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Original Article

The presence of rudimentary odontogenic structures in the mouse embryonic mandible requires reinterpretation of developmental control of first lower molar histomorphogenesis

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ABSTRACT In the mouse embryonic maxilla, rudimentary tooth primordia have been identified, which can be mistaken for the first upper molar. In order to determine whether such a situation might exist in the lower jaw as well, tooth development was investigated in the mouse mandibular cheek region during ED 12.5-15.0. A combination of histology, morphometry and computer-aided 3D reconstructions demonstrated the existence of rudimentary dental structures, whose gradual appearance and regression was associated with the segmental progress of odontogenesis along the mesio-distal axis of the jaw: 1) At ED 12.5, the mesial segment (MS) was the most prominent part of the dental epithelial invagination. It included an asymmetrically budding dental lamina. The MS, although generally mistaken for the lower first molar ($M_{1)}$ primordium, regressed and did not finally participate in M_1 cap formation. 2) At ED 13.5, a wide dental bud (called segment R2) appeared distally to the MS. Although the R2 segment transiently represented the predominant part of the dental epithelium at ED13.5, it participated only in the formation of the mesial end of the M_1 cap. 3) The top of the R2 segment at ED13.5 was not the precursor of the enamel knot (EK), contrary to what has been assumed. 4) The central segment of the M_1 cap as well as the EK developed later and distally to the R2 segment. 5) Time-space specific apoptosis correlated with the retardation in growth of the R2 segment as well as with strong regressive changes in the epithelium situated mesially to it. These highlight the need to reinterpret current molecular data on early M_1 development in the mouse in order to correlate the expression of signalling molecules with specific morphogenetic events in the appropriate antemolar or molar segments of the embryonic mandible.

KEY WORDS: *tooth, molar, development, mouse, apoptosis, 3D reconstruction*

Introduction

Mouse odontogenesis is one of the most frequently used models to study mechanisms involved in regulation of organ patterning and morphogenesis (e.g. Ruch, 1995; Linde, 1998). Until recently, classical descriptive data (Gaunt, 1955; Cohn, 1957) were the only sources used as the morphological basis to interpret molecular aspects of odontogenesis in the mouse. However, the modern approaches of morphology employing computer-aided 3D reconstruction techniques (Peterková *et al.*, 1995; Radlanski, 1995) have allowed a more complete understanding of tooth morphogenesis and have also led to new interpretations of the temporo-spatial dynamics of developing structures.

Analyses of histological sections and computer-aided 3D recons-tructions gradually revealed up to seven transitory ves-

Abbreviations used in this paper: ED, embryonic day; 3D, three dimensional; M_1, first lower molar; MS, mesial segment; wtc, weight class; EK, enamel knot.

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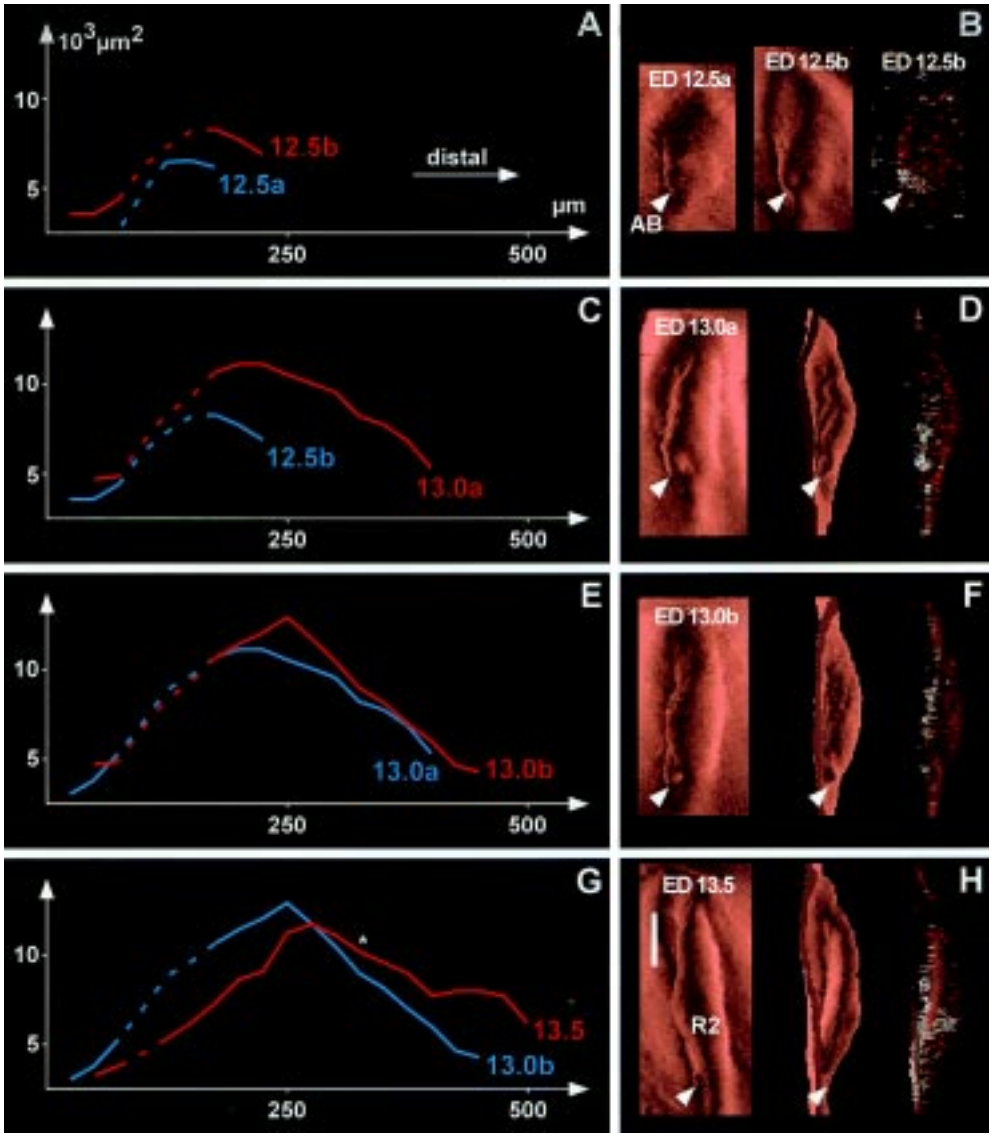

Fig. 1. Morphometry curves (A,C,E,G) documenting size of the dental epithelium measured in square micrometers (μm^2) and 3D representations (B,D,F,H) showing shape of dental epithelium in the mandibular cheek region at ED 12.5a (wtc. 76-100 mg), 12.5b (wtc. 101-125 mg), 13.0a (wtc. 126-150 mg), 13.0b (151-175 mg) and 13.5 (wtc. 151-175 mg). *X-axis, the mesio-distal length of the dental epithelium in µm. Shape of the dental epithelium is presented in aerial views* **(B)** *or in aerial and medial views* **(D,F,H).** *Mesial part of the dental epithelium is at the bottom of 3D pictures. The presence of the accessory bud in the mesial segment (MS) is indicated by a dashed line on curves and by a white arrow on 3D pictures. A star shows location of the posterior part of R2 segment (R2). Epithelial apoptoses are represented as white spots, epithelial metaphases as red spots. Bar, 100 µm.*

tigial tooth primordia in the ante molar region of mouse embryonic maxilla during embryonic day (ED) 12.5-13.5 (Peterková *et al.,* 1993, 1995, 1996; Lesot *et al.,* 1998). These rudimentary structures have a short life span and reach at most bud stage before they regress. Epithelial apoptosis associated with BMP2 and BMP4 expression is involved in the regression of these elements (Peterková *et al.*, 1996, 1998; Turecková *et al.*, 1996), and leads to delimitation of the mesial end of the first upper molar (M^1) cap, (Lesot *et al.*, 1996, 1998). Specific foci of degenerating cells have also been reported in the mouse mandible between ED 13.5-14.5 and related to the bud and cap staged molar epithelium (Nozue, 1971; Kindaichi, 1980; Vaahtokari *et al.*, 1996b; Viriot *et al.*, 1997). Apoptoses also accumulate at ED 12.5 in a small accessory bud protruding lingually from the mandibular dental lamina in the mouse cheek region (Viriot *et al.*, 1997).

The mouse model has provided considerable data on the expression of transcripts encoding for signalling molecules during mouse lower molar development from ED 12.5-14.5 (for review see Thesleff *et al.*, 1995; Maas and Bei, 1997; Thesleff and Jernvall, 1997; Thesleff and Sharpe, 1997; Peters and Balling, 1999; Tucker and Sharpe, 1999), however, their potential role in control processes still remains to be established. Very little is known about the exact sequence of morphological events taking place there during this period. In this study, tooth development was investigated in the mandibular cheek region in carefully staged mouse embryos during ED 12.5-15.0 using histology, morphometry and computer-aided 3D reconstructions. Specific attention was given to developmental relationships between the structures where apoptosis was concentrated. Our aims were 1) to understand the morphogenetic role of these areas of specifically localised cell death, and 2) to search for the possible existence of rudimentary dental structures in the mouse mandible.

Results

ED 12.5

The invagination of the dental epithelium in the mandibular cheek region was rather short in the mesio-distal direction (Fig.

1A,B). The most voluminous mesial segment (MS) was formed by a wide lamina. An accessory bud protruded from the lingual slope of the lamina towards the surrounding mesenchyme (Fig. 1B), giving a budding lamina shape to the dental epithelium on frontal sections (Figs. 2A,6C). The MS represented most of the dental epithelium where its size could be measured on sections (Fig. 1A). Compared to developmentally less advanced embryos at this stage (body weight lower than 100 mg), the accessory bud of the MS still extended along the mesio-distal axis and achieved its definitive length in ED 12.5 specimens weighing more than 100 mg (Fig. 1A,B). In distal direction, the thickness of the dental epithelium gradually decreased. It appeared as a thin and wide lamina vanishing distally in an epithelial thickening (Fig. B).

Apoptoses transiently accumulated in the accessory bud in specimens weighing 101-125 mg (Figs. 1B and 2A).

The morphology of the dental epithelium in ED 12.5 specimens weighing 126-150 mg represented a transition to the following chronological stage.

ED 13.0

The invagination of the dental epithelium elongated behind the MS (Fig. 1C,D). The accessory bud of the mesial segment became more accentuated, although its mesio-distal length remained unchanged (Fig. 1C). Compared to the situation at ED 12.5 (Fig. 1A,B), the epithelium increased in size distally to the MS, and gave rise to a bud shape appearance on frontal sections in the developmentally most advanced embryos at ED 13.0. Growth of the dental epithelium was reflected in the elevation and elongation of the curve depicting the size of the dental epithelium, and by distal movement of its maximum values from the MS (as seen at the previous stage, Fig. 1A) to the area of the bud shaped epithelium (Fig. 1C,E).

At ED 13.0, apoptosis was concentrated at the oral surface of the MS and of the bud shaped epithelium situated more distally (Fig. 1D,F).

ED 13.5

The distal part of the invagination of dental epithelium appeared more elongated increasing its size on frontal sections (Figs. 1G,H,2D,3A,B,C). In the MS, the budding dental lamina (i.e. the dental lamina as well as the accessory bud projecting from its lingual slope) decreased (compare Fig. 1F with Figs. 1H,2B). The accessory bud finally ceased to be apparent in the developmentally most advanced specimens at ED 13.5 (compare Figs. 1H and 3A).

The dental epithelium appeared most voluminous and prominent distally to MS, where a large bud was present on frontal sections at this stage (compare Figs. 2D,3C with 6B). We called this conspicuous segment R2 (Figs. 1H and 3A). It caused an apparent swelling in 3D reconstructions viewed from both aerial and medial side (Figs. 1H and 3A), and represented the high mesial peak on the morphometry curves (Fig. 4). Behind R2, the invagination of dental epithelium exhibited a narrow cylinder shape in 3D reconstructions and a smaller bud shape on frontal sections (Figs. 2E and 3B).

A wide lamina was present on sections (Fig. 2C) between the MS with remnant of the accessory bud (Fig. 2B) and the most voluminous epithelium of R2 segment (Fig. 2D) situated distally. As a result, the distance between the mesial end of the MS and the bud shaped epithelium increased (compare Fig. 1F and 1H) and the peak of the morphometry curve moved distally (Fig. 1G).

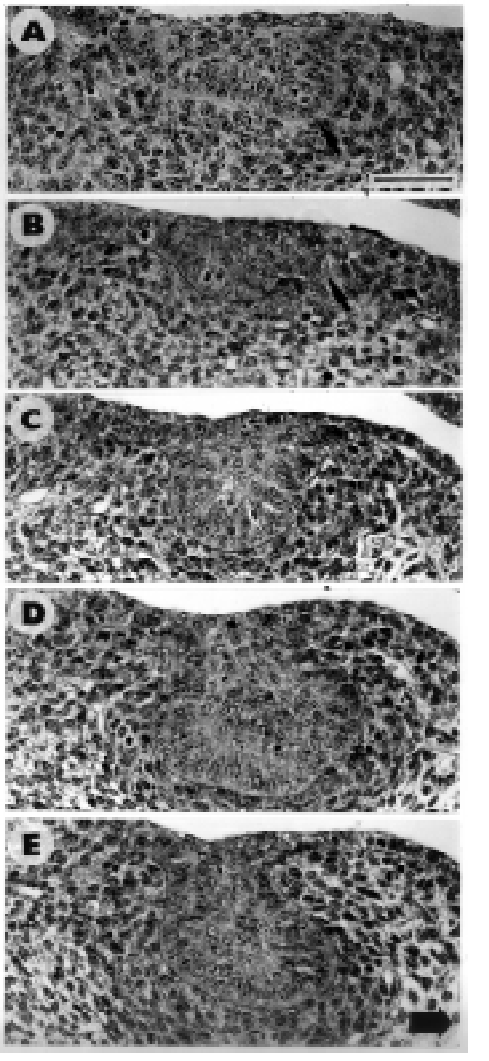

Fig. 2. Frontal histological sections depict the mandibular dental epithelium at ED 12.5, wtc. 101-125 mg (A) and 13.5, wtc. 151-175 mg (B-E). (A,B) *The mesial segment (MS), which exhibits in the documented section the budding lamina shape, i.e. the lamina with an accessory bud (slim arrow) protruding from its lingual side.* **(C)** *The lamina interconnecting the mesial segment and R2 segment.* **(D)** *The large (wide) bud shaped epithelium of R2 segment.* **(E)** *Epithelium located distally to R2 segment. Large arrow points lingually. Bar, 50 μm.*

Apoptosis still more affected superficial layers of the dental epithelium in place of the MS, interconnecting lamina and R2 segment. Furthermore the apoptosis was also concentrated in the internal part and at the top of the R2 segment (Figs. 1H,2D,3A,C).

ED14.0

Compared to the previous stage, the mesio-distal extent of the whole epithelial invagination exhibited minimal change (Fig. 4). However, its central part gradually extended in a bucco-lingual direction, where the cap of the M_1 was initiated (Fig. 3D) distally to R2 – in place where the epithelium exhibited the narrow cylinder shape in 3D representations (Figs. 1H and 3A) and the small bud shape on frontal sections (Figs. 2E,3B) at ED 13.5.

The R2 segment maintained its bud shape appearance on frontal sections (Fig. 3C,F). Medial views of 3D reconstructions

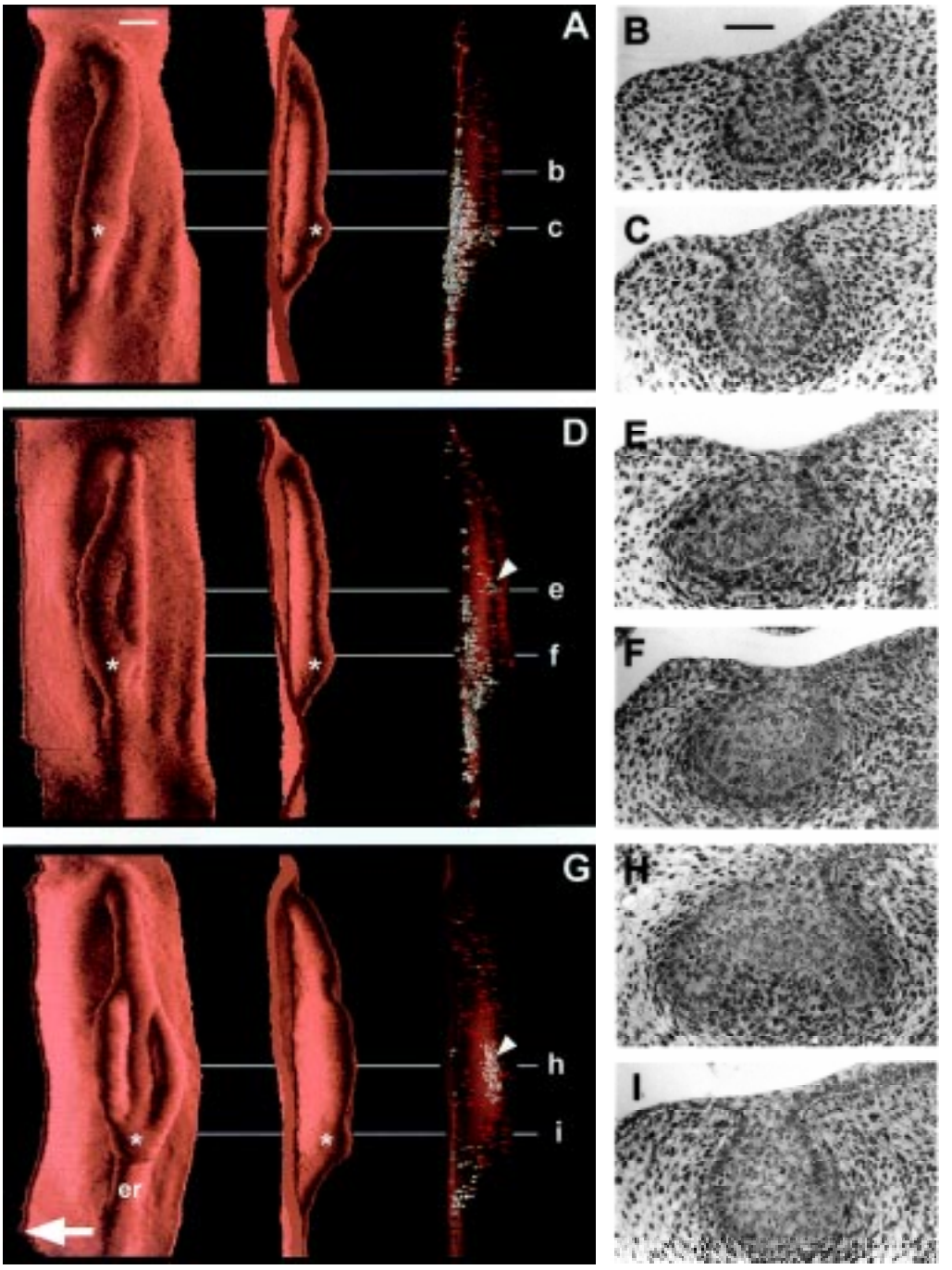

Fig. 3. Gradual growth and shaping of the dental epithelium are documented at ED 13.5, wtc. 251-275 mg (A,B,C), at ED 14.0, wtc. 251-275 mg (D,E,F), and at ED15.0, wtc. 426-450 mg (G,H,I) by 3D reconstructions presented in aerial and medial views (A,D,G), and by frontal histological sections recording R2 segment (C,F,I) or central part of molar epithelium (B,E,H). *Location of the histological section* **(B,C,E,F,H,I)** *on the corresponding 3D reconstruction is indicated by respective white horizontal lines (b,c,e,f,h,i). Mesial part of the dental epithelium is at the bottom of 3D pictures. A large arrow points lingually. A star indicates position of R2 segment. A white triangle points the enamel knot. er, epithelial ridge. Epithelial apoptoses are represented as white spots, epithelial metaphases as red spots. Bar, 100 μm (3D representations) or 25 μm (histological pictures).*

(Figs. 3A,D and 5) identified the persistence of the R2 segment at the mesial end of the M_1 cap. There, the R2 segment was involved in formation of a bulbous extremity of the enamel organ. Behind this region, the lateral and medial enamel grooves gradually appeared (Fig. 3).

At the initial cap stage, apoptosis ceased to concentrate in the internal parts of the R2 segment, although continued to be seen at the oral surface of R2 and in the dental epithelium situated mesially to R2 (Fig. 3D). Apoptosis was initiated in the enamel knot (EK) as soon as it started to form in the central part of the early M_1 cap (Fig. 3D,E).

Metaphases were ubiquitously distributed in the mandibular dental epithelium during early stages. They were less frequent, however, in regions where foci of apoptosis were observed. After cessation of apoptosis in the internal part of R2, the metaphases reappeared there at ED 14.0 (Fig. 3).

ED 15.0

The enamel cap of the M_1 was well formed. The lingual and buccal parts of the cervical loop were interconnected by the bulbous mesial extremity of the cap, which included the R2 segment (Figs. 3G and 4).

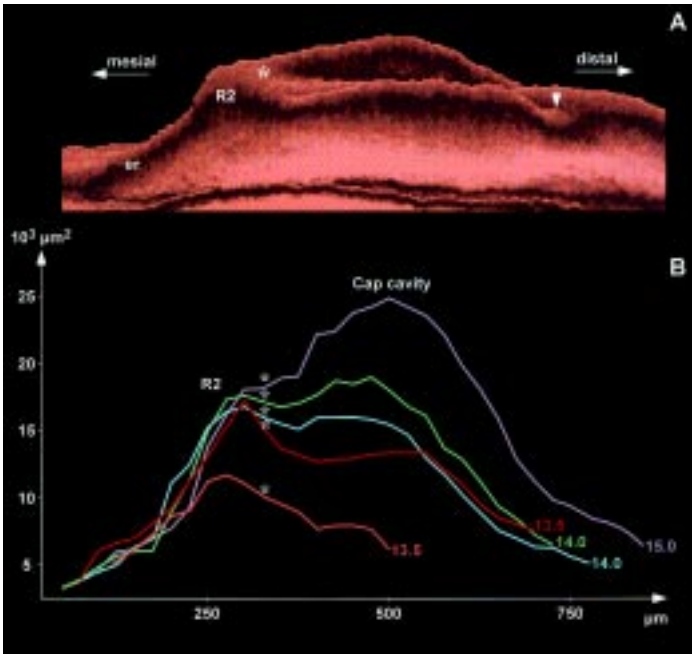

Fig. 4. The 3D reconstruction (A) shows the shape of the dental epithelium at ED 15.0 (wtc. 426-450 mg) viewed from the lingual side. *Morphometry curves* **(B)** *document size in square micrometers (µm²) of the dental epithelium in the mandibular cheek region at stages: ED 13.5, wtc.151-175 mg (pink); ED 13.5, wtc.251-275 mg (red); ED14.0, wtc.251-275 mg (blue); ED 14.0, wtc.276-300 mg (green); ED15.0, wtc.426-450 mg (violet). The curves were adjusted according to the posterior part (star) of R2 segment (R2). X-axis, mesio-distal length of the dental epithelium in µm. The same sections were used for morphometry and 3D reconstruction at ED 15.0. Due to it, the size and position of the 3D reconstruction could be precisely adapted according to x-axis of the corresponding curve (the star and triangle indicate examples of corresponding sections on both the curve and 3D reconstruction at ED 15.0). er, epithelial ridge.*

The dental epithelium situated in front of the R2 segment at previous stage (i.e. the MS and the wide lamina connecting it to R2) was transformed in the epithelial ridge extending mesially from the M_1 enamel organ (Figs. 3 and 4). There was an apparent reduction in number of apoptotic cells and bodies, which had strongly accumulated in this location at previous stages (Figs. 1 and 3).

Discussion

Segmental growth of the mandibular dental epithelium

An important observation of this study was the segmental growth of the mandibular dental epithelium. Segmental development has been previously reported during the cap to bell transition of the M_1 enamel organ in the mouse: the M_1 cap corresponds to the prospective mesial part of the crown (B1, L1 and B2, L2 cusps), while the distal part (B3, L3 regions) starts to develop as late as during the cap-bell transition and the most distal cusp (cusp 4) arises during the late bell stage (Lesot *et al.*, 1999).

Also during early stages, a segmental development of dental epithelium was shown to occur along the mesio-distal axis of the mandible. The thickest (greatest volume) part of the dental epithelium at each consecutive day was located distally to the most conspicuous epithelium of the previous day. Three segments developed sequentially during ED 12.5-14.5: the mesial segment (MS), the R2 segment, and the central part of the M_1 cap.

At ED 12.5, the epithelial invagination was thicker and developmentally more advanced at its mesial extremity. In this mesial segment (MS), the epithelium gave rise to a wide lamina bearing an accessory bud on its lingual slope. During further development, this MS lost its transient morphological dominance and was gradually reduced in size to an epithelial ridge (Fig. 6C,D). In the meantime, the dental epithelium situated more distally increased and demonstrated a large bud shape on frontal sections. As a result, the latter structure (R2 segment) represented the most voluminous and developmentally advanced part of the mandibular dental epithelium at ED 13.5. However, due to its growth retardation, even the R2 segment lost its dominance (similarly to the MS at previous stages) and became overshadowed by the rapidly growing epithelium situated still more distally (Figs. 4 and 5), where the central part of the M_1 cap developed. The R2 segment maintained its bud shaped appearance on sections and finally took part in the formation of the mesial bulbous extremity of the M_1 enamel organ (Fig. 6B,D). This structure interconnected the buccal and lingual parts of the cervical loop. In this way, delimitation of the prospective dental papilla was anticipated mesially in the M_1 cap, as previously reported (Viriot *et al.*, 1997; Lesot *et al.*, 1998).

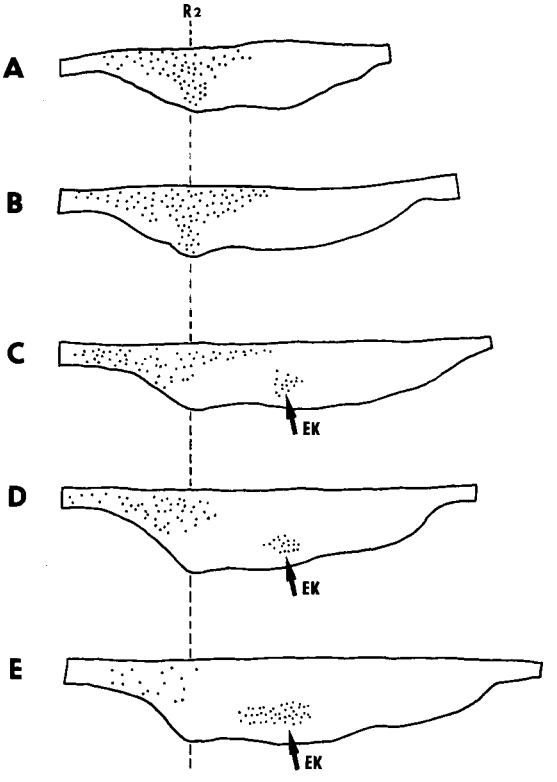

Fig. 5. Drawings of 3D reconstructions of the mandibular dental epithelium in profile views document persistence of R2 segment at the mesial end of the developing M_1 cap *(compare with Figs. 1 and 3).* **(A)** *ED 13.5 (wtc.151-175 mg);* **(B)** *ED 13.5 (wtc. 251-275 mg);* **(C)** *ED 14.0 (wtc. 251-275 mg);* **(D)** *ED 14.0 (wtc. 276-300 mg);* **(E)** *15.0 (426-450 mg). Adjustment has been made according to the top of R2 segment (dashed line). Apoptoses are introduced as black spots. Black arrow indicates the concentration of apoptosis in the enamel knot (EK).*

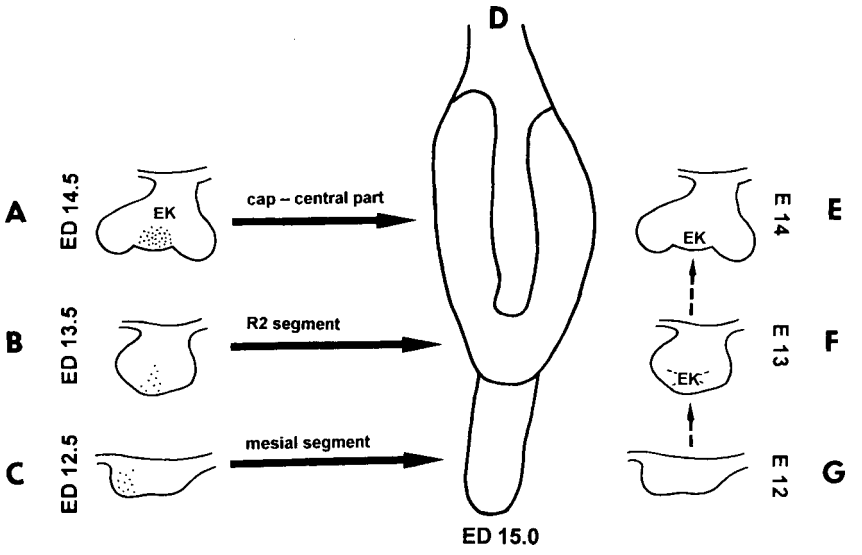

Fig. 6. Schematic drawings of the mandibular dental epithelium on frontal sections (A,B,C,E,F,G) and 3D reconstruction (D). (A-D) *Present study;* **(E-G)** *incorrect interpretation of the early M_1 development. Staging of corresponding embryos is introduced either in ED (midnight before morning detection of vaginal plug = ED 0.0), or in E (day of morning detection of vaginal plug = day 0).* **(A,B,C)** *Conspicuous segments consecutively appearing along the mesio-distal axis in the dental epithelium: at ED 12.5 – the budding dental lamina (i.e. the dental lamina with an accessory bud protruding from its lingual side) of the mesial segment (MS) (C) at ED 13.5 – the R2 segment developing a large (wide) bud (B); at ED 14.5 – the cap cavity of M_1 (A). Black spots demonstrate localisation of apoptoses, which could be detected in embryos at specific developmental stages. Full arrows indicate location of these epithelia (A,B,C) at ED 15.0* **(D)**. *The dashed arrows show the incorrect interpretation of succession of dental lamina* **(G)**, *bud* **(F)**, *and cap* **(E)** *stage during the M_1 development. EK, enamel knot, which did not arise from the top of the wide bud (F) at ED 13.5 (E13). er, epithelial ridge.*

Apoptosis and epithelium regression during early odontogenesis in mandible

Cell death has been recognised for decades as a normal feature of development (Saunders, 1966). Cell death (specified now as apoptosis) plays an important role in the morphogenesis of various organs (for review see Schwartz and Osborne, 1995).

The occurrence of cell death during tooth development was first reported in the mouse and human (Nozue, 1971). In the mouse mandible, cell death has been detected in the dental bud and in the enamel knot (EK) of the M_1 cap (Nozue, 1971; Kindaichi, 1980). As in the mouse maxilla (Lesot *et al.*, 1995, 1996), cell death in the bud- and cap-shaped dental epithelium of the mandible has also been identified as apoptosis (Vaahtokari *et al.*, 1996b). In the mouse lower jaw, apoptosis also transiently occurs in the small accessory budding at the mesio-lingual end of dental lamina at ED 12.5, and at the top of the wide bud-shaped epithelium at ED 13.5 (Viriot *et al.*, 1997).

Where the dental epithelium exhibited apoptosis in its deeper parts (at the mesenchymal surface), the present study demonstrated the existence of three temporo-spatially distinct structures: a) an accessory bud of the MS at ED 12.5 (Fig. 6C); b) the R2 segment at ED 13.5 (Fig. 6B); and c) the enamel knot in the central segment of the cap from ED 14.0 (Fig. 6A). The apoptotic cells and bodies also accumulated at the oral surface of the dental epithelium during ED 13.0-14.0, extending from the MS to the R2 segment (Fig. 5). The degree of regression of the different epithelial structures seemed to correlate with differences in the duration and intensity of the related apoptotic process: the dental epithelium situated mesially to R2 was affected by apoptosis for the longest period and consequently was regressed strongly to an epithelial ridge; the R2 segment, where the apoptotic period was shorter, exhibited only growth retardation and maintained a bud shape appearance at the mesial extremity of the raising M_1 cap (Fig. 6). The consequences of apoptosis in the enamel knot have been discussed in previous papers (Lesot *et al.*, 1995, 1996, 1999; Viriot *et al.*, 1997).

Signalling detected during early steps need not include prospective molar tissues

The present study has highlighted the need to reappraise data from previous studies in which expression of signalling molecules has been interpreted in relation to lower molar development. The thickest and developmentally most advanced dental epithelium in the mandibular cheek region in mouse embryos at ED 11.5, 12.5, 13.5 and 14.5 (E11, E12, E13, E14) has been generally assumed to correspond to the consecutive stages (epithelial thickening, dental lamina, bud and cap, respectively) of the M_1 development in the mouse (see also Fig. 6G,F,E). Thus, attempts have been made to correlate the molecular data obtained at these locations with the development of the molar itself. However, the present data demonstrated rudimentary structures were the most conspicuous at early stages and could thus be mistaken for the M_1 primordium, although they later underwent regression.

The morphological analysis of embryos ranked according to detailed staging allowed identification of the thickest and most conspicuous epithelium at specific stages as a) MS at ED 12.5 (compare with Fig. 6C), b) R2 segment at ED13.5 (Fig. 6B), and c) central part of the M_1 cap at ED 14.5 (Fig. 6A); the different fates of these distinct segments are shown in Figure 6D. (The embryos at ED 13.0 and 14.0 exhibited transitory stages). Since the MS represented the most developmentally advanced epithelium at the dental lamina stage on ED 12.5, the most conspicuous part of the epithelial thickening present at still earlier stages might be the precursor to the MS. All epithelium anterior to the R2 segment (i.e. also the MS) was finally situated outside of the M_1 cap and did not participate in its formation (Fig. 6).

Many transcription factors and growth factor genes (e.g. *BMP, FGF, Shh, Lef, Msx, Dlx*) have been detected during early mouse odontogenesis and suggested to regulate molar development (for reviews see e.g. Thesleff *et al.*, 1995; Maas and Bei, 1997; Thesleff and Sharpe, 1997; Peters and Balling, 1999; Tucker and Sharpe, 1999). The present findings raise questions as to which tissues have been investigated in various *in vivo* and *in vitro* tooth developmental studies, assuming exclusively molar epithelium and mesenchyme have been processed (analysed) at early stages in mouse embryos. Also, which of the available data indeed refer to the developmental control of the prospective M_1 in mouse, and which of these data can be related to the rudimental epithelium situated finally in front of the molar cap. There is a risk that the data obtained prior to ED 13.5 might not yet relate to the molar tissues, but the antemolar rudimental MS.

The data on the wide bud at ED 13.5 should be related rather to the mesial end than to the central part of the prospective M_1 cap (compare with Fig. 6). For this reason, it could be interesting to reinvestigate the expression of signalling molecules trying to correlate them with specific morphogenetic events in the appropriate antemolar or molar segments of the mandible.

Signalling centres during early tooth development

The enamel knot (EK) in the central part of the forming tooth cap, has been proposed as the signalling centre regulating tooth development (MacKenzie *et al.*, 1992; Jernvall *et al.*, 1994, 1998; Vaahtokari *et al.*, 1996a,b; Thesleff and Jernvall, 1997).

Due to its large size, the wide bud-shaped epithelium in day 13 (E13, ED 13.5) mouse embryos is generally assumed to be a precursor to the central part (bottom) of the first molar cap, while a cervical loop protrudes on its sides (compare with Fig. 6F,E). Probably for this reason, the up-regulation of *p21* and withdrawal of cells from the mitotic cycle at the top of the wide bud in day 13 (E13, ED 13.5) mouse mandibles have been interpreted as the onset of the EK formation (Vaahtokari *et al.*, 1996a; Thesleff and Jernvall, 1997; Jernvall *et al.*, 1998). The expression of genes encoding for signalling molecules at the top of the wide bud at day 13 (E13, ED 13.5) and at the bottom of the cap one day later have thus been attributed to the development of a unique structure – the primary EK of the M_1 (Vaahtokari *et al.*, 1996a; Åberg *et al.*, 1997; Thesleff and Jernvall, 1997; Jernvall *et al.*, 1998; Keränen *et al.*, 1998), (compare with Fig. 6F,E).

In the above-mentioned papers, however, both the cessation of mitotic activity as well as the gene expressions on day 13 (E13; ED13.5) have been documented from 3D reconstructions and/or in frontal sections in the wide bud-shaped epithelium, which most probably corresponds to the present R2 segment (Figs. 1H,2D,3A,C,6B). The present morphological investigation has revealed that the EK did not arise at the top of the R2 segment at ED13.5, but more distally and one day later (Figs. 3,5 and 6). The various transcripts detected in the wide bud epithelium on day 13 and attributed to the onset of signalling in the primary EK of the prospective centre of the M_1 cap (Vaahtokari *et al.*, 1996a; Åberg *et al.*, 1997; Thesleff and Jernvall, 1997; Jernvall *et al.*, 1998) might thus be related to a distinct structure (the present R2 segment) involved in the formation of only the mesial end of the M_1 enamel organ.

Keränen *et al.* (1998) have described the co-localised expression of signalling molecules *(Fgf-4, BMP-2, BMP-4, Shh)* and transcription factors *(Lef-1, Msx-1, Msx-2, p21)* in signalling centres suggested to regulate the M_1 development in mouse. Two signalling centres have been identified before day 15 and correlated with specific morphological features of the M_1: a) the early centre with the budding dental lamina at E12 (ED 12.5) and b) the enamel knot centre with the late bud at E13 (ED 13.5) and with the cap at E14 (ED 14.5), (Keränen *et al.*, 1998).

The results presented here demonstrate the existence of similar specific morphological features in three topographically distinct structures: a) the budding lamina of the MS at ED 12.5; b) the large bud of the R2 segment at ED 13.5; and c) the middle of the M_1 cap at ED 14.5 (Fig. 6). These observations strongly support the hypothesis that three active zones for specific signalling ("signalling centres") might exist in the mouse mandible before day 15, which appear sequentially in front (MS), at the mesial end (R2) and in the central part (EK) of the prospective M_1 cap (compare with Fig. 6A-D). It will be necessary to verify whether the cells of the active signalling zones arise independently, or are interrelated as a result of cell migration according to the recent hypothesis from Coin *et al.* (1999). Functional investigations (Kingsley, 1994) should elucidate the molecular basis and tissue interactions leading to the different fates of these three segments (MS, R2, central part of the M_1 cap).

Conclusion

Developmental studies have used the developing tooth as a model to investigate not only dental formation, but also the general mechanisms regulating organ development. The correct interpretation of results from tooth developmental studies is thus of broad importance. The present descriptive results suggest that different interpretations may exist for classical molecular data gained in the tooth model. The temporo-spatial correlation of the available molecular data with the corresponding mesio-distal segments of the mandibular odontogenic tissues at specific stages could help to determine which genes can be co-localised and indeed linked to the same signalling cascade, and which of them might regulate progressive or regressive events during organ development.

Materials and Methods

Staging of embryos

The ICR females were mated overnight and the midnight before the morning, detection of the vaginal plug was determined as embryonic day (ED) 0.0. The embryos were harvested at noon and midnight from ED 12.5 till ED 15.0. The developmental stage of specimens of the same chronological age was specified in more detail by the wet body weight of embryos before fixation (Peterková *et al.*, 1993). According to their weight, the specimens were distributed in 25 mg weight classes (wtc.). In such a way, a detailed succession of stages documenting tooth development could be made using weight specification (in mg) of embryos of the same chronological age (in ED).

Histology

The embryos were fixed in Bouin-Hollande fluid. The head of at least one specimen from each weight class at each chronological stage was processed histologically. The 5 μm frontal sections from paraffin embedded heads were stained with alcian blue-hematoxylin-eosin. Epithelial thickening, dental lamina and bud were distinguished on frontal histological sections according to the shape of the dental epithelium and morphology and arrangement of its cells (Peterková *et al.*, 1996).

3D reconstructions

Three dimensional analysis including apoptosis distribution was performed in 12 specimens. One specimen from each of several weight classes at each chronological stage was analysed: ED 12.5 (wtc. 76-100 mg, 101-125 mg, 126-150 mg), ED 13.0 (wtc. 126-150 mg, 126-150 mg, 151-175 mg), ED 13.5 (wtc. 126-150 mg, 151-175 mg, 251-275 mg), and ED 14.0 (wtc. 251-275, 276-300 mg). At ED 15.0 only 1 specimen (wtc. 426-450 mg) was processed. Contours of the dental and adjacent oral epithelium were drawn from histological sections in 5 μm intervals using a JENAVAL microscope equipped with a drawing chamber at a magnification of 320x. Apoptoses and metaphases in the epithelium were recorded in the drawings. Apoptotic cells and bodies, whose nature has previously been confirmed using the TUNEL method (Turecková *et al.*, 1996), were identified from histological sections on the basis of morphological criteria (Kerr *et al.*, 1995; Turecková *et al.*, 1996).

The digitalisation of the serial drawings and correlation of successive images (Olivo *et al.*, 1993) have previously been described (Lesot *et al.*, 1996). Software packages allowing image acquisition and treatment were developed and adapted to this work in the lab. Three-dimensional images were generated using a volume-rendering programme (Sun Voxel, Sun Microsystems).

Morphometry

The morphometry evaluation was performed in the dental epithelium processed for 3D reconstructions. The cross sectional surface area of the dental epithelium in the right mandibular cheek region was measured in each 5th drawing (i.e. at 25 μm intervals) using a planimeter (REISS). The measurements could not be performed in the most distal mandible region, where the demarcation of the dental epithelium was not distinct. The values in square micrometers were plotted on graphs (Figs. 1 and 4).

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References

ÅBERG, T., WOZNEY, J. and THESLEFF, I. (1997). Expression patterns of bone morphogenetic proteins *(Bmps)* in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev. Dyn. 210:* 383-396.

COHN, S.A. (1957). Development of the molar teeth in the albino mouse. *Am. J. Anat. 101:* 295-320.

COIN, R., LESOT, H., VONESCH, J-L., HAIKEL, J. and RUCH, J-V. (1999). Aspects of cell proliferation kinetics of the inner dental epithelium during mouse molar and incisor morphogenesis: a reappraisal of the role of the enamel knot area. *Int. J. Dev. Biol. 43:* 261-267.

GAUNT, W.A. (1955). The development of the molar pattern of the mouse *(Mus musculus). Acta Anat. 24:* 249-268.

JERNVALL, J., ÅBERG, T., KETTUNEN, P., KERÄNEN, S. and THESLEFF, I. (1998). The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. *Development 125:* 161-169.

JERNVALL, J., KETTUNEN, P., KARAVANOVA, I., MARTIN, L.B. and THESLEFF, I. (1994). Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating *Fgf-4* gene. *Int. J. Dev. Biol. 38:* 463-469.

KERÄNEN, S.V.E., ÅBERG, T., KETTUNEN, P., THESLEFF, I. and JERNVALL, J. (1998). .Association of developmental regulatory genes with the development of different molar tooth shapes in two species of rodents. *Dev. Genes Evol. 208:* 477-486.

KERR, J.F.R., GOBÉ, G.C., WINTERFORD, C.M. and HARMON, B.V. (1995). Anatomical methods in cell death. In *Cell Death. Methods in Cell Biology, Vol. 46* (Eds. L.M. Schwartz and B.A. Osborne). Academic Press, London, pp. 1-27.

KINDAICHI, K. (1980). An electron microscopic study of cell death in molar tooth germ epithelia of mouse embryos. *Arch. Histol. Jpn. 43:* 289-304.

KINGSLEY, D.M. (1994). The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev. 8:* 133-146.

LESOT, H., PETERKOVÁ, R., SCHMITT, R., MEYER, J-M., VIRIOT, L., VONESCH, J-L., SENGER, B., PETERKA, M. and RUCH, J-V. (1999). Initial features of the inner dental epithelium histo-morphogenesis in the first lower molar in mouse. *Int. J. Dev. Biol. 43:* 245-254.

LESOT, H., PETERKOVÁ, R., VONESCH, J-L., VIRIOT, L., TURECKOVÁ, J., PETERKA, M. and RUCH, J-V. (1998). Early stages of tooth morphogenesis in mouse analyzed by 3-D reconstructions. *Eur. J. Oral Sci.106:* 64-70.

LESOT, H., VONESCH, J-L., PETERKA, M., PETERKOVÁ R. and RUCH, J-V. (1995). Mouse molar morphogenesis: Spatial distribution of mitoses and apoptosis. *Proceedings of the 10th Internat. Symp. Dent. Morph., Berlin, Sept. 6-10.* (Eds. R.J. Radlanski and H. Renz). "M" Marketing Services C.& M. Brüne, pp. 27-32.

LESOT, H., VONESCH J-L., PETERKA, M., TURECKOVÁ, J., PETERKOVÁ, R. and RUCH, J-V. (1996). Mouse molar morphogenesis revisited by three dimensional reconstruction: II) Spatial distribution of mitoses and apoptosis in cap to bell stages first and second upper molar teeth. *Int. J. Dev. Biol. 40:* 1017-1031.

LINDE, A. (Ed.) (1998). *Odontogenesis and Craniofacial Development (Conference proceedings).* Eur. J. Oral. Sci., Vol. 106, Suppl. 1. MUNKSGAART, Copenhagen, Denmark.

MAAS, R. and BEI, M. (1997). The genetic control of tooth development. *Crit. Rev. Oral. Biol. Med. 8:* 4-39.

MACKENZIE, A., FERGUSON, M.W.J. and SHARPE, P. (1992). Expression patterns of the homeobox gene, *Hox-8*, in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development 115*: 403-420.

NOZUE, T. (1971). Specific spindle cells and globular substances in enamel knot. *Okajimas Folia Anat. Jpn. 48:* 139-152.

OLIVO, J.C., IZPISUA-BELMONTE, J.C., TICKLE, C., BOULIN, C. and DUBOULE, D. (1993). Reconstruction from seriál sections: a tool for developmental biology. Application to Hox genes expression in chicken wing buds. *BioImaging 1:* 151-158.

PETERKOVÁ, R., LESOT, H., VONESCH, J-L., PETERKA, M. and RUCH, J-V. (1996). Mouse molar morphogenesis revisited by three dimensional reconstruction: I) Analysis of initial stages of the first upper molar development revealed two transient buds. *Int. J. Dev. Biol. 40:* 1009-1016.

PETERKOVÁ, R., PETERKA, M. and RUCH, J-V. (1993). Morphometric analysis of potential maxillary diastemal dental anlagen in three strains of mice. *J. Craniofac. Genet. Dev. Biol. 13:* 213-222.

PETERKOVÁ, R., PETERKA, M., VONESCH, J-L. and RUCH, J-V. (1995). Contribution of 3-D computer assisted reconstructions to the study of initial steps of mouse odontogenesis. *Int. J. Dev. Biol. 39:* 239-247.

PETERKOVÁ, R., PETERKA, M., VONESCH, J-L., TURECKOVÁ, J., VIRIOT L., RUCH, J-V. and LESOT, H. (1998). Correlation between apoptosis distribution and BMP-2 and BMP-4 expression in vestigial tooth primordia in mice. *Eur. J. Oral Sci. 106:* 667-670.

PETERS, H. and BALLING, R. (1999). Teeth - where and how to make them. *Trends Genet. 15*: 59-65.

RADLANSKI, R. (1995). Morphogenesis of human tooth primordia: the importance of 3-D computer-assisted reconstruction. *Int. J. Dev. Biol. 39:* 249-256.

RUCH, J-V. (Ed.) (1995). *Odontogenesis: Embryonic Dentition as a Tool for Developmental Biology (Conference proceedings).* Int. J. Dev. Biol., Vol. 39. UBC Press, Leioa.

SAUNDERS, J.W. (1966). Death in embryonic systems. *Science 154:* 604-612.

SCHWARTZ, L.M. and OSBORNE, B.A. (Eds.) (1995). *Cell Death. Methods in Cell Biology,* Vol. 46. Academic Press, London.

THESLEFF, I. and JERNVALL, J. (1997). The enamel knot: a putative signaling center regulating tooth development. *Cold Spring Harbor Symp. Quant. Biol. 62:* 257-267.

THESLEFF, I. and SHARPE, P. (1997). Signalling networks regulating dental development. *Mech. Dev. 67:* 111-123.

THESLEFF, I., VAAHTOKARI, A. and PARTANEN, A-M. (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int. J. Dev. Biol. 39:* 35-50.

TUCKER, A.S. and SHARPE, P.T. (1999). Molecular genetics of tooth morphogenesis and patterning: The right shape in the right place. *J. Dent. Res. 78:* 826-834.

TURECKOVÁ, J., LESOT, H., VONESCH, J-L., PETERKA, M., PETERKOVÁ, R. and RUCH, J-V. (1996). Apoptosis is involved in disappearence of the diastemal dental primordia in mouse embryo. *Int. J. Dev. Biol. 40:* 483-489.

VAAHTOKARI, A., ÅBERG, T., JERNVALL, J., KERÄNEN, S. and THESLEFF, I. (1996a). The enamel knot as a signaling center in the developing mouse tooth. *Mech. Dev. 54:* 39-43.

VAAHTOKARI, A., ÅBERG, T. and THESLEFF, I. (1996b). Apoptosis in the developing tooth: association with an embryonic signaling center and suppression by EGF and FGF-4. *Development 122:* 121-129.

VIRIOT, L., PETERKOVÁ, R., VONESCH, J.-L., PETERKA, M., RUCH, J-V. and LESOT, H. (1997). Mouse molar morphogenesis revisited by three-dimensional reconstruction. III. Spatial distribution of mitoses and apoptoses up to bell-stages first lower molar teeth. *Int. J. Dev. Biol. 41:* 679-690.

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