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Role of *Dlx-1* and *Dlx-2* genes in patterning of the murine dentition

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SUMMARY

The molecular events of odontogenic induction are beginning to be elucidated, but until now nothing was known about the molecular basis of the patterning of the dentition. A role for *Dlx-1* and *Dlx-2* genes in patterning of the dentition has been proposed with the genes envisaged as participating in an 'odontogenic homeobox gene code' by specifying molar development. This proposal was based on the restricted expression of the genes in molar ectomesenchyme derived from cranial neural crest cells prior to tooth initiation.

Mice with targeted null mutations of both *Dlx-1* and *Dlx-2* homeobox genes do not develop maxillary molar teeth but incisors and mandibular molars are normal. We have carried out heterologous recombinations between mutant and wild-type maxillary epithelium and mesenchyme and show that the ectomesenchyme underlying the maxillary molar epithelium has lost its odontogenic potential. Using molecular markers of branchial arch neural crest (*Barx1*) and commitment to chondrogenic differentiation (*Sox9*), we show that this population alters its fate from odontogenic to become chondrogenic. These results provide evidence that a subpopulation of cranial neural crest is specified as odontogenic by *Dlx-1* and *Dlx-2* genes. Loss of function of these genes results in reprogramming of this population of ectomesenchyme cells into chondrocytes. This is the first indication that the development of different shaped teeth at different positions in the jaws is determined by independent genetic pathways.

Key words: homeobox, Dlx, patterning, tooth development, neural crest, mouse, dentition, odontogenic induction

INTRODUCTION

Tooth development is known to occur through a series of reciprocal epithelial-mesenchymal interactions. The initiation of tooth development involves an interaction between epithelium of the first arch and the underlying neural-crest-derived ectomesenchyme. Following initiation, the epithelium invaginates forming a tooth bud around which the ectomesenchyme condenses. During these stages and subsequent stages many potentially regulative molecules are expressed. Key components of tooth initiation signalling pathways have been shown to be Bmp-4, Lef1 and Msx-1 (Vainio et al., 1993; van Gederen et al., 1994; Satokata and Maas, 1994). Targeted mutations of *Lef1* or *Msx-1* result in the arrest of all tooth development at the bud stage. Despite an increasing wealth of information regarding tooth initiation from recombination and knock-out studies, no molecular evidence has been reported that may contribute to an understanding of odontogenic patterning. Recombination experiments have been carried out by many workers to attempt to elucidate whether the patterning is regulated by the epithelium or the mesenchyme. It appears from this work that the control of morphogenesis fluctuates between these two tissue layers at different stages of tooth development, but there is still no firm evidence confirming which tissue sets up the initial patterning signals (Lumsden, 1988; Kollar and Baird, 1969; Miller, 1969). Thus, despite over 40 years of discussion, there is no experimental evidence to explain what determines the precise site of tooth development or what type of tooth will develop at that site. We describe here the first molecular data relating to patterning of the dentition, showing that the development of maxillary molars requires the regional specification of a population of cranial neural crest cells by the *Dlx-1* and *Dlx-2* homeobox genes.

Dlx-1 and *Dlx-2* expression in neural-crest-derived mesenchyme of the mandibular and maxillary processes is restricted to proximal regions where molar teeth will develop. *Dlx-2* is also expressed in the epithelium of the distal aspects of the first branchial arch at this stage, prior to the initiation of tooth development (Fig. 1). *Dlx-1* and *Dlx-2* are members of a family of genes orthologous to the *Drosophila distal-less* homeobox gene. These genes are located on the same chromosome (chromosome 2 in mouse), separated by 10 kb and are transcribed in oppposite directions (Ozcelick et al., 1992; McGuiness et al., 1996). Interestingly, *Dlx-5* and *Dlx-6* have been found on a different chromosome in a similar arrangement (Simeone et al., 1994), suggesting an initial duplication event, followed by a second, tandem duplication. *Dlx-5* and

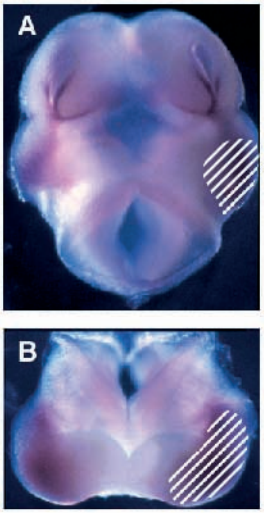

Fig. 1. Expression of *Dlx-2*. Digoxigenin-labelled whole-mount in situ hybridisation of E10.5 embryos showing *Dlx-2* expression from the oral aspect in the maxilla and frontonasal process (A) and mandibular process (B). White shading indicates the areas of mesenchymal *Dlx-2* expression.

Dlx-6 are expressed in very similar domains to *Dlx-1* and *Dlx-2* in the mandibular division of the first branchial arch, but are not expressed in the maxillary division (Qiu et al., 1997). Thus, prior to the initiation of tooth development, *Dlx-1*, *Dlx-2*, *Dlx-5* and *Dlx-6* are all expressed in the ectomesenchyme of the presumptive mandibular molar region, but only *Dlx-1* and *Dlx-2* are expressed in the ectomesenchyme of the presumptive maxillary molars.

A mechanism for patterning of the dentition, based on spatially restricted expression of homeobox genes in first branchial arch mesenchyme has previously been proposed (Sharpe, 1995; Thomas and Sharpe, 1997). This 'odontogenic homeobox code' proposes *Dlx-1* and *Dlx-2* genes to be involved specifically in patterning of molar tooth development. Furthermore, the fact that *Dlx-1* and *Dlx-2* are the only Dlx genes expressed in the presumptive maxillary molar mesenchyme, suggested that these genes would play a specific role in maxillary molar development.

Targeted mutations in either *Dlx-1* or *Dlx-2* affect development of cranial neural-crest-derived skeletal elements derived from the proximal parts of the first and second branchial arches but have no effect on tooth development (Fig. 2A,B) (Qiu et al., 1995, 1997). Mice generated with mutations in both genes, however, have a profound tooth phenotype where new-born mice have no maxillary molars but all other teeth are present (Fig. 2C-L). Analysis of specific molecular markers of tooth development shows this tooth phenotype is primary and not related to other structural changes in jaw development, most of which also occur in the *Dlx-2* single mutants, which have maxillary molars (Qiu et al., 1995, 1997). This is the first targeted mutation identified that produces a tooth patterning defect and shows that the presence of either *Dlx-1* or *Dlx-2* is sufficient for normal tooth development but both genes are required specifically for development of maxillary molars.

We have analysed the molecular basis of the failure of maxillary molar tooth development in the *Dlx-1/Dlx-2* mutants. The phenotype appears to result from a defect in the mesenchyme where odontogenic cells are reprogrammed to become chondrogenic resulting in ectopic cartilage replacing maxillary molar teeth.

MATERIALS AND METHODS

Generation of mutant mice

Targeted mutations in *Dlx-1* or *Dlx-2* and both *Dlx-1* and *Dlx-2* genes were produced as described (Qiu et al., 1997). Mutations in *Dlx-1* or *Dlx-2* were produced using targeting vectors which deleted exons two and three of *Dlx-1* or exons two, three and part of exon one of *Dlx-2*. These both resulted in complete removal of the homeobox following homologous recombination. Since *Dlx-1* and *Dlx-2* are separated by only 10 kb of intergenic sequence, targeted disruption to produce double mutants was carried out using a targeting vector with deletion of exons two and three of *Dlx-1* and exons two, three and part of exon one of *Dlx-2*. JM-1 ES cells were electroporated, selected and characterised as described (Qiu et al., 1995). Genotyping of ES cells and mouse tails was performed using Southern blots and PCR assays. Primers for the PCR assays were 5′-AAGGCGGGGCAG-CTCTGGAG-3′, 5′-AGGGAGACGGGCAGGAAGCG-3′ for *Dlx-1*, which amplified a fragment in the third exon, and 5′-TCCGAAT-AGTGAACGGGAAGCCAAAG-3′, 5′CAGGGTGCTGCTCGGTG-GGTATCTG-3′ for Dlx-2, which amplified a fragment in the 3′ coding region. The PCR conditions were: 1 cycle 94°C; 35 cycles of 94°C 1 min, 65°C 1 min, 72°C 1 min and 1 cycle 72°C, 1 min for the *Dlx-1* primers and 1 cycle 94°C; 35 cycles of 94°C 1 minute, 58°C 1 minute, 72°C 1.5 minutes and 1 cycle 72°C 1.5 minutes for the *Dlx-2* primers. For the PCR assay to genotype the double mutants, the same primers and conditions were used as for the *Dlx-1* mutants.

Embryo preparation

Embryos were obtained from matings between *Dlx-1/Dlx-2* heterozygous mice. Day E0 was taken as midnight prior to finding a vaginal plug.

Heterotypic recombinations

Recombinations were carried out at E12.5 and E13.5. Heterozygous females and males were mated to give a 1 in 4 chance of a homozygous mutant. At these stages, there is no obvious difference in gross morphology between the wild types and mutants. Since the frontonasal process was not included in the dissection, the epithelium used in the culture excluded all presumptive incisor epithelium. The molar region of the maxillas were dissected out in D-MEM as indicated in Fig. 3A. The rest of the embryo was used for genotyping. Epithelium was removed using Dispase in calcium- and magnesium-free PBS at 2 units per ml. The E12.5 maxillas were incubated for 12 minutes at 37°C, the E13.5 maxillas for 15 minutes at 37°C. After incubation the maxillas were washed in D-MEM with 10% fetal calf serum (FCS) and the epithelium was dissected off using fine tungsten needles. The mesenchyme was placed on membrane filters supported by metal grids following the Trowel technique as modified by Saxén (Trowel, 1959; Saxén, 1966).

It was important that the mesenchyme was orientated on the filter so that the oral surface faced upwards. To visualise the epithelia, they were weakly dyed with neutral red before being placed over the mesenchyme on the grids. Most of the presumptive incisor region of the maxilla was removed during the dissection. This is important as maxillary incisors develop as normal in the mutant. In addition to this, the epithelia were placed only over the mesenchyme of the most proximal part of the cultures. In this way, if a tooth bud was seen, it could only represent a rescue of a molar bud. The recombinations were cultured for 3 to 5 days in D-MEM with 10% FCS and 50 μg/ml transferrin, the medium being changed every 2 days. All solutions used contain penicillin and streptomycin at 20 i.u./ml. A standard incubator was used at 37°C with an atmosphere of 5% CO_2 in air and 100% humidity. After the period of culture, recombinations were washed in ice-cold methanol for 5 minutes then fixed in fresh 4% paraformaldehyde for 1 hour at room temperature. Cultures were embedded in paraffin for sectioning and stained with haematoxylin-

eosin or were used for whole-mount digoxigenin in situs. Whole-mount in situs were later sectioned and adjacent sections counter-stained with haematoxylin-eosin to show the morphology.

In situ hybridisation

Heads from time-mated embryos were fixed in paraformaldehyde, then processed for whole-mount in situs or paraffin wax embedded for section in situs. Genotypes were determined by Southern analysis and PCR analysis of genomic DNA prepared from the embryo bodies. Serial frontal sections were cut at 8 μm. Radioactive in situ hybridisation and digoxigenin in situ hybridisation were carried out as described by Wilkinson (1995), with a few modifications.

The RNA probes were synthesised from linearised DNA templates using T3 [*Dlx-2* (Bulfone et al., 1993), *Sox9* (Wright et al., 1995), *Fgf8* (Mahmood et al., 1995), *Ptc* (Goodrich et al., 1996)], T7 [*Dlx-1* (Bulfone et al., 1993), *Barx1* (Tissier-Seta et al., 1995), *Shh* (Bitgood and McMahon, 1995)] or SP6 [*Msx-1* (Mackenzie et al., 1991), *Bmp4* (Lyons et al., 1990)] RNA polymerase.

RESULTS

Dlx-1 and *Dlx-2* have a redundant role in development of maxillary molar teeth

Targeted mutations in *Dlx-1*, *Dlx-2* and double mutants in *Dlx-1* and *Dlx-2* were generated and frontal sections of new-born mice analysed. Normal tooth development was observed in *Dlx-1* and *Dlx-2* mutants heads (Fig. 2A,B) (Qui et al., 1995, 1997). However, sections of *Dlx-1/Dlx-2* double mutant heads revealed the complete absence of all maxillary molar teeth (M1, M2 and M3), but mandibular molars and all incisors were present and cytodifferentiation was histologically normal (Fig. 2C-L). In addition to missing maxillary molars, ectopic pieces of cartilage were observed in the mutants in the positions where maxillary molars should have developed (Fig. 2C,O). The maxillary phenotype was 100% penetrant, being observed in all 30 mutants studied so far.

In order to determine at which stage of development maxillary molar tooth development was arrested in the double mutants, frontal sections of embryo heads from E11.5 to E15.5 were examined. Early stage mutants showed epithelial thickening formation as in the wild-type embryos (Fig. 2M). At later stages, mutant embryos showed normal development of the incisors and mandibular molars but maxillary molar development failed to proceed past the epithelial thickening stage (Fig. 2N).

Dlx-1/*Dlx-2* mutant mesenchyme is unable to support tooth development

Tooth development is characterised by a series of interactions between epithelium and mesenchyme. At the epithelial thickening stage, signals pass from the epithelium to the underlying mesenchyme to initiate mesenchymal condensation, followed by a reciprocal signal from the mesenchyme to the epithelium resulting in bud formation (Thesleff et al., 1995).

In order to investigate the tissue origin of the *Dlx-1/Dlx-2* knock-out tooth defect, tissue recombinations were

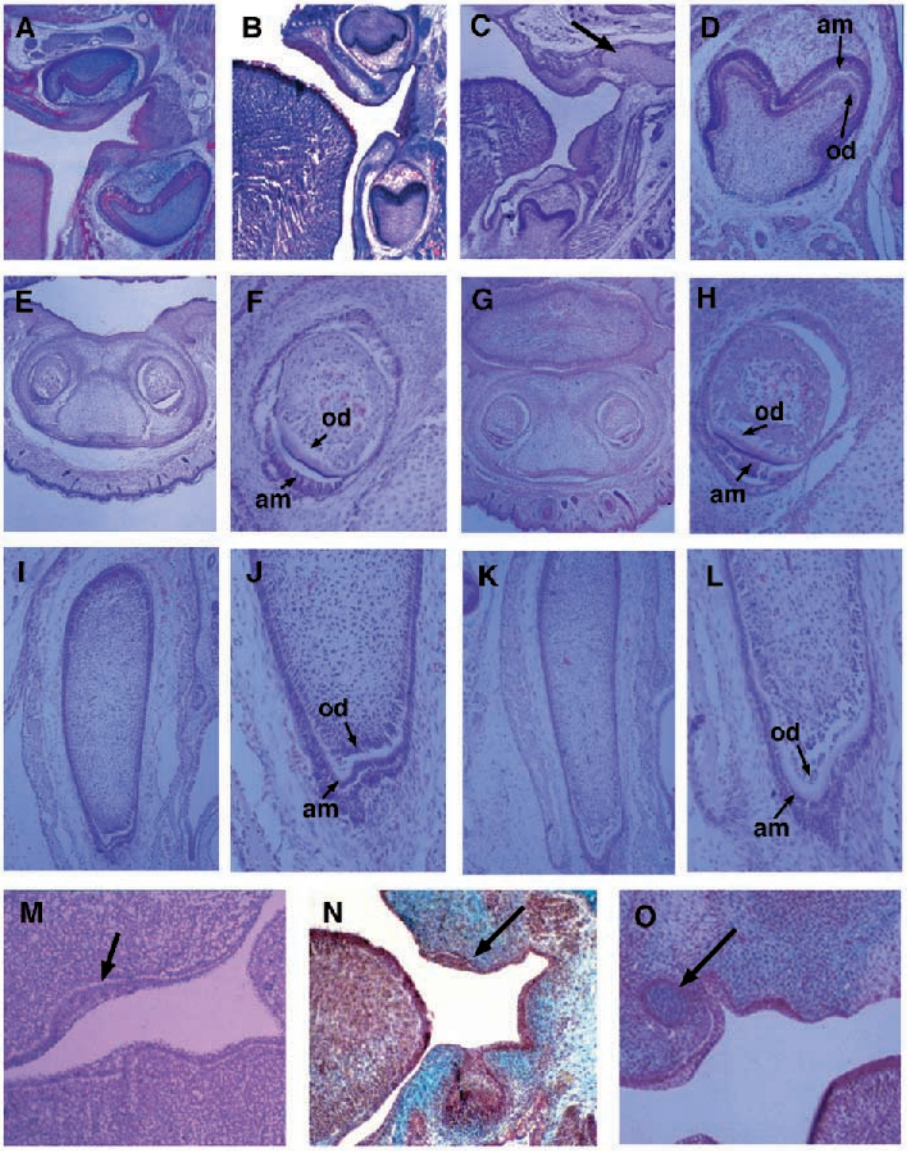

Fig. 2. Knock-out phenotypes. Frontal sections of new born mice null mutant for *Dlx-1* (A), *Dlx-2* (B) and *Dlx-1/Dlx-2* (C) showing the presence of upper and lower molars in the single gene mutants and only lower molars in the double mutant. Arrow shows area of ectopic cartilage in new born double mutants. Higher magnification of lower molar in the double mutant showing normal cytodifferentiation (D). Comparison of incisor phenotypes in the double mutant and wild types. Double mutant lower incisors (E,F) and upper incisors (I,J). Wild-type lower incisors (G,H) and upper incisors (K,L). Normal cytodifferentiation can be seen at the higher magnification (F,J). Frontal sections of *Dlx-1/Dlx-2* mutant embryos at E11.5 (M), E14.5 (N) and E15.5 (O) showing the epithelial thickening in the maxillary molar area at E11.5 and at E14.5, indicated by an arrow, and the ectopic cartilage seen at this site in later stage embryos, indicated by an arrow. am, ameloblasts; od, odontoblasts.

performed between mutant and wild-type maxillary tooth-forming epithelium and mesenchyme (Fig. 3A). Between E12.5 and E13.5, the maxillary molar tooth germ proceeds from an epithelial thickening to a bud stage in wild-type embryos. At the stages used therefore, the maxillary molar tooth germs are at the stage when the epithelium is signalling to the mesenchyme.

Recombination of mutant mesenchyme with wild-type epithelium failed to produce tooth buds but did produce excessive cartilage (Fig. 3B), whereas the reciprocal recombination of wild-type mesenchyme with mutant epithelium consistently produced tooth buds (Fig. 3C; Table 1). These buds were shown to express *Msx-1* in the condensing mesenchyme (Fig. 3E,F). The defect in maxillary molar development thus resides in the ectomesenchyme.

Table 1. Number of tooth buds formed after recombination of oral epithelium and mesenchyme

	E12.5	E13.5
Wt epi Wt mes	7/14	2/4
Mutant epi Wt mes	4/6	3/6
Wt epi Mutant mes	0/5	0/4
Mutant epi Mutant mes	0/0	0/2

Recombinations carried out at E12.5 and E13.5 and cultured for 3-5 days.

Expression of epithelial-mesenchymal signalling molecule genes is normal in *Dlx-1*/*Dlx-2* mutants

To confirm that the arrest in maxillary molar tooth development at the epithelial thickening stage did not occur due to an inability of the thickened epithelium to signal to the underlying mesenchyme, the expression of epithelial signalling molecules was examined. *Shh* and *Fgf-8* are expressed in tooth epithelial thickenings at E11.5 and together with *Bmp-4* are candidates for signals that induce gene expression in the underlying mesenchyme to stimulate tooth bud formation (Bitgood and McMahon, 1995; Neubüser et al., 1997; Vainio et al., 1993). Expression of *Shh* and *Fgf-8* at E11.5 in tooth epithelium was found to be indistinguishable between mutants, heterozygous littermates and wild-type embryos indicating that at least with respect to production of these signalling molecules the dental epithelium of the double mutants is normal (Fig. 4A-D).

The ability of the mesenchyme underlying the epithelial thickenings to respond to epithelial signals was examined using the expression of *Bmp-4*, *Ptc* and *Msx-1* as markers of response to Bmp-4 and Shh epithelial signals. Bmp-4 secreted from the thickened epithelium acts as a signal to the mesenchyme and activates *Msx-1* expression which in turn activates *Bmp-4* expression in the mesenchyme (Vainio et al., 1993; Chen et al., 1996). Thus Bmp-4 participates first as an epithelial-to-mesenchymal signal and, subsequently, at the early bud stage as a mesenchymal-to-epithelial signal. The expression of *Bmp-4* in the bud mesenchyme is essential for tooth development to proceed (Chen et al., 1996). Likewise, *Msx-1* expression is also required for a progression past the bud stage as shown in mice with targeted mutation of *Msx-1* (Satokata and Maas, 1994). Thus, early mesenchymal expression of *Bmp-4* and *Msx-1* is a marker for mesenchyme that has responded to epithelial signals. Similarly the expression of the Shh receptor, *Ptc*, in tooth germ mesenchyme is a marker for reception of Shh signals. Expression of *Ptc*, *Msx-1* and *Bmp-4* in the underlying mesenchyme of mutant teeth from E12.5-14.5 was found to be normal (Fig. 4E-J). It is clear therefore that the epithelial-mesenchymal signalling pathways for Shh and Bmp4 are not disrupted but there is a failure in subsequent development of maxillary molar tooth germs. *Dlx-1* and *Dlx-2* genes are therefore not required for initiating these early odontogenic signals but have a specific role in patterning by allowing development of maxillary molars to proceed beyond an epithelial thickening.

Maxillary molar odontogenic mesenchyme is respecified in *Dlx-1/Dlx-2* mutants

Since mesenchymal cells can respond to early odontogenic epithelial signals by expressing *Bmp-4*, *Msx-1* and *Ptc*, the tooth defects in the mutants are unlikely to be caused by failure of neural crest cell migration. Given that the maxillary molar ectomesenchyme is unable to undergo odontogenesis, we wished to know the alternative fate of these ectomesenchymal cells. To investigate this, we used the expression patterns of two marker genes. Expression of the homeobox gene, *Barx-1* was used as a marker of a subpopulation of the ectomesenchyme contributing to the developing face. *Barx-1* is expressed in ectomesenchymal cells in the proximal regions of the first branchial arch where molar teeth will develop but is absent from distal regions where incisors will develop (Tissier-Seta et al., 1995). It is not expressed in cells destined to become cartilage, such as Meckel's cartilage or the nasal septum. *Barx-1* expression is thus expressed in the region of the first branchial arch that gives rise to molar teeth and later becomes restricted specifically to the mesenchymal component of the molars (Tissier-Seta et al., 1995).

In wild-type maxillary molar tooth germs and mutant mandibular molar tooth germs, *Barx-1* expression was present in a large area of ectomesenchyme around the molar tooth germs (Fig. 5A). However, in mutant maxillary molars, a circular patch of ectomesenchymal cells, immediately underlying the thickened epithelium was found to be *Barx-1* negative (Fig. 5D). Since *Barx-1* expression is restricted to non-chondrogenic cells in facial mesenchyme, ectopic cartilages in the maxilla are a feature of the *Dlx-1/Dlx-2* phenotype and excessive cartilage was formed when mutant mesenchyme was recombined with wild-type epithelium, we investigated the possibility of a chondrogenic fate of this patch of *Barx-1*-negative cells.

The HMG-box transcription factor gene *Sox9* has been shown to be expressed in the early development of chondrogenic cells and to regulate the expression of *Type II collagen* gene expression (Wright et al., 1995; Ng et al., 1997). *Sox9* is thus an early marker for chondrogenic cell differentiation (Wright et al., 1995). At E13.0, *Sox9* expression in facial mesenchyme of wild-type embryos was strong in the forming chondrogenic regions but was excluded from the mesenchyme immediately adjacent to the epithelial thickenings in the tooth-forming regions. In double mutant embryos, a very obvious ectopic patch of *Sox9*-expressing cells was observed in the

maxilla adjacent to the epithelial thickening and coincided with the patch of cells that had lost *Barx-1* expression (Fig. 5C,F). We conclude from this that the ectomesenchymal cells that would normally be odontogenic in maxillary molar teeth have been reprogrammed to become chondrogenic in the absence of *Dlx-1* and *Dlx-2*.

DISCUSSION

Two contrasting models have been proposed to explain patterning of the dentition. Butler (1956) proposed a gradient model where concentration gradients of unspecified molecules across the mandible and maxilla might determine incisor and molar tooth fields. Osborne (1978) suggested a clonal model of patterning where different clones of mesenchymal cells (ectomesenchyme) populated the first arch giving rise to molar- and incisor-specified mesenchyme. Based on the highly restricted domains of homeobox gene expression in maxillary and mandibular ectomesenchyme, we proposed an extension of Osborne's model whereby patterning was controlled by the combinations of these homeobox genes expressed in facial mesenchyme; the odontogenic homeobox code (Sharpe, 1995; Thomas and Sharpe, 1997). Until now no experimental data has been produced to support either of these models.

Interactions between cranial neural crest cells and oral epithelium are required for tooth development. Pattern must be determined either by a prepatterning of the epithelium into molar and incisor regions or by patterning of ectomesenchyme cells as incisor and molar populations. Recombination experiments have so far proved inconclusive in identifying the origin of dental pattern (Lumsden, 1988, Kollar and Baird, 1969, Miller, 1969). The results that we present here suggest that it is the ectomesenchyme that is patterned by specific populations (or clones) of cells.

Based on the odontogenic code, we proposed that *Dlx-1* and *Dlx-2* genes would have a role specifically in molar development because their expression in first arch mesenchyme is restricted to presumptive molar regions (Fig. 1A,B). We tested this hypothesis by generating mutations by gene targeting. As previously reported, we found no obvious tooth abnormalities in either *Dlx-1* or *Dlx-2* mutant mice in which all teeth were present in new-born mice (Fig. 2A,B). These mice do however show abnormalities in development of specific facial skeletal elements (Qui et al., 1995, 1997). *Dlx-1* and *Dlx-2* are co-localised in facial mesenchyme; therefore, it seemed likely that there may be functional redundancy between the genes for a role in tooth patterning. We generated double mutants that lack both functional *Dlx-1* and *Dlx-2* genes. Histology of the teeth in the double mutant showed normal development and cyto-differentiation of the incisors and mandibular molars in new-born mice, but there was no evidence of any maxillary molar teeth (Fig. 2C-L). *Dlx-1* and *Dlx-2* thus appear to have a functionally redundant role in development of maxillary molar teeth but are not required for development of incisors or mandibular molars. This phenotype is in marked contrast to other tooth development phenotypes produced by mutations in *Msx-1* and *Lef-1*, which affect development of all teeth.

In principle, tooth development can be arrested at any stage of development so we analysed mutant embryos at different stages between E11.5 and E15.5 to determine exactly when the arrest occurred (Fig. 2M-O). Development of incisor and mandibular molar tooth germs was found to progress in a normal temporal pattern in the mutants. Maxillary molar tooth development was halted at the epithelial thickening stage and no bud- or cap-stage tooth germs were identified. This indicates that arrest of tooth development occurs very early in these mutants, at the time when tooth germ development is first initiated. This again contrasts with the *Lef-1* and *Msx-1* knock-outs in which tooth development is arrested at the bud stage.

The tooth phenotype of the *Dlx-1*/*Dlx-2* mutants supports the odontogenic code model since loss of *Dlx-1/Dlx-2* expression in neural-crest-derived mesenchymal cells that populate the presumptive maxillary molar region results in inhibition of tooth development from a very early stage. Since maxillary molar tooth development was arrested at the thickening stage when reciprocal signals between the epithelium and the underlying mesenchyme are known to be required for progression to the bud stage, it was important to determine the tissue origin of the defect. To investigate this, recombinations of mutant epithelium with wild-type mesenchyme and the reciprocal recombinations were performed at E12.5 and E13.5, the epithelial thickening stage of the maxillary molars. Only recombinations of mutant epithelium with wild-type mesenchyme were able to induce formation of a tooth bud, implying that the defect originates in the ectomesenchyme (Fig. 3; Table 1).

The exact molecular role of Dlx-1 and Dlx-2 in the maxillary ectomesenchyme is not clear but one possibility is that these Dlx proteins regulate the expression of molecules downstream of the early epithelial/mesenchymal signalling pathways, therefore tooth development arrests before bud formation. Regulation of these downstream genes, or equivalent genes in development of other teeth, must be regulated by genes other than *Dlx-1* or *Dlx-2*. Possible candidates for this role include other Dlx genes such as *Dlx-3*, *Dlx-5* and *Dlx-6* which are all expressed in mesenchyme of the developing mandible but are not expressed in the maxilla (Qiu et al., 1997). It is possible therefore that the lack of phenotype in the mandibular molars of the mutants is explained by functional redundancy between *Dlx-1*/*Dlx-2* and other Dlx genes.

Dlx-1 and Dlx-2 do not appear to be required for initiation of maxillary molar tooth development since epithelial thickenings do form in the mutants. Moreover, these epithelia are capable of producing important signals to the mesenchyme such as Fgf-8 and Shh (Fig. 3). Similarly, although the odontogenic capacity of the mesenchymal cells has been lost, as shown by recombination experiments, they are able to carry out their initial molecular responses to epithelial signals. Thus *Bmp-4* expression is induced, presumably in response to Bmp-4 signals from the epithelium and *Ptc* expression is also induced, indicating an intact Shh pathway. Fgf-8 is capable of inducing *Dlx-2* expression in tooth mesenchyme along with other homeobox genes such as *Msx-1* (unpublished observations; Thesleff and Sharpe, 1997). The normal expression of *Msx-1* in the mutants would suggest that the Fgf-8 pathway is intact but, because Bmp-4 can also induce *Msx-1* expression in mesenchyme, the expression of other Fgf-8 targets, once identified, will need to be studied to confirm this.

The potential role of *Dlx-1/Dlx-2* genes in specification of a subpopulation of neural-crest-derived cells was investigated using the homeobox gene *Barx-1* as a molecular marker of pre-

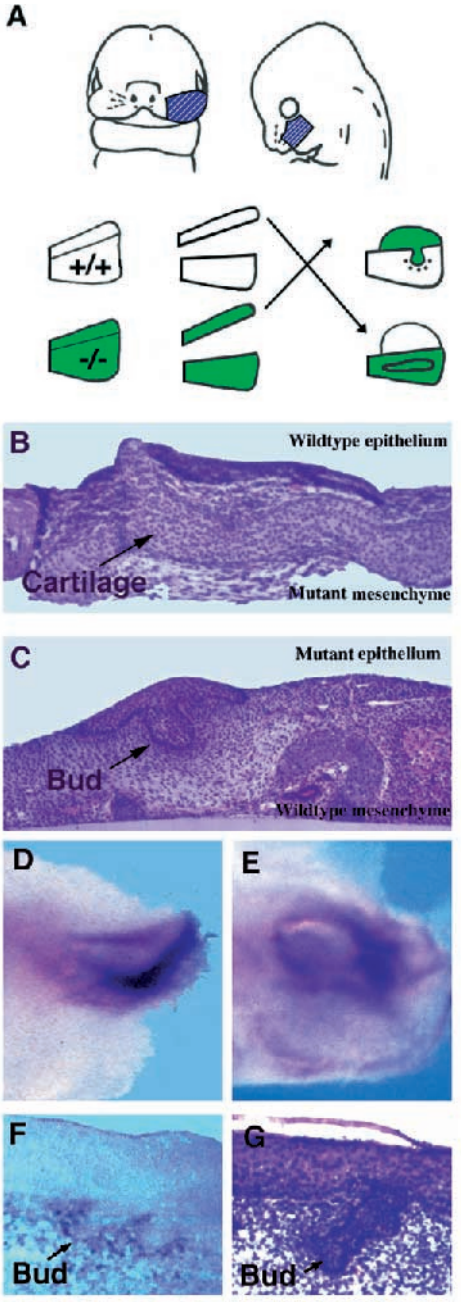

Fig. 3. Recombinations of oral epithelium and mesenchyme at E12.5. (A) Diagram illustrating region of the maxilla dissected, and recombinations of wild-type and mutant epithelium and mesenchyme. (B) Wild-type epithelium recombined with *Dlx-1/2* mutant mesenchyme. No tooth buds are seen, and the mesenchyme has formed large regions of cartilage. (C,E-G) *Dlx-1/2* mutant epithelium recombined with wild-type mesenchyme. (C) The epithelium has invaginated and formed a bud while the surrounding mesenchyme has started to condense. (D) Whole-mount digoxigenin in situ hybridisation showing *Msx-1* expression in the condensing mesenchyme around a wild-type maxilla molar tooth bud at E13.5. (E) Whole-mount digoxigenin in situ hybridisation showing *Msx-1* expression around a tooth bud after recombination of mutant epithelium with wild-type mesenchyme. (F) Section through a digoxigenin whole-mount in situ hybridisation showing *Msx-1* expression in the mesenchyme around the invaginating epithelium. (G) Same specimen further through the bud showing clear tooth morphology.

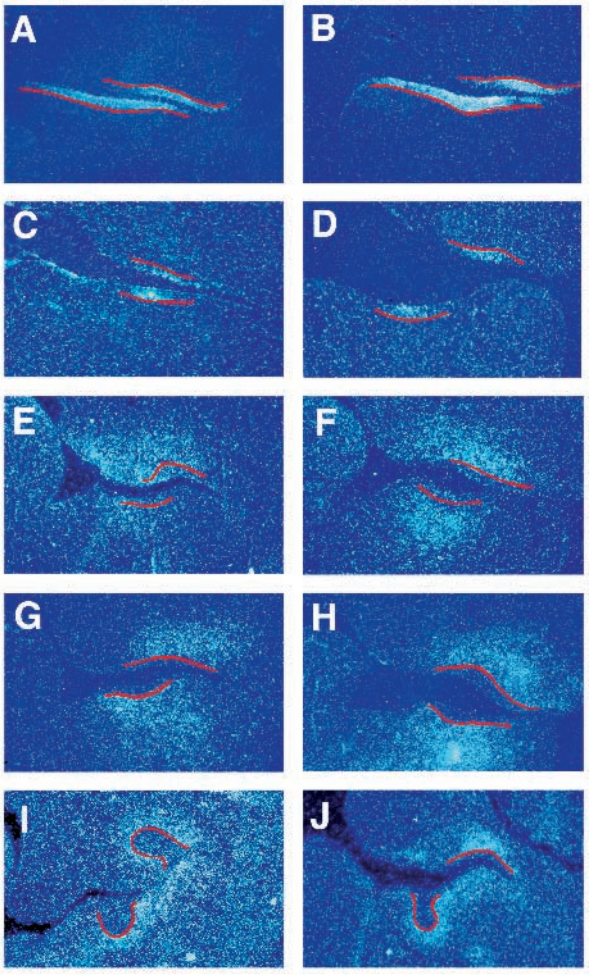

Fig. 4. Expression of marker genes of epithelial-mesenchymal signalling in early tooth development. Tooth germ epithelium is outlined in red. (A-D) Close up of molar epithelial thickening at E11.5. (A) Wild-type expression of *Fgf8* in the epithelial thickening. (B) Expression of *Fgf-8* in the mutant. (C) Wild-type expression of *Sonic hedgehog* in the epithelial thickening. (D) Expression of *Sonic hedgehog* in the mutant. Note normal expression in the maxillary molar epithelium. (E-H) Close up of molar late-epithelial thickening at E12.5. (E) Wild-type expression of *Ptc* in the mesenchyme. (F) Expression of *Ptc* in the mutant. (G) Wild-type expression of *Msx-1* in the mesenchyme. (H) Expression of *Msx-1* in the mutant. Note normal expression in the maxillary molar mesenchyme. (I-J) Close up of molar buds at E14.5. (I) Wild-type expression of *Bmp4* in the mesenchyme surrounding the tooth bud. (J) Expression of *Bmp4* in the mutant. Note that although the maxillary molar has failed to form a tooth bud expression of *Bmp4* is normal in the mesenchyme.

sumptive molar ectomesenchyme. In wild-type embryos, *Barx-1* is expressed in the ectomesenchyme of the proximal regions of the first branchial arch and becomes strongly expressed in the mesenchyme around the tooth buds during early stages of tooth development. In *Dlx-1/Dlx-2* mutants, we identified an area of mesenchymal cells immediately underlying the maxillary epithelial thickenings that had lost expression (Fig. 5). *Barx-1* expression in the mandible was normal in the mutants. The mesenchymal cells underlying the epithelial thickenings are those that will participate in odontogenesis and

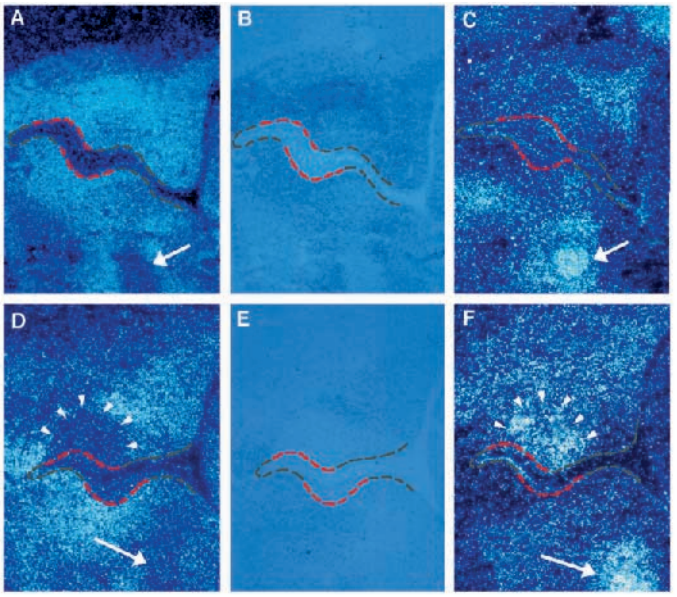

Fig. 5. Respecification of odontogenic cells in the molar region. Expression of *Barx-1* (A,D) and *Sox9* (C,F) adjacent to the appropriate light field views (B,E) in wild-type (A-C) and mutant (D-F) molar tooth germs at E13.0. Tooth epithelium is outlined by a red dashed line. The area of *Barx-1*-negative cells in the mesenchyme underlying the maxillary molar tooth germ (D) is coincident with the area of ectopic expression of *Sox9* (F), as indicated by the arrowheads. Arrows indicate Meckel's cartilage.

eventually form odontoblasts. The highly specific loss of *Barx-1* expression in these cells in the mutants, together with the fact that tooth development arrested suggested that these cells had lost their odontogenic capacity. Thus *Dlx-1* and *Dlx-2* are responsible for specifying a subpopulation of neural crest derived mesenchymal cells as odontogenic for the maxillary molar region.

Barx-1 expression is consistently absent in chondrogenic cells in the developing face. The localised loss of *Barx-1* expression in the mutants might therefore be indicative of a reprogramming of the odontogenic cells into a chondrogenic population. The appearance of ectopic pieces of cartilage in the maxillary molar region in the double mutants is consistent with this. The change in potential of these cells was confirmed by the observation of ectopic expression of *Sox9* in the *Barx-1*-negative cells (Fig. 5). Thus, in the absence of *Dlx-1* and *Dlx-2*, maxillary molar odontogenic mesenchyme cells become reprogrammed into chondrogenic cells, suggesting that the redundant function of *Dlx-1* and *Dlx-2* is to specify a distinct population of cranial neural crest cells.

Grafting experiments have provided evidence for prespecification of cranial neural crest cells by showing that presumptive premigratory first arch crest grafted in a more caudal region resulted in a duplication of first arch structures in the second arch (Noden, 1983). Birds do not develop teeth, therefore it is impossible to say from these experiments whether odontogenic neural crest cells are prespecified. Targeted mutation of the *Hoxa-2* gene in mice produces a similar branchial arch phenotype to the Noden experiments but, since Hox genes are not expressed in cranial neural crest cells that populate the first arch, this is not very informative for odontogenesis (Rijli et al., 1993). Lineage mapping of the origin of odontogenic mesenchymal cells is not sufficiently detailed to determine the precise origin of maxillary molar ectomesenchymal cells but suggests that cells populating different regions of the jaws have different temporal and spatial origins in the neural tube (Imai et al., 1996). Transient *Dlx-2* expression has been observed in the hindbrain of E8.5 embryos and subsequently in migrating cranial neural crest cells, thus odontogenic cells could be specified prior to or during migration (Robinson and Mahon, 1994, and J. L. R. R. unpublished observations).

The tooth phenotype in the double mutants is clearly a patterning phenotype, affecting only the development of maxillary molars. This is in marked contrast to the other knock-outs in transcription factors that give tooth abnormalities, namely *Msx-1* and *Lef-1*. Knock-outs of these genes affect development of all teeth. Significantly, the other unique feature of the Dlx mutants is the development of ectopic cartilage, which does not happen in the *Msx-1* or *Lef-1* mutants. This suggests that the function of the Dlx genes is markedly different to these other transcription factors, which are implicated in epithelial-mesenchymal signalling pathways.

Dlx-1 and Dlx-2 have non-redundant functions in development of specific regions of the facial skeleton (Qui et al., 1995, 1997), but a redundant function in development in maxillary molar teeth. Interestingly the effect of the absence of both genes is a conversion of cells from odontogenic to chondrogenic. Expression of other Dlx genes in the development of the limb has been correlated with cartilage differentiation but there is no functional data to support this role in these tissues (Ferrari et al., 1995).

Dlx-1 and *Dlx-2* are the first genes to be identified that have a role in odontogenic patterning. Moreover their specific role in controlling maxillary molar development indicates that there are separate genetic pathways directing the development of different teeth. Specification of different populations of neural crest for individual tooth types by different genetic pathways would make evolutionary sense allowing changes in shape and relative positions of the teeth to be easily generated. The specification of neural crest cells into separate odontogenic populations determined by a molecular code may explain the variations of heterodonty exhibited amongst many species of mammals as well as lower vertebrates.

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