Mouse Otx2/RIEG Expression in the Odontogenic Epithelium Precedes Tooth Initiation and Requires Mesenchyme-Derived Signals for Its Maintenance

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The mouse Otx2 gene is a new member of the paired-like family of homeobox genes whose human homologue, RIEG, is involved in Rieger syndrome, an autosomal-dominant disorder. One of the cardinal features of Rieger syndrome is dental hypoplasia, indicating that Otx2/RIEG activity is essential for normal tooth development. Here, we analyzed the expression of Otx2 during mouse tooth development and studied its regulation in dental explants. Otx2 expression distinguishes stomatodeal from other ectoderm as early as Embryonic Day 8.5, well before tooth initiation. Thereafter, its craniofacial expression becomes restricted to the tooth-forming areas and to the epithelial components of molar and incisor primordia. Although Otx2 induction precedes the specification of odontogenic mesenchyme, tissue recombination experiments show that the maintenance of its expression requires signals from the mesenchyme and that dental mesenchyme has the capacity to induce ectopic expression of Otx2 in nondental epithelium. Finally, we compare Otx2 expression with that of the recently identified homeodomain transcription factor Barx1 expressed in molar mesenchyme. Their strictly complementary expression patterns in the epithelial and mesenchymal components suggest that both genes participate in the reciprocal tissue interactions which are a hallmark of odontogenesis.

INTRODUCTION

Inductive interactions between epithelial and mesenchymal tissues initiate odontogenesis and govern later differentiation events. The first morphological sign of tooth development is the formation of the dental placodes, local thickenings of the stomatodeal epithelium which then invaginate to form the dental laminae. Through subsequent reciprocal interactions, the epithelial and mesenchymal components of the tooth anlage proceed through well-characterized morphological stages (successively bud, cap, and bell stages). The dental lamina eventually develops into the enamel organ, whereas the underlying neural crest-derived mesenchyme (ectomesenchyme) gives rise to the dental papilla (for reviews, see Ruch, 1995; Thesleff et al., 1995).

Tissue recombination experiments have shown that the stomatodeal ectoderm, but no other ectoderm tested, possesses the capacity to initiate tooth formation (Lumsden, 1988). On the other hand, only cranial ectomesenchyme is competent to participate in tooth development (Lumsden, 1988). The potential for tooth initiation resides in the epithelium even prior to dental placode formation and persists until Embryonic Day 12 (E12), after which it gradually shifts to the condensing mesenchyme (Mina and Kollar, 1987). The induced mesenchyme now becomes capable of instructing nondental epithelium to participate in tooth formation (Kollar and Baird, 1969; Ruch et al., 1973; Ruch, 1987). Reciprocal inductive interactions also govern later stages of odontogenesis, including root development (Ruch, 1987; Thomas, 1995). These interactions seem to have a position-specific component, since different types of teeth, i.e., molars and incisors in the mouse, are generated at different locations, and tissue recombination experiments suggest that epithelium and mesenchyme are regionally specified to give rise to either molars or incisors (Kollar and Baird, 1969; Lumsden, 1988).

To gain insight into the underlying molecular mecha-
nisms, different groups have analyzed the expression patterns of candidate regulatory genes encoding transcription factors and secreted signaling molecules during mouse odontogenesis (reviewed by Sharpe, 1995; Thesleff et al., 1995; Thesleff and Nieminen, 1996). Many of these genes are expressed in patterns suggesting their participation in induction and differentiation of tooth primordia. Signaling molecules such as retinoic acid, bone morphogenetic protein-4 (BMP-4), and fibroblast growth factor-4 (FGF-4) were found to induce specific gene expression in the mesenchyme of tooth germ explants (Vainio et al., 1993; Mitsiadis et al., 1995a, 1996; Kratochwil et al., 1996; Chen et al., 1996). Moreover, genetic evidence showing that the homeodomain transcription factors Msx1 and Lef1 are essential for tooth development in vivo has been obtained (Satokata and Maas, 1994; van Genderen et al., 1994; Vastardis et al., 1996). A conspicuous feature of several transcription factors, such as Lef1 and Msx2, is that their expression shifts between mesenchymal and epithelial compartments during tooth development (MacKenzie et al., 1992; Kratochwil et al., 1996). Such shifts in expression probably reflect shifts in inductive capacity. Hence, these transcriptional regulators appear to control expression of signaling molecules involved in tissue interactions.

The stomatodeal ectoderm can be distinguished from other ectoderm by its tooth-inducing capacity as early as E9.5 (Mina and Kollar, 1987; Lumsden, 1988), but candidate regulators responsible for this ectodermal specification have not been identified so far. Similarly, the molecular mechanisms involved in the specification of tooth shape, i.e., molar or incisor shape, have remained largely unknown (Sharpe, 1995). Most growth factors and transcription factors are not tooth-type specific, with the exception of the recently identified homeodomain transcription factor Barx1, which is specific for molar mesenchyme (Tissier-Seta et al., 1995).

In a screen designed to identify members of the paired-like family of homeobox genes in the mouse, we have cloned Otx2 (Mucchielli et al., 1996), a new member of this family, which is most closely related to mouse Ptx1/P-Otx/Otx1 (Lamonterie et al., 1996; Szeto et al., 1996; Mucchielli et al., 1996) and unc-30 from Caenorhabditis elegans (Jin et al., 1994). Using a positional cloning strategy, Semina et al. (1996) have isolated the same gene in man and mouse under the name RIEG. Their results show that RIEG most likely is the gene responsible for Rieger syndrome, an autosomal-dominant human disorder of variable expressivity whose cardinal features are anomalies of the anterior chamber of the eye, a protuberant umbilicus, and dental hypoplasia. In the mouse embryo, Otx2/RIEG has been reported to be expressed in the forming pituitary gland, periocular mesenchyme, epithelium of the first branchial arch, limb mesenchyme, and specific regions of the pros- and mesencephalon (Semina et al., 1996; Mucchielli et al., 1996). Here we present a detailed analysis of Otx2 expression during odontogenesis. We show that Otx2 expression distinguishes stomatodeal from other ectoderm already at E8.5, well before the appearance of other known markers, and remains confined to the epithelial components of molar and incisor primordia throughout odontogenesis. Although Otx2 induction precedes the specification (and probably the arrival) of odontogenic ectomesenchyme, the maintenance of its expression in explants requires signals from the mesenchyme.

**MATERIALS AND METHODS**

**Animals and Tissue Preparation**

Swiss and C57Bl/6 mice were used at embryonic and postnatal stages. The age of the mouse embryos was determined according to the appearance of the vaginal plug (Day 0.5) and confirmed by morphological criteria. Animals were killed by cervical dislocation and the embryos were surgically removed. Whole embryos or dissected heads from mouse embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA).

**Probes and in Situ Hybridization**

Digoxigenin-labeled (Boehringer Mannheim) antisense riboprobes for Otx2 (Mucchielli et al., 1997) and Barx1 (Tissier-Seta et al., 1995) were synthesized following the manufacturer's instructions. Whole-mount in situ hybridization and in situ hybridization on cryosections and paraffin-embedded sections were performed as described (Tissier-Seta et al., 1995; Tiveron et al., 1996; Mitsiadis et al., 1995a).

**Tissue Recombination Experiments**

From mandibles of E13–E14 mouse embryos, nonodontogenic oral areas and odontogenic areas containing the primordia of the first molars were dissected, as well as the distal parts of the developing hindlimbs of E11 embryos. After dissection, tooth germs and oral and limb tissues were incubated for 3 min in 2.25% trypsin and 0.75% pancreatin on ice, and the epithelia were mechanically separated from mesenchyme in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum (Gibco), as previously described (Mitsiadis et al., 1995a,b). The isolated epithelia were placed in contact with isolated mesenchyme in various homo- and heterotypic combinations and cultured for 24 to 48 hr on a polycarbonate membrane (Nuclepore Corp.) as previously reported (Mitsiadis et al., 1995a,b). After culture, the explants were fixed for 2 hr in 4% PFA, dehydrated in ethanol, and embedded in paraffin wax. Serial sections (5 μm) were mounted on silanized slides, dried, and stored at 4°C until use.

**RESULTS**

Otx2 is expressed at several sites during mouse embryogenesis, at the base of the limb buds, in specific areas of the CNS, in periocular mesenchyme, and in the developing pituitary gland (Semina et al., 1996; Mucchielli et al., 1996), but in this report, we focus on the oral region and the developing teeth.
Early Expression of Otlx2 in Oral Epithelium and Dental Primordia

The earliest stages of Otlx2 expression were explored by whole-mount in situ hybridization. We first detected Otlx2 transcripts at presomitic stages (E7.5) in the mesenchyme of the headfold (Figs. 1A and 1B). Much of this expression seems to be transient, since 1 day later, the mesenchymal expression in the head was limited to the optic eminence (Fig. 1C). Already at E8.5 (12-somite stage), expression was strong in the stomatodeal ectoderm. Expression in the ectoderm stopped abruptly at the edges of the stomatodeal cavity, and the ectoderm covering the outer aspects of the frontonasal, maxillary, and mandibular processes was negative (Figs. 1C and 1D). The ectoderm that will form the oral epithelium and its annexes, the teeth and the anterior pituitary, is thus molecularly distinct from the epidermis of the face and neck as early as E8.5. This expression pattern was basically unchanged at E9.5 (Fig. 1E). The mesenchymal expression in the eye region (Fig. 1E and Mucchielli et al., 1996) continued and at later stages in the pericocular mesoderm (not shown). Other sites of expression become visible at E10.5, in Rathke’s pouch, which develops as an invagination of the stomatodeal epithelium, and in the future diencephalon (Mucchielli et al., 1996).

The following stages of Otlx2 expression in the oral epithelium and during odontogenesis were examined by in situ hybridization with digoxigenin-labeled probes on cryosections. By E11.5, the previously homogenous labeling of the oral epithelium had become nonuniform. The signal continued to be strong in the dental placodes visualized as local thickenings of the epithelium, but was fading out elsewhere in the oral epithelium (Fig. 2A). Hence, Otlx2 expression prefigures the sites of future odontogenesis and is restricted to the epithelium. Because of their later presence in the vestibular lamina, a transient epithelial structure outlining the future oral vestibule, Otlx2 transcripts may be assumed to be present also in placodes giving rise to this structure, but it is not clear whether the dental and vestibular laminae arise from separate placodes.

At E13.5, the dental epithelium has invaginated into the underlying mesenchyme and forms a bud around which the mesenchyme condenses. Otlx2 was strongly expressed in all cells of the tooth bud and the thickened dental epithelium and somewhat more weakly in the vestibular lamina, whereas in the surrounding epithelium, the labeling was very faint or absent. Figure 2B shows the bud-stage upper and lower incisor anlagen; at this stage, the pattern observed in the first molar tooth germs is virtually indistinguishable (not shown). Transient Otlx2 labeling was also detected in the myogenic mesenchyme of the tongue at this stage.

Otlx2 Expression during Morphogenesis of the First Molars and the Incisors

At the late cap stage (E15.5), the outer and inner enamel epithelium and the stellate reticulum can be distinguished
FIG. 2. Pattern of Otx2 expression during early tooth development. (A) At E11.5, Otx2 transcripts are exclusively detected in the thickened presumptive dental epithelium of the mandibular and maxillary processes (arrows). (B) At E13.5, Otx2 is expressed in the epithelium of the bud-stage incisor germ. Transcripts are also observed in the vestibular lamina (arrow). The myogenic mesenchyme of the tongue (asterisk) is weakly stained. (C) Otx2 expression is restricted to dental epithelial cells at the late cap stage of the first molars (E15.5). Note that the signal is stronger in the enamel knot and in the outer enamel epithelium. (D) At E16.5 (early bell stage), the Otx2 signal has become faint in the stellate reticulum. (E) In an E16.5 upper incisor, Otx2 is expressed throughout the epithelial derivatives except for the inner enamel epithelium at the labial side. (F) In an E16.5 lower incisor, the Otx2 signal is strong in the outer enamel epithelium, but weak or absent in the stratum reticulum and the inner enamel epithelium along the labial and lingual surfaces. Abbreviations: a, preameloblast/ameloblast layer; dl, dental lamina; ie, inner enamel epithelium; k, enamel knot; la, labial surface; li, lingual surface; md, mandibular process; mx, maxillary process; oe, outer enamel epithelium; p, dental papilla mesenchyme; si, stratum intermedium; sr, stellate reticulum; tg, tongue. Scale bars, 200 μm.

in the epithelial compartment of the molar anlage. Otx2 transcripts remained restricted to these epithelial derivatives and the adjacent dental laminae (Fig. 2C). Particularly strong expression was seen in the enamel knot, a transient thickening of the enamel epithelium, proposed to be an important signaling center (Jernvall et al., 1994; Vahtokari et al., 1996), and in the outer enamel epithelium. At the early bell stage (E16.5), the dental epithelium of the first
Otx2 in Odontogenesis

Otx2 and Barx1 Expression during Later Stages of Tooth Morphogenesis and Cytodifferentiation

The early expression pattern of Barx1 in the facial ectomesenchyme and its progressive restriction to the dental papilla of the primordium of the first molar has been described previously (Tissier-Seta et al., 1995). Here we compared Otx2 and Barx1 expression in the molar anlage on serial sections from E16.5 to Postnatal Day 4 (P4). At E16.5, the Barx1 pattern in the first molar was a mirror image of that of Otx2 (Figs. 3A and 3B). The Barx1 signal was confined to the condensed mesenchyme of the dental papilla and of the follicle. As reported previously for earlier stages (Tissier-Seta et al., 1995), the incisor anlagen were Barx1 negative (not shown, but see below for a later stage).

At birth (P0), cytodifferentiation has started in the first molars, which are now at the late bell stage: cells of the inner enamel epithelium are about to differentiate into preameloblasts/ameloblasts, while the cells of the dental papilla facing the inner enamel epithelium at the tip of the cusps are differentiating into odontoblasts. Development of the second and especially of the third molar is considerably delayed, as the second molar is still at the early bell and the third molar at the bud stage. Ameloblast differentiation at the tip of the cusps coincided with down-regulation of Otx2 expression, while expression persisted in developmentally less advanced areas of the inner enamel epithelium, in the intercuspal folds and in the cervical loop (Fig. 3C). A particularly strong signal was observed in the stratum intermedium and outer enamel epithelium, whereas the labeling was very faint in the stellate reticulum. Otx2 expression was also strong in the epithelial components of the second and the third molar anlagen. Expression of Barx1 in newborn mice was confined to the mesenchymal derivatives of the forming molars (Fig. 3D). The mesenchymal compartment of the posterior part of an incisor primordium, whose epithelial components were strongly labeled by the Otx2 probe (Fig. 3C), was completely Barx1 negative (Fig. 3D), showing that Barx1 expression continues to be restricted to the molars after birth. The signal was particularly strong in the differentiating odontoblasts of the first molar and in the papilla of the second molar. By contrast, Barx1 expression was still very weak in the mesenchyme adjacent to the strongly Otx2-positive epithelial bud of the third molar anlage.

At P4 (stage of mineralization), the ameloblasts and odontoblasts of the first and second molars are now starting to deposit enamel and dentin, respectively. Hertwig’s epithelial sheet of the first two molars has formed the epithelial diaphragm delimiting the future pulp chamber, and the third molar has advanced to the early bell stage. Otx2 expression was still strong in the growing epithelial diaphragm and in the stratum intermedium of the first and second molar, moderate in the outer enamel epithelium, and weak or absent in preameloblasts/ameloblasts and in the stellate reticulum (Fig. 3E). In the first and second molar, Barx1 expression had been down-regulated in the dental mesenchyme forming the pulp, but weak expression remained in the differentiating odontoblasts of the crown (Fig. 3F). In the third molar tooth germ, now at the late cup stage, Barx1 expression had become much stronger in the condensed mesenchyme of the future papilla underlying the Otx2-positive epithelium (compare with Fig. 3D). This result shows that, during third molar development, the Barx1 gene is induced in the mesenchyme in contact with the already Otx2-expressing dental epithelium. Otx2 and Barx1 thus show complementary expression patterns in all three molars during comparative developmental stages.

Otx2 Expression in Dental Epithelium Is Regulated by Mesenchyme-Derived Signals

While expression of Otx2 in the oral epithelium is initiated before dental mesenchyme has been induced, the maintenance of Otx2 expression may depend on signals from the ectomesenchyme, at stages when it has acquired odontogenic potential. To explore this issue, we recombined microdissected epithelial and mesenchymal components from odontogenic and nonodontogenic regions and examined Otx2 ex-

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FIG. 3. Comparison between Otx2 and Barx1 expression during later stages of molar tooth development. (A) At E16.5, Otx2 is strongly expressed in the enamel epithelium, whereas the signal is weaker in the stellate reticulum and the epithelium covering the dental lamina. (B) In an adjacent section, Barx1 transcripts are exclusively detected in mesenchymal cells of the dental papilla and follicle. (C) At P0, Otx2 mRNA is down-regulated in preameloblasts and the stellate reticulum of the first molar, while the gene is strongly expressed in cells of the stratum intermedium and of the outer enamel epithelium. Transcripts are also detected in the epithelium of the second and third molars. Note that the enamel epithelium of the incisor (posterior end) is positive for Otx2. (D) On an adjacent section, Barx1 transcripts are detected in the differentiating preodontoblasts/odontoblasts of the first molar, while the gene is down-regulated in the dental papilla and follicle. Barx1 is strongly expressed in dental papilla mesenchyme of the second molar, whereas expression is hardly detectable in the mesenchyme of the third molar. Note that the gene is absent from the incisor mesenchyme. Labeling of the condensing mesenchyme of the third molar is still very faint. (E) At P4, Otx2 expression is down-regulated in preameloblasts/ameloblasts of the second molar, but persists in the stratum intermedium of the enamel organ and in the epithelial diaphragm during root formation. A strong signal is detected in the enamel epithelium of the third molar. (F) Barx1 expression is lost in the second molar from dental papilla mesenchyme and from fully differentiated odontoblasts. Barx1 transcripts are abundant in the papilla of the third molar. Abbreviations: 1m, 2m, and 3m, first, second, and third molar, respectively; a, ameloblasts; dl, dental lamina; e, epithelial compartment of the third molar; ed, epithelial diaphragm; f, dental follicle; i, incisor; ie, inner enamel epithelium; o, odontoblasts; oe, outer enamel epithelium; p, dental papilla and forming pulp; si, stratum intermedium; sr, stellate reticulum. Scale bars in A and B, 200 \( \mu m \); in C and D, 150 \( \mu m \); in E and F, 400 \( \mu m \).
expression in the homo- and heterotypic recombinants by in situ hybridization after 1–2 days in culture.

In homotypic recombinants from E13 molar tooth germs, strong Otlx2 expression was observed in epithelial cells contacting the mesenchyme (Fig. 4A). Hence, when cultured in the presence of dental mesenchyme, the dental epithelium maintains Otlx2 expression in culture. Fewer transcripts were detected in epithelial cells separated from the mesenchyme by several cell layers. This may reflect the in vivo situation, where expression is strong in the enamel epithelium and the stratum intermedium overlying the mesenchyme and faint or absent in cells of the stellate reticulum. To investigate whether maintenance of Otlx2 expression required the presence of odontogenic mesenchyme or could be achieved by any mesenchyme, we examined Otlx2 expression in heterotypic recombinants. When E13 oral mesenchyme from outside the tooth-forming region (Fig. 4B) or E11 limb mesenchyme (not shown) was recombined with E13 dental epithelium, Otlx2 expression in the epithelium was very faint or absent after 48 hr in culture. Hence, dental mesenchyme either emits signals required to maintain Otlx2 expression or signals from nonodontogenic mesenchyme shut off Otlx2 expression.

We then asked whether dental mesenchyme could induce Otlx2 expression in epithelium which does not have odontogenic potential. E13 dental mesenchyme was cultured for 48 hr together with E13 oral epithelium, in which the Otlx2 gene is not normally expressed at this stage (see Fig. 2B). After a 24-hr culture period, the epithelium had invaded the dental mesenchyme and acquired a bud-like configuration. Otlx2 transcripts were found throughout the epithelial cells (Fig. 4C), indicating that dental mesenchyme has the capacity to induce Otlx2 expression in an epithelium which does not express it normally. By contrast, oral mesenchyme from outside the tooth region did not induce the gene in nonodontogenic epithelium (Fig. 4D). Together, these data strongly suggest that positive signals derived from dental mesenchyme maintain Otlx2 expression in the epithelial components of the tooth germ.

**DISCUSSION**

**Otlx2 and Tooth Initiation**

Initiation of mouse tooth formation becomes morphologically evident at around E10.5, when the oral epithelium

**FIG. 4.** Localization of Otlx2 transcripts in explants of recombined epithelium and mesenchyme from different regions. (A) Explants of recombined E13 dental epithelium and mesenchyme after 24 hr of culture. Otlx2 transcripts are detected in the epithelium, but are absent from epithelial cells farther away from the dental mesenchyme. (B) Explants of recombined E13 dental epithelium and E13 oral mesenchyme from outside the tooth region after 48 hr of culture. Otlx2 transcripts are absent from both tissues. (C) Explants of recombined E13 oral epithelium from outside the tooth region and E13 tooth mesenchyme after 48 hr of culture. A strong Otlx2 signal is observed in epithelial cells. (D) Explants of recombined E13 oral epithelium and mesenchyme from outside the tooth region. Otlx2 mRNA is absent from both tissues. Abbreviations: e, epithelium; fl, filter; m, mesenchyme. Scale bar in A, 50 μm; in B and D, 100 μm; in C, 200 μm.
which does not give rise to ameloblasts. Apparently, \textit{Otlx2} in the lingual inner enamel epithelium of the incisors, of events leading to tooth formation. This down-regulation is also observed the notion that enamel epithelium, at the time they start to differentiate acquires tooth-inducing potential, adding further weight to \textit{Otlx2} cap stage, the forming teeth throughout odontogenesis. Starting at the forming region. Hence, dental mesenchyme is capable of \textit{Otlx2} (MacKenzie et al., 1992), Dlx-2 (Robinson and Mahon, 1994), and Lef1 (Kratochwil et al., 1996), are expressed in the presumptive dental epithelium together with the secreted signaling molecules BMP-4 (Vainio et al., 1993), FGF-8 (Heikinheimo et al., 1994), and sonic hedgehog (Bitgood and McMahon, 1995; Kronmiller et al., 1995). However, already at E9.5, mandibular epithelium has acquired the capacity of inducing odontogenic properties in cranial neural crest (Lumsden, 1988) or the ectomesenchyme of the second branchial arch (Mina and Kollar, 1987; Kollar and Mina, 1991). In fact, the capacity to initiate tooth formation is restricted to the stomatodeal ectoderm, since rostral (oral) but not caudal (aboral) mandibular epithelium has tooth-initiating potential (Lumsden, 1988). However, molecules that distinguish oral from aboral jaw epithelium at such early stages have not yet been identified. Here we show that \textit{Otlx2}, a new homeobox gene of the paired-like family, is expressed as early as E8.5 in the stomatodeal, but not in any other ectoderm. Tlx-1 is another homeobox gene expressed at a comparatively early stage in the mandibular epithelium, but it is also expressed in the nonodontogenic epithelium of the first and the second branchial arches (Raju et al., 1993).

\textit{Otlx2} represents the earliest known marker for sites of dental initiation and its product may therefore be causally involved in the initiation of tooth formation. Consistent with such a role, \textit{Otlx2} expression in the oral epithelium becomes progressively restricted to the dental placodes, the sites of future odontogenesis. The recent identification of mutations in \textit{RIEG}, the human homologue of \textit{Otlx2}, as a likely cause of Rieger syndrome (Semina et al., 1996) provides strong evidence that \textit{Otlx2} is indeed essential for proper odontogenesis. Tooth anomalies consisting of anodontia vera, microdontia, or abnormally shaped teeth are main characteristics of Rieger syndrome (Fitch and Kaback, 1978; Semina et al., 1996). The available evidence suggests haploinsufficiency as the likely mechanism causing this autosomal-dominant disorder (Semina et al., 1996), although a dominant-negative effect of the mutated gene remains a possibility. It is not known at which stage tooth development is affected in patients with Rieger syndrome. Our results suggest that \textit{Otlx2}/\textit{RIEG} may already be required for the early specification of the odontogenic epithelium.

In contrast to other transcription factor genes such as \textit{Msx2} (MacKenzie et al., 1992) or \textit{Lef1} (Kratochwil et al., 1996), \textit{Otlx2} is specific for the epithelial compartment of the forming teeth throughout odontogenesis. Starting at the cap stage, \textit{Otlx2} is down-regulated in the cells of the inner enamel epithelium, at the time they start to differentiate into preameloblasts. This down-regulation is also observed in the lingual inner enamel epithelium of the incisors, which does not give rise to ameloblasts. Apparently, \textit{Otlx2} is required during morphogenesis, but not for later differentiation events.

### Barx1 Expression during Molar Development

The homeobox gene \textit{Barx1} is expressed in the molar mesenchyme in a pattern strictly complementary to that of \textit{Otlx2}. We have reported on the early expression pattern of \textit{Barx1} in the branchial arch ectomesenchyme and the mesenchyme of the developing first molar (Tissier-Seta et al., 1995). In contrast to all other known transcription factor genes expressed during odontogenesis, \textit{Barx1} was expressed only in the molar, but not in the incisor anlagen. Here we show that this is true for later stages of tooth development, and that \textit{Barx1} expression remains confined to the mesenchymal compartment throughout molar development. We initially interpreted the \textit{Barx1} expression sequence as a progressive restriction of a broad domain in the posterior branchial arch to the papilla and follicle of the molar tooth germ (Tissier-Seta et al., 1995). However, we already noted that, after strong expression in the branchial arch mesenchyme, \textit{Barx1} was first down-regulated before again becoming strongly expressed in the molar mesenchyme. Our present results show that in the anlage of the third molar, \textit{Barx1} expression is in fact induced in the dental papilla within a \textit{Barx1}-negative territory. This result suggests that \textit{Barx1} expression comprises two distinct phases which are driven by different mechanisms: a first phase of position-dependent expression in branchial arch mesenchyme is followed by a second phase of expression in the molar mesenchyme, probably initiated by signals from the dental epithelium.

As is the case for \textit{Otlx2}, \textit{Barx1} expression is down-regulated during terminal differentiation of the odontoblast layer and the pulp chamber.

### \textit{Otlx2} Expression Is Controlled by Signals from the Dental Mesenchyme

Our evidence suggests that not only \textit{Barx1}, but also \textit{Otlx2} expression comprises two distinct phases involving different control mechanisms. While \textit{Otlx2} expression in the oral epithelium well before the underlying mesenchyme acquires odontogenic potential, the maintenance of its expression requires signaling from dental mesenchyme, as shown by two observations. First, \textit{Otlx2} expression in the dental epithelium is shut off in recombinants with nonodontogenic mesenchyme. Second, E13 dental mesenchyme, which is competent to instruct nonodontogenic epithelia to form enamel organ (Kollar and Baird, 1969; Ruch et al., 1973; Kollar and Fisher, 1980), induces \textit{Otlx2} expression in \textit{Otlx2}-negative epithelium taken from outside the tooth-forming region. Hence, dental mesenchyme is capable of inducing the \textit{de novo} expression of \textit{Otlx2} at the time it acquires tooth-inducing potential, adding further weight to the notion that \textit{Otlx2} is an obligatory early step in the chain of events leading to tooth formation.

Previous studies using tissue recombinations have shown that the maintenance of \textit{Msx1} and \textit{Msx2} expression in the mesenchyme requires signals from the dental epithelium
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