenous expression domains (Fig. 2D-G, arrows), and there was no overlap in the cells induced to express Msx1 and Barx1. FGF8 beads placed distally (i.e. in presumptive incisor ectomesenchyme) could no longer induce Barx1 in surrounding ectomesenchymal cells (Fig. 2G) and when beads were placed proximally (i.e. in presumptive molar ectomesenchyme), Msx1 expression was found to be restricted to those mesenchymal cells that were more distally located (Fig. 2D,F). Beads that were soaked in PBS or BSA were used in control experiments and these had no effect on Barx1 or Msx1 expression (Fig. 2H,I). The ability of a localised exogenous source of FGF8 to restore the correct spatial expression domains of Barx1 or Msx1 at E10.5, identifies the long range signalling properties of this protein. Removal of the epithelium from E11-11.5 explants had no effect on expression of Msx1 or Barx1 in the ectomesenchyme. Expression remained localised to the endogenous domains indicating that maintenance of spatial expression no longer required epithelial signals.

Maxillary and mandibular ectomesenchyme responds differently to epithelial signals

Dlx genes show differential expression patterns in mandibular and maxillary primordia ectomesenchyme. Dlx1 and Dlx2 are expressed in proximal ectomesenchyme of both primordia whereas other Dlx genes such as Dlx3, Dlx5 and Dlx6 are predominantly expressed in the mandibular primordia with only very small expression domains in the most proximal ectomesenchyme of the maxillary primordia at the junction with the mandible (Qui et al., 1997). The lack of expression of Dlx5 in maxillary arch ectomesenchyme at this time is consistent with the absence of any primary abnormalities in maxilla skeletal structures derived from these cells in Dlx5 mutant mice (Acampora et al., 1999; Depew et al., 1999). Double mutation of Dlx1 and Dlx2 genes results in failure of maxillary molar tooth initiation, but mandibular molars develop normally (Thomas et al., 1997). The most surprising tooth phenotype is that obtained from mutation of the activin βA gene that is expressed in patches of ectomesenchyme which mark the sites of initiation of all teeth (Ferguson et al., 1998). Mice lacking a functional activin βA gene and hence activin A protein develop normal maxillary molars but development of all other teeth is arrested at the bud stage.

These data suggest that molar tooth specification on the maxillary and mandibular primordia involves different genetic pathways. In order to investigate whether the differences in ectomesenchymal gene expression patterns reflect differences in epithelium or ectomesenchyme of the mandibular and maxillary processes, these primordia were explanted and analysed for Dlx2 and Dlx5 expression in the mesenchyme in response to epithelial signals (Fig. 3A-H).

At E9.5 mandibular ectomesenchyme expresses Dlx2 and Dlx5, whereas maxillary ectomesenchyme is characterised by expression of Dlx2, but a lack of Dlx5 expression (Fig. 3A,B) (Qui et al., 1997). As has been reported for Dlx2, Dlx5 expression in the mandible is restricted to the presumptive molar region at this time (Fig. 3C) (Thomas et al., 1997). To test whether the ectomesenchymal expression of these genes is controlled by epithelial signals in the same way as shown for Msx1 and Barx1, Fgf8 beads were implanted into distal mandibular arch ectomesenchyme at E10.25. Dlx5 expression (and Dlx2 – data not shown) was ectopically induced around the beads as shown for Msx1 and Barx1 (Fig. 3C,D). Having established that at E10.25 Fgf8 was capable of inducing Dlx2 and Dlx5 expression in mandibular arch ectomesenchyme, the expression of these two genes following implantation of FGF8 beads was compared in mandibular and maxillary primordia ectomesenchyme at E10.25.

Dlx2 and Dlx5 expression was induced around the FGF8 bead in mandible explants (Fig. 3D-F). In maxillary explants, however, only Dlx2 was induced around the bead (Fig. 3G,H). Thus despite removing maxillary epithelium, that may serve as a source of Dlx5 inhibitory signals, FGF8 is unable to induce Dlx5 expression in maxillary ectomesenchyme.

To further compare the competence of maxillary and mandibular ectomesenchyme to respond to epithelial signals, recombinations were carried out on explants at E10.25 such that maxillary epithelium was replaced with mandibular epithelium (containing the Dlx5 inducing signals) and vice versa (Fig. 3I-L). After 24 hours in culture the explants were fixed and assessed for Dlx5 expression. Dissected mandibular and maxillary primordia prior to culture showed that Dlx5 was expressed widely in the proximal ectomesenchyme of the mandible (Fig. 3I), in the nasal process but not in the maxillary primordium except at the extreme proximal edge which belongs to the hinge region with the mandible (Fig. 3I). In recombined explants, maxillary epithelium was able to induce Dlx5 in the underlying mandibular ectomesenchyme (Fig. 3K). However, in the reciprocal explants, Dlx5 was not upregulated in the maxillary ectomesenchyme lying beneath the transplanted mandibular epithelium (Fig. 3L). Thus mandibular epithelium was not able to induce Dlx5 expression
in maxillary ectomesenchyme, which is consistent with the lack of induction by Fgf8.

These results indicate that the ectomesenchyme cells of the maxillary and mandibular primordia differ in their competence to respond to epithelial signals. Cells of both the maxillary and mandibular primordia are capable of responding to Fgf8 and expressing Dlx2, but cells of the maxilla are unable to express Dlx5.

**Cell autonomous regulation of Dlx5 expression by ectomesenchyme cells of maxillary and mandibular primordia**

Since there are no reports of differences in epithelial gene expression patterns in maxillary versus mandibular primordia, the above results suggest that the neural crest-derived mesenchyme cells of the maxilla and mandible behave differently to each other. To test whether this is a result of intrinsic differences between the populations of cranial neural crest-derived cells that populate the maxillary and mandibular primordia or whether extrinsic differences are present in the developing primordia, small groups (plugs) of presumptive molar ectomesenchymal cells were reciprocally transferred between mandibular and maxillary explants at different times (Fig. 4). To identify the transferred ectomesenchyme, the groups of cells were either labelled with Dil prior to transplantation or were derived from ROSA-26 mouse embryos (Zambrowicz et al., 1997). After 24 or 45 hours in culture the explants were fixed, embedded and consecutive sections of each explant were analysed using expression of different genes as markers of cell identity. Visualisation of Dil (Fig. 4A,B) or expression of lacZ (data not shown) was used to identify the transferred cells. Expression of Dlx2 and Dlx5 was used to determine the maxillary or mandibular ‘nature’ of the transferred ectomesenchymal cells (Fig. 4E,H,K-O) and Barx1 (Fig. 4F,I) and Lhx7 (data not shown) were used as markers of presumptive molar ectomesenchyme and oral ectomesenchyme (Grigoriou et al., 1998), respectively, to assist with orientation of the explants. Groups of maxilla ectomesenchymal cells (Dlx2 and Barx1 positive; Dlx5 negative) at E9.5 or 10.25 that were transplanted and cultured in the presumptive molar regions of mandibular explants (Dlx2 and Barx1 positive; Dlx5 positive), continued to express Dlx2 and Barx1 but unlike the surrounding mandibular ectomesenchymal cells did not express Dlx5 (Fig. 4A-F,J-L). Likewise, groups of mandibular ectomesenchymal cells (Dlx2 and Barx1 positive; Dlx5 positive) that were cultured within the presumptive molar regions of maxillary explants (Dlx2 positive; Dlx5 negative), retained their original expression repertoire, such that Dlx5-expressing cells derived from the transplanted plug could be seen in the maxillary primordia (Fig. 4G-I,M-O). The results were the same whether the explants were cultured for 24 hours or 45 hours. Thus between E9.5-10.25 ectomesenchymal cells of the maxillary and mandibular primordia retain their donor characteristics and when transplanted, do not take on expression patterns characteristic of their host, despite the fact that at these times mandibular primordia cells are equally responsive to epithelial signals such as Fgf8. The ectomesenchyme cells of the maxillary and mandibular primordia thus appear to be intrinsically different in their responses to epithelial signals.

**DISCUSSION**

Reciprocal epithelial-mesenchymal signals act to control tooth development from the initiation of tooth germs through to mineralisation stages (Thesleff and Sharpe, 1997). The first instructive signals required for tooth germ initiation come from the oral epithelium which when recombined with neural crest-derived mesenchyme of a non-first branchial arch origin can instruct tooth development (Mina and Kollar, 1987; Lumsden, 1988). The extent to which the responses of underlying CNC-derived ectomesenchyme cells are regionally prespecified has been controversial because of conflicting results obtained from recombination experiments and more recently from the tooth phenotypes produced from gene targeting.

Having previously established that expression of a number of genes showing early, regionally restricted expression in first branchial arch ectomesenchymal cells is regulated by specific epithelial signals (Neubuser et al., 1997; Ferguson et al., 1998; Grigoriou et al., 1998; Kettunen and Thesleff, 1998; Tucker et al., 1998b), we set out to determine whether such early signals between epithelium and ectomesenchyme cells might reveal the nature of the interactions that control tooth patterning. Using expression of Barx1, Dlx2 and Dlx5 as proximal (presumptive molar) markers and Msx1 as a distal (presumptive incisor) mandibular ectomesenchyme marker we showed that prior to E11.5, expression of these genes is dependent on signals from the epithelium but from E11.5, expression is independent of epithelium. Between E9.5-E10.25, the response of ectomesenchyme cells to epithelial signals was found to be unrestricted, thus all cells were competent to respond and express Msx1, Dlx2, Dlx5 and Barx1. By E10.5 however, this changed dramatically such that competence to express these genes in response to epithelial signals became regionally restricted. Only ectomesenchyme cells that had expressed these genes before E10.5 retained competence, whereas cells that had not expressed a particular gene were not competent to express that gene by E10.5 (summarised in Fig. 5). These results were found to be identical for all the five homeobox genes studied.

Three important conclusions can be drawn from these results. The first is that tooth patterning, namely the determination of tooth type, is specified by the regional expression of ectomesenchymal genes but this expression is produced in response to epithelial signals. Thus reciprocal incisor/molar region recombination carried out prior to E10.5 will show that the epithelium determines tooth type (Miller, 1969; Lumsden, 1988; Kollar and Mina, 1991) whereas the same recombination carried out after E10.5 will show that the ectomesenchyme determines tooth type (Kollar and Baird, 1969). Our data therefore identifies some of the molecular mechanisms that underlie the findings from previous recombination studies. The second conclusion is that since all mandibular arch ectomesenchyme cells are equally competent to respond to Fgf8 and activate expression of Msx1, Barx1, Dlx2 or Dlx5, before E10.5, regardless of position, this suggests that CNC cells populating the mandibular arch are not prespecified with respect to proximodistal position and odontogenic fate. The third conclusion is that Fgf8 appears to have both short and long range signalling properties that change between E10 and E10.5. At E10, addition of FGF8 beads to ectomesenchyme resulted in localised gene expression.
around the bead. Identical experiments carried out at E10.5 showed only restoration of gene expression in endogenous domains and not around the bead. Thus the changes in cell competence between E10 and E10.5 correspond to a change from short-range to long-range Fgf8 signalling. Since Fgfs have a very limited diffusion range (Storey et al., 1998) the mechanism of long-range signalling remains unexplained.

The apparent lack of odontogenic prespecification of CNC cells agrees with the results of experiments which show that tooth identity can be changed by inhibition of Bmp signalling and the accompanied misexpression of a proximal gene, Barx1, in presumptive incisor mesenchyme at E10 (Tucker et al., 1998b). It also agrees with experiments showing that teeth can develop when non-oral ectomesenchyme from the mandibular arch is recombined with oral epithelium and where oral epithelium is recombined with non-first branchial neural crest-derived mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). CNC cells are thus not prespecified with respect to their odontogenic potential. It is likely that all CNC cells would be capable of odontogenesis when presented with the appropriate instructive signals, namely those produced by oral epithelium. This odontogenic plasticity of CNC cells seems to contradict CNC grafting experiments carried out in avian embryos, the results of which have generally been interpreted as providing evidence for skeletogenic prespecification of CNC cells. Thus grafts of premigratory CNC cells destined to form first branchial arch skeletal elements (from r1 and r2) into the position from where second arch CNC cells would normally migrate (r4/6) resulted in development of first arch skeletal structures in the second arch (Noden, 1983). The interpretation of these results as being indicative of prespecification of premigratory CNC cells is however unlikely to be correct since similar grafts of forebrain and midbrain CNC cells also produced first arch skeletal structures in place of second arch structures and not duplications of frontonasal structures as expected if these cells are prespecified (Noden, 1978, 1983).

A general conclusion from these grafting experiments is that CNC cells rostral to r2/3 have the capacity to form lower jaw skeletal structures when provided with a ‘permissive’ branchial arch environment. Since the main obvious molecular difference between these cells and those caudal to r2/3, is that the former do not express Hox genes, this suggests that Hox gene expression in CNC cells is an important determinant of branchial arch patterning (Hunt et al., 1991).

The role of Hox genes and particularly Hoxa2 in skeletal patterning of the second branchial arch is well established. Targeted mutations in Hoxa2 produce duplications of first arch skeletal elements similar to those obtained by grafting experiments in chick embryos (Noden, 1983; Gendron-Maguire et al., 1993; Rijli et al., 1993). Hoxa2 appears to function by repressing chondrogenesis, consistent with ectopic expression of Hoxa2 in the first branchial arch repressing...
development of first arch skeletal elements (Couly et al., 1998; Kanzler et al., 1998). Because the ectopic expression of Hoxa2 in the first branchial arch was achieved by manipulations of avian embryos it is not possible to determine if this would result in repression of odontogenesis. However this seems unlikely since oral epithelium is clearly able to induce

Fig. 4. Dlx5 and Dlx2 expression in explants after heterotopic transplantation of small plugs of mesenchymal cells. Transplantations of E9.5 plugs of ectomesenchyme cells into E10 host are shown in D-I. Transplantations carried out at E10.25 are shown in A-C and J-O.

(A-C) Tracing the transplanted plugs of maxillary ectomesenchymal cells into the presumptive molar region of a mandibular primordium explant. Fluorescent microscopy revealed a Dil-labelled maxillary plug (A) after transplantation (B). DIG wholemount in situ hybridisation shows that Dlx5 expression is missing in the maxillary plug (C). Arrows indicate the position of the transplanted plug in the mandible explant.

(D-F,J-L) Transplantation of a plug of maxillary ectomesenchymal cells into the presumptive molar region of a mandibular explant. The transplanted plug is Dlx5 negative (E,K) and Barx1/Dlx2 positive (F,L). The corresponding light-field photomicrographs are shown in D and J.

(G-I,M-O) Transplantation of a plug of mandibular mesenchymal cells to the presumptive molar region of a maxillary explant. The transplanted mandibular cells are both Dlx5 positive and Dlx2/Barx1 positive. (G-I) Maxillary explant probed with Dlx5 (h) and Barx1 (I). The corresponding light-field photomicrograph is shown in (G). (M) Dark-field photomicrograph showing low power view of Dlx5 expression in a maxillary explant. (N,O) Higher power view of the same explant probed with Dlx5 (N) and Dlx2 (O). Arrow indicates the position of a developing maxillary molar. Note the molar mesenchyme underlying the invaginating molar epithelium is Dlx2 positive but Dlx5 negative.
development of molar teeth on the maxillary and mandibular primordia, which required further investigation.

Expression of \(Dlx2\) and \(Dlx5\) were both found to be regulated by epithelial signals at E10.25. Fgf8 was identified as one of the molecules responsible for inducing this gene expression. However whereas both maxillary and mandibular epithelial and exogenous FGF8 were all capable of inducing \(Dlx2\) expression in maxillary and mandibular ectomesenchyme, none of these sources of epithelial signals were able to induce expression of \(Dlx5\) in maxillary ectomesenchyme. Thus despite being able to receive and respond to epithelial signals and express \(Dlx2\), maxillary ectomesenchyme cells are incapable of expressing \(Dlx5\) in response to the same signals. Since these experiments were carried out at a time when we had established that all ectomesenchymal cells of the mandibular primordium are equally competent to respond to Fgf8 and the maxillary primordia are developmentally 12 hours behind the mandibular primordium, they suggest that the ectomesenchymal cells of the maxillary and mandibular primordia are in some way fundamentally different. By reciprocally recombining maxillary epithelium and mandibular ectomesenchyme at E10.25, we confirmed that maxillary epithelium is capable of inducing \(Dlx5\) expression in mandibular ectomesenchyme. Despite being able to induce \(Dlx5\) expression in mandibular ectomesenchyme, mandibular epithelium was unable to do so in maxillary ectomesenchyme. These experiments establish that maxillary epithelium does not produce signals that repress \(Dlx5\) expression.

Since our experiments show that maxillary and mandibular epithelium appear to behave identically in regulating \(Dlx5\) expression we investigated the possible effects of the ectomesenchymal cell environment on regulating \(Dlx5\) expression. Cell transplantation were used to investigate the responses of maxillary and mandibular ectomesenchyme cells to epithelial signals. \(Dlx5\)-expressing cells transplanted from the mandibular into the maxillary primordium at E10.25 retained their expression whilst maxillary cells transplanted into the mandibular primordium did not express \(Dlx5\) despite being surrounded by \(Dlx5\)-expressing cells (summarised in Fig. 6). These results imply that the ectomesenchymal cells of the first branchial arch have intrinsic properties related to their rostrocaudal positions. Thus maxillary ectomesenchymal cells behave differently in their responses to epithelial signals than mandibular ectomesenchymal cells.

Our data and those from recombination experiments shows that first branchial arch epithelium is unique in containing instructive signals for odontogenesis (Mina and Kollar, 1987; Lumsden, 1988; Tucker et al., 1999) and that these signals are capable of overriding any prepatterning information present in the CNC cells. If this is the case then it follows that cells receiving these instructive signals must follow identical differentiation pathways. However, the mandibular and maxillary primordia develop obviously different skeletal structures and subtly different teeth yet both are covered by the same oral epithelium. In addition, the molecular and genetic differences identified between the ectomesenchymal cells of these two processes i.e. the differences in \(Dlx\) gene expression, the different knock-out phenotypes observed for maxillary and mandibular molars and skeletal elements and the results from our cell transplantation studies, are indicative of different
Fig. 6. A schematic diagram summarising the results obtained from manipulations of maxillary and mandibular explants. (A) Sagittal view of an embryonic head at E10-E10.5, showing the extent of Dlx5 and Dlx2 expression in the mesenchyme of maxillary and mandibular primordia. (B) A higher power sagittal view of an embryonic head showing that an Fgf8 signal supplied endogenously by the oral epithelium or exogenously by beads, is able to induce both Dlx5 and Dlx2 expression in mandibular mesenchyme, but only Dlx2 expression in maxillary mesenchyme. (C) A sagittal view of an embryonic head showing that when mesenchymal cells from the maxilla were transplanted into the mandible and vice versa, the transplanted cells retained the expression profile characteristic of their origin and were not competent to take on the characteristics of their new position in the embryo, indicating intrinsic differences between maxilla and mandibular mesenchyme.

 patterning processes. The fact that maxillary and mandibular epithelia are interchangeable as regulators of ectomesenchymal gene expression indicates that the specificity of responses to the instructive epithelial signals must be a property intrinsic to the ectomesenchymal cells. Thus in order for these cells to develop their maxilla-specific skeletal structures they may be able retain an element of prespecification to prevent development as mandible skeletal elements.

It is tempting to speculate that the different properties of the mandibular and maxillary ectomesenchymal cells are related to the origins of the neural crest cells that populate these components of the first branchial arch. Fate mapping in avian and mouse embryos shows that the mandible is mainly composed of CNC cells that migrate from the midbrain with some contribution from rhombomeres 1 and 2 (Imai et al., 1996; Kontges and Lumsden, 1996). The maxillary ectomesenchymal cells are derived from CNC cells migrating from both the midbrain and the forebrain (Osumi-Yamashita et al., 1994). Such a difference in axial origin might thus explain the different responses of these cells to epithelial signals. Alternatively, the CNC cells might acquire their rostrocaudal specification – mandibular versus maxillary – during migration where signals from the cranial mesoderm are likely to be involved (Paul Trainor and Robb Krumlauf, personal communication).

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