Eur J Oral Sci 1998; 106 (suppl 1): 112–116
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EUROPEAN JOURNAL OF
ORAL SCIENCES
ISSN 1397-7555
ISBN 87-16-15628-5

Expression of the transcription factors *Otlx2*, *Barx1* and *Sox9* during mouse odontogenesis

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Mitsiadis TA, Mucchielli M-L, Raffo S, Proust J-P, Koopman P, Goridis C: Expression of the transcription factors Otlx2, Barx1 *and* Sox9 *during mouse odontogenesis. Eur J Oral Sci 1998; 106 (suppl 1): 112–116.*

The molecular mechanisms governing the decision between molariform and incisiform patterns of rodent dentition are not yet known. Transcription factors are regulators of regionally specific morphogenesis and key co-ordinators of gene activity during developmental processes. Here, we analysed the expression of several transcription factors during mouse tooth development. *Otlx2/Rieg* is a homeobox gene involved in Rieger syndrome, a human disorder characterized by dental hypoplasia. *Otlx2/Rieg* expression distinguishes stomatodeal epithelium well before tooth initiation, and thereafter its expression becomes restricted to the epithelia of both molar and incisor primordia. The recently identified homeodomain transcription factor *Barx1* is first expressed in mesenchyme of the first branchial arch, but during advanced developmental stages the gene is exclusively expressed in the mesenchyme of molar primordia. Finally, the *Sry*-related transcription factor *Sox9* is expressed in epithelial components and to a lesser degree in condensed mesenchyme of the developing teeth. These results suggest that *Otlx2/Rieg*, *Barx1*, and *Sox9* participate in the hierarchical cascade of factors involved in the regulation of tooth morphogenesis.

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Key words: *Barx1*; *Otlx2*; Rieger syndrome; *Sox9*; tooth

Accepted for publication July 1997

Tooth development results from sequential inductive interactions between epithelial and mesenchymal tissues. Tissue recombination experiments have shown that only the stomatodeal ectoderm possesses the capacity to initiate tooth formation, while neural crest-derived mesenchyme is competent to participate in tooth development (1). The odontogenic potential resides in the epithelium until embryonic day 12 (E12) and shifts thereafter to the condensing mesenchyme (2), which becomes capable of instructing non-dental epithelium to participate in tooth formation (3). Tissue recombination experiments also suggested that the mesenchyme is regionally specified to give rise to either molars or incisors (1, 3), indicating that these inductive interactions have a position-specific component.

The progression of tooth morphogenesis is characterized by sequential changes in the expression of a number of structural and regulatory molecules (4, 5). Signalling molecules such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) are found to induce specific gene expression in dental tissue explants (6–8). Moreover, the recent identification of gene defects responsible for dental agenesis and tooth abnormalities in mouse and man has shown that transcription factors such as Msx1 and Lef1 are important for proper tooth development (9–11).

While great progress has been made towards identifying molecular mechanisms involved in tooth development, candidate regulators responsible for the specification of tooth shape remain largely unknown. Most growth factors and transcription factors are not tooth type-specific, with the exception of the homeodomain transcription factor *Barx1*, which is specific for molar mesenchyme (12).

Here we present an analysis of the expression

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pattern of the recently identified transcription factors *Barx1*, *Otlx2*/*Rieg* and *Sox9* during mouse odontogenesis. *Otlx2*/*Rieg* is a member of the *paired*-like family of homeobox genes (13, 14), which is responsible for Rieger syndrome (14), an autosomal-dominant human disorder characterized by dental hypoplasia. The *Sry*-related gene *Sox9* is important for sex determination and chondrogenesis in mammals (15, 16). *Sox9* encodes a highly conserved protein with the characteristic features of a transcription factor (17, 18).

Material and methods

Animals and tissue preparation

Swiss and C57Bl/6 mice were used at embryonic stages. The age of the mouse embryos was determined according to the vaginal plug (embryonic day 0.5; E0.5) 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) in phosphate-buffered saline (PBS), pH 7.4. Some of the E10.5 embryos were washed in PBS and stored in absolute methanol (MeOH) at −20°C until analysis by whole mount *in situ* hybridization. For *in situ* hybridization on tissue sections, E10.5–18.5 embryos were equilibrated with 30% sucrose/PBS and were then embedded in Tissue-Tek OTC (Miles Lab., USA) and rapidly frozen. Cryostat sections (12 μm) were stored at −70°C.

Probes and *in situ* hybridization

Digoxigenin-labelled (Boehringer Mannheim, Meylan, France) antisense riboprobes for *Barx1* (12), *Otlx2*/*Rieg* (13), and *Sox9* (15) were synthesized following the manufacturer's instructions. Whole mount *in situ* hybridization and *in situ* hybridization on cryosections were performed as described (8, 13).

Results

Expression of *Barx1* and *Otlx2*/*Rieg* before and during the initiation of tooth development (E9.5–10.5) was explored by whole-mount *in situ* hybridization (Fig. 1A, B). *Otlx2*/*Rieg* expression was strong in the E9.5–10.5 stomatodeal ectoderm, while the ectoderm covering the outer parts of the fronto-nasal, maxillary and mandibular processes was negative (Fig. 1A and data not shown). *Barx1* expression was strong in regions of the E10.5 ectomesenchyme of the maxillary and mandibular processes and of the second branchial arch (Fig. 1B).

Barx1, Otlx2/*Rieg*, and *Sox9* expression during the subsequent stages of tooth development were examined by *in situ* hybridization with digoxigenin-labelled probes on cryosections. At E13.5, the dental epithelium forms a bud around which the mesenchyme condenses. *Otlx2*/*Rieg* was strongly expressed in all cells of the tooth bud of both incisor and first molar anlagen, but was fading out elsewhere in the oral epithelium (not shown). Transcripts for *Barx1* were restricted to the mesenchyme of the posterior part of the jaws, including the condensed mesenchyme of the first molar (not shown). *Sox9* transcripts were observed in the epithelial bud and to a lesser degree in the condensed mesenchyme of first molar and incisor germs (Fig. 2A).

At the early bell stage (E16.5), the dental epithelium differentiates into the enamel organ, while the underlying condensed mesenchyme forms the dental papilla. *Otlx2*/*Rieg* was strongly expressed in the outer and inner enamel epithelia and the stratum intermedium of the first molar anlage, whereas the stellate reticulum was only weakly labelled (not shown). In the incisor, *Otlx2*/*Rieg* signal was observed in the epithelial derivatives, except for the inner enamel epithelium, both in the labial and in the lingual side (data not shown). At E16.5, the expression pattern of *Barx1* in the molar was complementary to that of *Otlx2*/*Rieg*: the *Barx1* signal was confined to the mesenchyme of both dental papilla and follicle. Incisors were *Barx1*-negative (not shown, but see below for a later stage). An interesting *Sox9* expression pattern was observed at E16.5. The *Sox9* signal was strong in epithelial derivatives and faint in dental papilla mesenchyme of the molar. However, while inner enamel epithelial cells of the labial side were expressing the gene, transcripts were absent from the same cells of the lingual side (Fig. 2B).

During the late bell stage (E18.5), cytodifferentiation starts at the tip of the cusps of the first molars: cells of the inner enamel epithelium differentiate into preameloblasts, while cells of the dental papilla differentiate into odontoblasts. Preameloblast differentiation coincided with down-regulation of *Otlx2*/*Rieg* expression, while expression persisted in the developmentally less advanced areas of the intercuspal folds and in the cervical loop (Fig. 1C). A particularly strong signal was detected in the stratum intermedium and outer enamel epithelium. *Otlx2* expression was also observed in the epithelium of the developing second molar. Expression of *Barx1* was confined to the mesenchyme of the forming molars (Fig. 1D). The

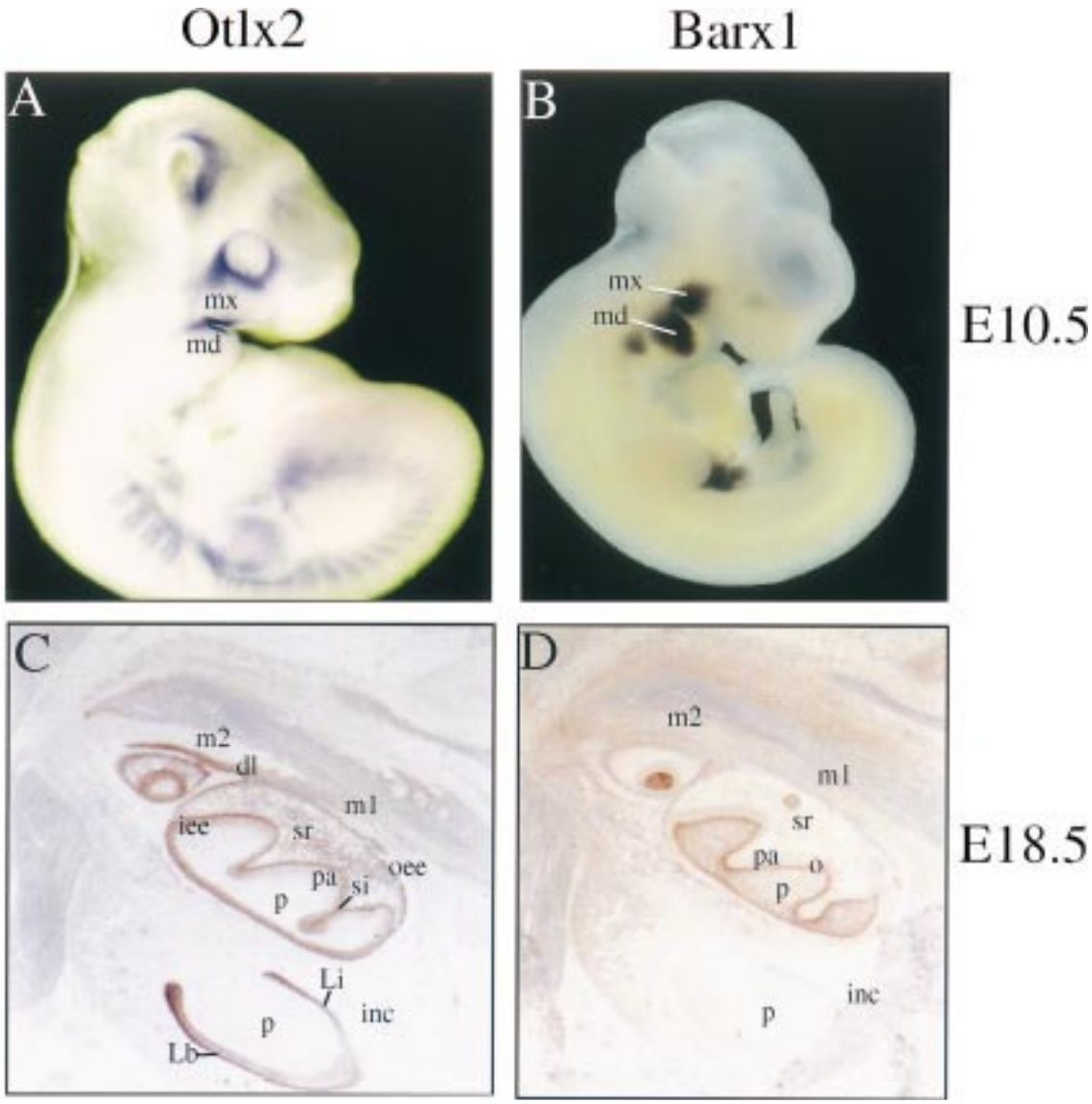

Fig. 1. Patterns of *Otlx2*/*Rieg* and *Barx1* expression during embryonic tooth development. *In situ* hybridizations on whole-mount preparations of E10.5 mouse embryos (A and B) and on cryosections of E18.5 mouse embryos (C and D) using digoxigenin-labelled probes. (A) At E10.5, *Otlx2*/*Rieg* transcripts are exclusively detected in the stomatodeal epithelium of the mandibular (md) and maxillary (mx) processes. (B) *Barx1* expression is seen in posterior positions of the first branchial arch. (C) At E18.5, *Otlx2*/*Rieg* is strongly expressed in cells of the stratum intermedium (si) and of the outer enamel epithelium (oee), while expression is downregulated in preameloblasts (pa) and the stellate reticulum (sr) of the first molar (m1). *Otlx2*/*Rieg* transcripts are also detected in the epithelium of the second molar (m2) and in dental lamina (dl). Note that the epithelium of the incisor (inc) is positive for *Otlx2*/*Rieg* in both lingual (Li) and labial (Lb) sides. Transcripts are not detected in dental papilla mesenchyme (p). (D) On an adjacent section, *Barx1* transcripts are observed in the differentiating preodontoblasts/odontoblasts (o) of the first molar, while the gene is downregulated in the dental papilla. *Barx1* is strongly expressed in the dental papilla of the second molar. Transcripts are not found in the mesenchyme of the incisor.

signal was particularly strong in the differentiating odontoblasts of the first molar and in the dental papilla mesenchyme of the second molar. In contrast, the mesenchyme of the incisors was *Barx1*-negative (Fig. 1D). At E18.5, *Sox9* expression was strong in preameloblasts/ameloblasts and stratum intermedium, while in dental papilla transcripts were observed only in cells of the sub-odontoblastic layer (Fig. 2C, D).

Discussion

Mouse tooth formation starts at E10.5 as local thickenings of the oral epithelium at the sites of future odontogenesis. Transcription factor genes such as *Msx2*, *Dlx2* and *Lef1*, and secreted signalling molecules such as BMP4 and FGF8, are expressed in the presumptive dental epithelium at E10.5 (5). All these factors are potentially involved

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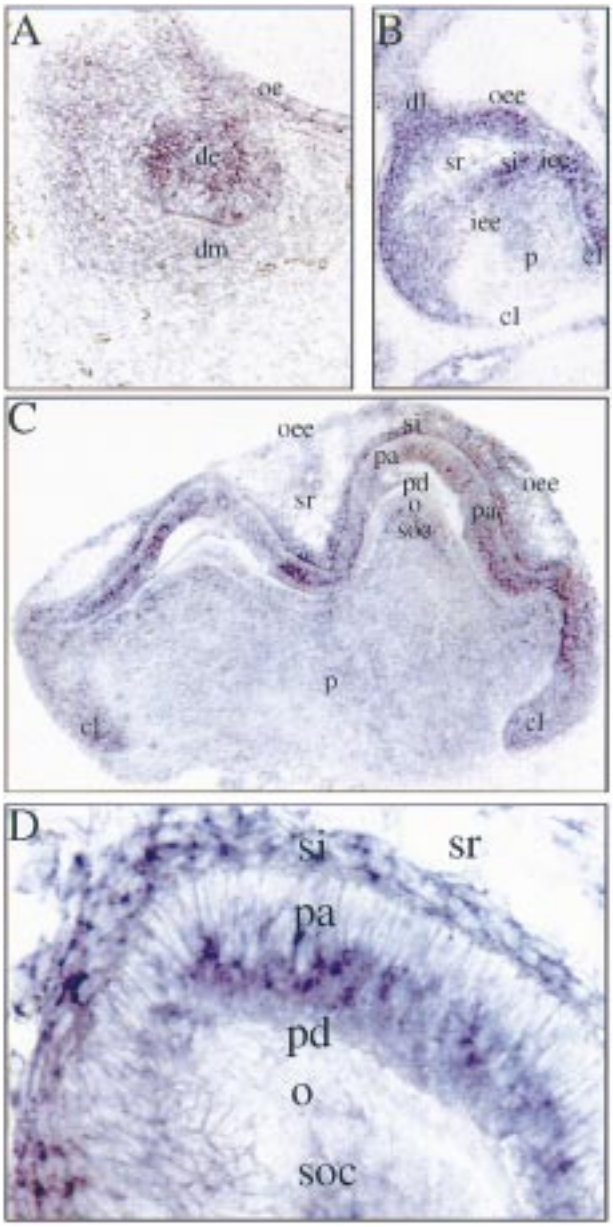

Fig. 2. Pattern of *Sox9* expression during embryonic tooth development. *In situ* hybridizations on cryosections using a digoxigenin-labelled probe. (A) At E13.5, *Sox9* transcripts are detected in the epithelium (de) of the bud-stage molar tooth germ. Fewer transcripts are observed in the condensed dental mesenchyme (dm) and in oral epithelium (oe). (B) *Sox9* is expressed in dental epithelial cells at the early bell stage of a molar (E16.5). Note that the transcripts are detected only in cells of the inner enamel epithelium (iee) of the labial side, while they are absent in inner enamel epithelial cells of the lingual side. (C) At the late bell stage (E18.5), *Sox9* is expressed in the differentiating preameloblasts (pa) and the stratum intermedium (si) of the first molar, while the transcripts are not observed in stellate reticulum (sr) and outer enamel epithelium (oee). In dental papilla mesenchyme (p), *Sox9* transcripts are detected in cells of the sub-odontoblastic layer (soc), whereas transcripts are absent in odontoblasts (o). (D) Higher magnification of the cuspal area of an E18.5 molar. dl, dental lamina. cl, cervical loop. pd, predentine.

in the morphogenesis of the tooth germ and likely cross-regulate each other directly or through autocrine or paracrine loops. However, the stomatodeal ectoderm has the capacity to initiate tooth formation in neural crest-derived mesenchyme (1, 2) already at E9.5. Molecules expressed specifically in the stomatodeal ectoderm at such early stages have not yet been identified. Expression of the new homeobox gene of the *paired*-like family, *Otlx2/Rieg,* distinguishes the stomatodeal from any other ectoderm at E9.5, suggesting that its product may be involved in the initiation of odontogenesis. During tooth initiation, the recently identified homeobox gene *Barx1* (12) is exclusively expressed in the mesenchyme where molars will develop, suggesting that Barx1 may be a key regulator of regionally specific tooth morphogenesis.

During early odontogenesis, the pattern of *Otlx2/Rieg* expression becomes progressively specific for the epithelial compartment of the forming teeth. *Otlx2/Rieg* is down-regulated in the cells of the inner enamel epithelium, at the time they start to differentiate into preameloblasts, suggesting that *Otlx2/Rieg* is required for tooth morphogenesis but not for cytodifferentiation. Recently, Semina *et al.* (14) reported mutations in the *Otlx2/Rieg* gene as a likely cause of Rieger syndrome, a human disorder characterized by tooth abnormalities such as anodontia vera, hypodontia or abnormally shaped teeth (14, 19). However, it is not known at which stage tooth development is affected in patients with Rieger syndrome. Taken together, these findings strongly suggest that *Otlx2/Rieg* is essential for proper tooth development.

During tooth morphogenesis, *Barx1* expression remains confined to the mesenchymal compartment of molars, in a pattern strictly complementary to *Otlx2/Rieg*. *Barx1* is not expressed in the incisor anlagen, in contrast to all other known transcription factors expressed during odontogenesis (4, 5). *Barx1* downregulation in dental mesenchyme of molars coincides with the completion of crown formation, suggesting that *Barx1* is involved in both early and late morphogenetic events.

The *Sry*-related gene *Sox9* is important for chondrogenesis and sex-determination in mammals (15, 16). The property of the Sox9 protein to activate transcription (18) and its tissue-specific expression pattern indicate that Sox9 may regulate gene expression in specific cell lineages. *Sox9* is expressed in both incisor and molar germs throughout odontogenesis. *Sox9* expression in preameloblasts/ameloblasts suggests a role for the product of this gene in ameloblast specification and/or terminal differentiation. Interestingly, in cells of the inner enamel epithelium (future preameloblasts) *Sox9* is

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expressed only at the labial side of the molar, but not at the lingual side, suggesting that different regulatory mechanisms distinguish the lingual from the labial sides of molars.

In conclusion, we have shown that *Otlx2/Rieg* expression remains confined to the epithelial components of the developing molars and incisors, while *Barx1* expression is restricted to the mesenchyme of developing molars. *Sox9* is expressed in epithelial components and to a lesser degree in condensed mesenchyme of both molar and incisor primordia. These results suggest that combinations of various transcription factors may be a prerequisite for the generation of tooth diversity.

Acknowledgements – This work was supported by institutional grants from CNRS and Université de la Méditerranée and by specific grants from the "Ministère de l'Education Nationale", the "Fondation pour le Recherche Medicale" (T.M.) and the "Association pour la Recherche sur le Cancer" (T.M.).

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