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Mouse odontogenesis *in vitro*: the cap-stage mesenchyme controls individual molar crown morphogenesis

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ABSTRACT Day 14 ICR mouse first lower (M_1) and upper molars (M^1) as well as heterotopic recombinations of M^1 epithelium/M_1 mesenchyme and M_1 epithelium/M^1 mesenchyme were cultured for 6, 8 and 10 days on semi-solid medium. Computer-assisted 3D reconstructions were performed to follow the *in vitro* development of these explants. *In vitro* culture of cap-stage molars allowed for the emergence of unequivocal morphological features distinctive for M^1 versus M_1 including the cusp pattern, cusp inclination and tooth specific chronology for odontoblast and ameloblast terminal differentiations. Both M_1 epithelium/M^1 mesenchyme and M^1 epithelium/M_1 mesenchyme recombinations developed according to the known developmental fate of the mesenchyme. Our data demonstrate that the cap-stage dental ecto-mesenchyme not only directs tooth class specific morphogenesis, but also individual molar crown features. Furthermore, the mesenchyme apparently also controls the typical mirror symmetry of right and left handed teeth.

KEY WORDS: *mouse molar, mesenchyme, heterotopic recombinations, crown morphogenesis, 3D reconstructions*

Introduction

The mouse first lower and upper molars are distinguished by specific morphological features (Gaunt, 1955, 1961). The first lower molar (M_1) crown is composed of seven cusps having a marked biserial antero-posterior arrangement: two series of three buccal and three lingual cusps essentially of a paired nature and one single posterior cusp. The first upper molar (M^1) demonstrates a marked triserial arrangement of eight cusps in both longitudinal and transverse directions. However, there is no lingual cusp in the posterior part of the crown (Gaunt, 1961). Furthermore, the initiations of terminal differentiation of odontoblasts followed by ameloblasts, about 24 h later, are not synchronous in both teeth. Respective terminal differentiations in the lower molar start about 24 h in advance of the upper molar (Moullec, 1978).

Mouse tooth germ development *in vitro* has received much attention (Hay, 1961; Kollar and Baird, 1969, 1970a,b; Ruch *et al.,* 1970; Fisher, 1971; Thesleff, 1976; Boukari and Ruch, 1981) and the morphodifferentiation of the M_1 *in vitro* has been investigated using 3D reconstructions (Fisher, 1971). This author demonstrated that the number, shape and arrangement of the cusps showed similarities to the *in vivo* situation. However, *in vitro* small teeth of abnormal spherical shape developed and the cusps were more angular with partial fusions. The *in vitro* growth and cell proliferation kinetics were also investigated by Ahmad and Ruch (1987) who demonstrated a significant lengthening of the cell cycle duration *in vitro*. Jowett and Ferguson (1991) suggested the existence of two phases of proliferation, the first being responsible for defining the tooth outline and cuspal pattern and the second for the increase in size to its final form. *In vitro*, this last phase appeared to be limited.

The respective roles of dental epithelium and dental mesenchyme in tooth morphogenesis have been evaluated by classical tissue recombinations (Kollar and Baird, 1969, 1970a,b). Heterotopic recombinations of cap-bell stage mouse incisor and molar mesenchyme and enamel organs demonstrated that the incisor mesenchyme directs incisor-like tooth development whilst molar mesenchyme recombined with incisor epithelium gives rise to "molariform" teeth bearing several cusps. These data were confirmed by Osman *et al.* (1977) using heterotopic interspecific rabbit/mouse dental tissue-recombinations.

However, these investigations did not address whether the dental mesenchyme controls the specific, individual, molar tooth type cusp pattern. To answer this question we have analysed by 3D reconstructions 1) the morphology of first lower and upper mouse molars cultured *in vitro* and 2) the cusp pattern of heterotopic recombinations of first lower/upper molar dental mesenchyme and

Abbreviations used in this paper: 3D, three dimensional; M_1, first lower molar; M^1, first upper molar; ep, epithelium; mes, mesenchyme.

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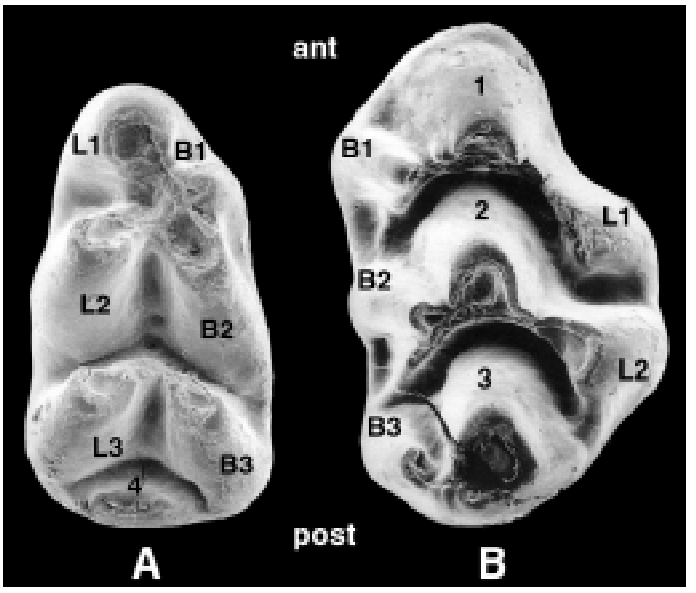

Fig. 1. Scanning electron micrographs of 23 days post-natal first right handed lower (A) and upper (B) mouse molars. *The cusps were named according to the terminology of Gaunt (1955, 1961).* **(A)** *A biserial arrangement of three buccal (B1, B2, B3) and three lingual cusps (L1, L2, L3) exists. Cusps L1, B1, B2 and L2 express a "trefoil" like pattern. Cusp 4 has a median and posterior localization. The crest connecting L1, B1, B2 and L2 has a lingual discontinuity.* **(B)** *A triserial arrangement of three buccal (B1, B2, B3), three sagittal (1, 2, 3) and two lingual (L1, L2) cusps exists. The buccal cusps are less prominent. The lingual cusps give rise to a bulge on the molar. The corresponding left handed teeth present a mirror symmetry. ant, anterior; post, posterior.*

first upper/lower molar dental epithelium cultured *in vitro*. The present data demonstrate the role of the dental mesenchyme in controlling individual molar crown morphogenesis.

Results

The physiological cusp patterns of M_1 and M^1 and the nomenclature of the cusps are indicated in Figure 1A and B according to Gaunt (1955, 1961). The crown table of the M_1 is broken up by a longitudinal cleft which separates the lingual and buccal ridges with each ridge showing a three fold subdivision due to an anterior and median transverse valley. All cusps slope in an anterior direction. In the M^1, the crown table is broken up by a pair of longitudinal and a pair of transverse valleys. All the cusps have a pronounced backward tilt.

M_1 in vitro

Twenty E-14 cap-staged M_1 were cultured for 6 (n=11), 8 (n=5) or 10 (n=4) days. Randomly selected teeth cultured for 6 and 8 days were analysed by 3D reconstructions.

After 6 days culture, seven cusps were visible (Fig. 2A): two anterior, two median and two posterior cusps. Cusp 4 appeared in the posterior part of the tooth. A curved nearly closed crest linked the four anterior and median cusps (Fig. 2A). The crown displayed crests rather than tubercles after six days of culture. The tooth was subdivided by a deep median transverse fissure into a larger anterior portion including four cusps (L1, B1, B2, L2) and a smaller posterior one with cusps L3, B3 and 4 (Fig. 2A,C). After 8 days culture, seven cusps existed (Fig. 3A). The single posterior cusp 4 was separated from the cusps in front (L3, B3) by a third posterior transverse cleft (Fig. 3D). Cusp L1, increased in size, took up a median position and fused partially with B1. The median transverse valley penetrated more deeply and the tooth was curved in shape (Fig. 3A,D).

After 6 days culture, the cusps had a very slight forward tilt (Fig. 2C). After 8 days in culture the cusps demonstrated a more vertical orientation (Fig. 3D).

After 8 days culture (Fig. 5A) functional odontoblasts were present in all the teeth (6/6). After 8 days culture, functional ameloblasts were observed in five out of six teeth. After 10 days culture, enamel was seen in the four teeth.

M^1 in vitro

Fourteen E-14 cap-staged M^1 were cultured for 6 (n=4), 8 (n=4) or 10 (n=6) days. Randomly selected teeth cultured for 6 and 8 days were analysed using 3D reconstructions.

After 6 days culture, the crown pattern demonstrated three median rounded cusps (1, 2, 3), three less developed buccal cusps (B1, B2, B3) and two lingual cusps (L1, L2) which nearly fused. L1-

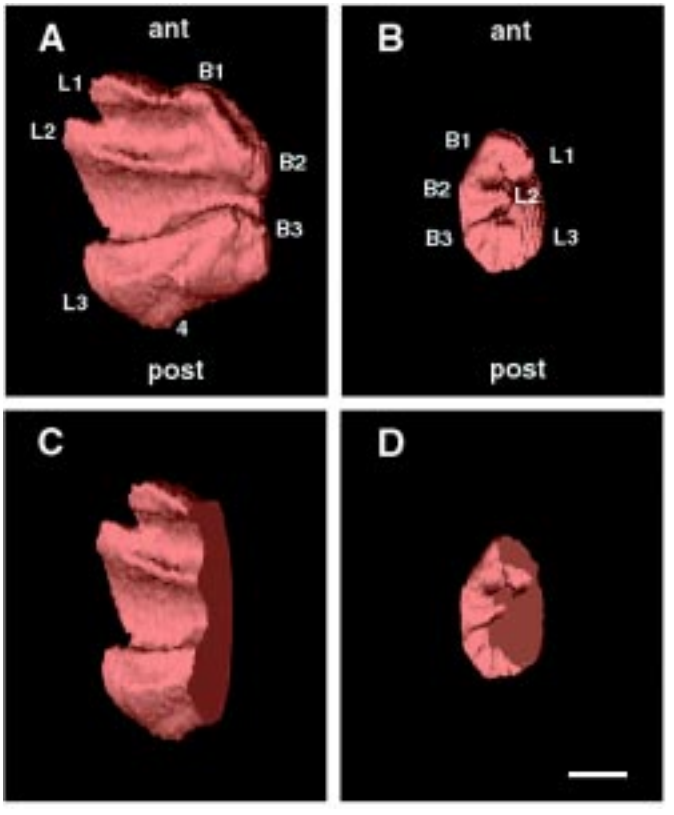

Fig. 2. Three-D reconstructions of dental papilla of a E-14 M_1 (A,C) and a M^1ep/M_1mes recombination (B,D) cultured respectively for 6 days. **(A)** *The physiological cusp pattern (seven cusps: L1, L2, L3, B1, B2, B3, 4) of a right handed M_1 is evident.* **(C)** *The computer assisted section of the same tooth (A) reveals a slight forward tilt to the cusps.* **(B)** *The recombinant led to the development of a small left handed M_1 with six identified cusps: L1, L2, L3, B1, B2, B3.* **(D)** *The computer assisted section reveals the rather vertical orientation of the cusps. ant, anterior; post, posterior. Bar, 100 μm.*

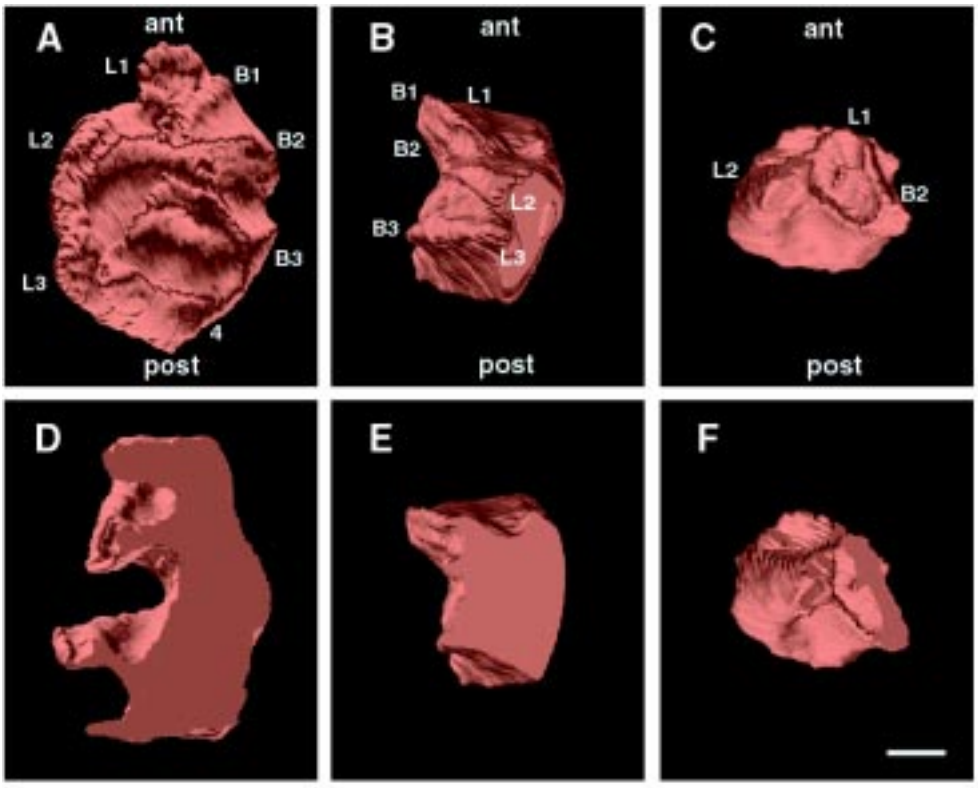

Fig. 3. Three-D reconstructions of the dental papilla of a E-14 M_1 (A,D) and of two M^1ep/M_1mes recombinations (B,C,E,F) after 8 days of culture. **(A)** *The physiological cusp pattern of a right handed M_1 exists: seven characteristic cusps (L1, L2, L3, B1, B2, B3, 4) have developed.* **(D)** *The computer assisted section of the tooth shows that these cusps have a rather vertical orientation.* **(B)** *This recombinant displays a typical cusp pattern of a left handed M_1: L1, L2, L3, B1, B2 and B3 are identified. Cusp 4 is missing.* **(E)** *The orientation of the cusps is more or less vertical.* **(C)** *This recombinant gave rise to an incomplete M_1: the anterior cusps (L1, L2, B1, B2) developed with a characteristic trefoil-like arrangement.* **(F)** *These cusps have a vertical orientation. ant, anterior; post, posterior. Bar, 100 μm.*

L2 were separated from cusp 2 by a deep cleft (Fig. 4A). The crown table was broken up by a pair of transverse valleys (Fig. 4A,D).

After 8 days culture, the outline of the tooth was more mature (Fig. 4B). The crests penetrated more deeply and the cusps increased in size (Fig. 4B,E).

After 6 days culture, cusps 1, L1 and L2 showed a backward tilt whilst cusps 2 and 3 had a more vertical orientation (Fig. 4D). Cusps inclination increased during cultivation: after 8 days all cusps showed a backward tilt except for cusp 3 which maintained a more vertical orientation (Fig. 4E).

Furthermore, after 8 days culture, functional odontoblasts were present in all teeth (6/6) however no functional ameloblasts were observed (Fig. 5C). After 10 days, secretion of enamel was observed in seven teeth out of twelve.

Heterotopic tissues recombinations

M^1 epithelium/ M_1 mesenchyme (M^1ep/M_1mes)

Twenty four M^1ep/M_1mes recombinations were performed and cultured for 6 (n=3) or 8 (n=21) days. For 3D reconstructions, one 6 day and six 8 day specimens were selected according to histological criteria (i.e., complete serial sections).

After 6 days in culture (Fig. 2B) the recombinant was very small but demonstrated a characteristic cusp pattern: the tooth was subdivided into a larger mesial portion with four cusps (L1, B1, B2, L2) and a smaller distal one with two cusps (L3, B3). After 8 days culture, one tooth demonstrated the same cusp pattern (Fig. 3B) as observed after 6 days of culture (Fig. 2A). The crown displayed crests rather than tubercles. The crown table was broken up by a median transverse valley and a posterior one. Another incomplete tooth demonstrated the presence of cusps L1, L2 and B2 with a M_1 specific trefoil arrangement (Fig. 3C).

All anterior cusps had a slight forward tilt and median and posterior cusps had a more vertical orientation (Figs. 2D and 3E,F).

Furthermore, after 6 days culture, predentin was present in one out of three recombinants. After 8 days, predentin was observed in all sixteen recombinants (Fig. 5B). After 8 days, enamel secretion was observed in four out of sixteen recombinants.

M_1 epithelium/ M^1 mesenchyme (M_1ep/M^1mes)

Twenty four M_1ep/M^1mes recombinations were performed and cultured for 6 (n=4) or 8 (n=20) days. For 3D reconstructions one 6 day and four 8 day specimens were selected.

After 6 days culture, three buccal (B1, B2, B3), three sagittal rounded cusps 1, 2, 3, and two fused lingual cusps L1, L2 were identified (Fig. 4C). The crown table was broken up by a pair of transverse valleys. Cusps L1-L2 were separated by a fissure from the neighbouring cusp 2.

All cusps had a backward tilt except for cusp B3 which had a more vertical orientation (Fig. 4F).

Furthermore, after 6 days of culture, no functional odontoblasts were present in two out of three recombinants (Fig. 5D). After 8 days, predentin was observed in nine out of twelve recombinants. Enamel was never seen.

Discussion

The *in vitro* culture of intact first lower and upper cap-stage mouse molars permits reproducible development of tooth specific cusp patterns. The culture techniques may affect tooth morphogenesis in diverse ways as previously stressed by Boukari and Ruch (1981) and Ahmad and Ruch (1987, 1988 and references therein). Agar solidified medium was found to provide a permissive

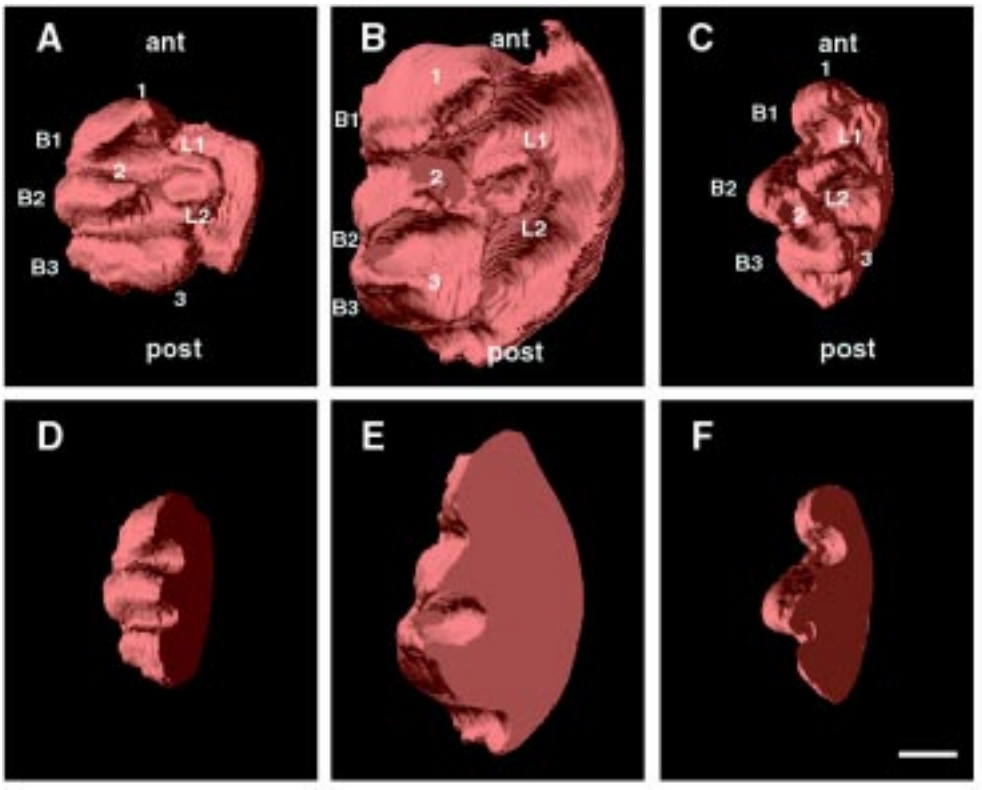

Fig. 4. Three-D reconstructions of the dental papilla of a E-14 M^1 cultured for 6 (A,D) or 8 (B,E) days and of a M_1ep/M^1mes recombination (C,F) cultured for 6 days. **(A)** *The physiological cusp pattern of a right handed M^1 is expressed. Eight cusps develop: B1, B2, B3 on the buccal side, prominent L1 and L2 on the lingual side and sagittal cusps 1, 2 and 3.* **(B)** *After 8 days a complete M^1 cusp pattern also exists. The particular orientation of the 3D reconstruction allows for the identification of cusps 1, 2, 3, L1, L2, B1, B3.* **(D,E)** *The respective computer assisted sections illustrate the generally backward tilt of the cusps.* **(C)** *The recombinant demonstrates a complete typical, right, handed M^1 cusp pattern.* **(F)** *These cusps display a backward tilt. ant, anterior; post, posterior. Bar, 100 μm.*

substrate for the unrolling of developmental morphogenetic processes. However the well known constraints of organotypic culture lead to reduced growth and partial fusion of some cusps may also occur. Nevertheless, our data demonstrated that after *in vitro* culture of cap-stage molars unequivocal morphological features of M^1 versus M_1 were achieved including the cusp pattern, cusp inclination and tooth specific chronology of odontoblast and ameloblast terminal differentiation, i.e., acceleration of cytodifferentiations in the M_1.

Both M_1ep/M^1mes and M^1ep/M_1mes recombinations develop according to the developmental fate of the mesenchyme. In comparison with cultured intact tooth germs, even if all the anticipated cusps did not develop, the pattern of the existing cusps, their inclination and the chronology of terminal differentiation confer M^1 versus M_1 specificities to the explants. Acceleration or delay of terminal cytodifferentiation occur according to the source of dental mesenchyme. Sometimes the recombined teeth were incomplete resulting most probably from recombination with incomplete epithelium.

The data presented in this paper demonstrate that the cap-stage dental ecto-mesenchyme not only directs tooth class specific morphogenesis but also individual molar crown features. Furthermore, the recombinants demonstrated a left or right handedness to the cusp pattern. The mesenchyme apparently also controls the typical mirror symmetry of right and left handed teeth.

How the dental ecto-mesenchyme controls the growth and folding of the epithelial-mesenchymal junction in a tooth specific manner is not yet clear although many cellular and molecular data have led to different hypotheses (Jernvall *et al.*, 1994; Ruch, 1995; Weil *et al.*, 1995; Lesot *et al.*, 1996; Slavkin and Diekwisch, 1996; Tabata *et al.,* 1996; Thesleff *et al.*, 1996; Aberg *et al.*, 1997; Maas and Bei, 1997; Peterkova *et al.*, 1997; Stock *et al.*, 1997; Thesleff and Jernvall, 1997; Kettunen and Thesleff, 1998; Tucker *et al.*, 1998). Differential mitotic activities, apoptoses, cell-cell and cell-matrix interactions are involved (Lesot, 1981; Fausser *et al.*, 1998; Lesot *et al.*, 1998; Yoshiba *et al.*, 1998; Coin *et al.*, 1999 in this issue). To understand the role of epithelial/mesenchymal interactions in morphogenetic processes at the molecular level, further investigations are required.

Materials and Methods

Tissues

Mouse embryos were obtained by mating ICR mice. The morning of the appearance of the vaginal plug was designated as day 0 of embryonic development. First lower (M_1) and upper (M^1) molars were dissected on day 14 of gestation (E-14).

Heterotopic tissues recombinations

E-14 M^1 and M_1 enamel organ and mesenchyme were enzymatically dissociated with a mixture of 1% trypsin (Gibco) and 0.9 u/mg of dispase (Gibco) in PBS at 4°C for 30 min. Dental epithelium and mesenchyme were isolated. We reassociated respectively lower dental epithelium with upper dental mesenchyme and upper dental epithelium with lower dental mesenchyme. The tissue components were positioned so that the epithelium made close contact with the clearly identifiable dental papilla. The recombinants were cultured for 6 or 8 days.

Organ culture

The intact teeth and the recombinations were cultured for 6, 8 or 10 days on 2 ml of semi-solid medium per Petri dish (Nunc, Roskilde, Denmark; 35x10 mm). The medium consisted of BGJ-B (Gibco, Fitton Jakson modified) supplemented with ascorbic acid (0.18 mg/ml, Merck), L-Glutamin (2 mM, Seromed), fetal calf serum (20%, Boehringer Bioproducts), kana-

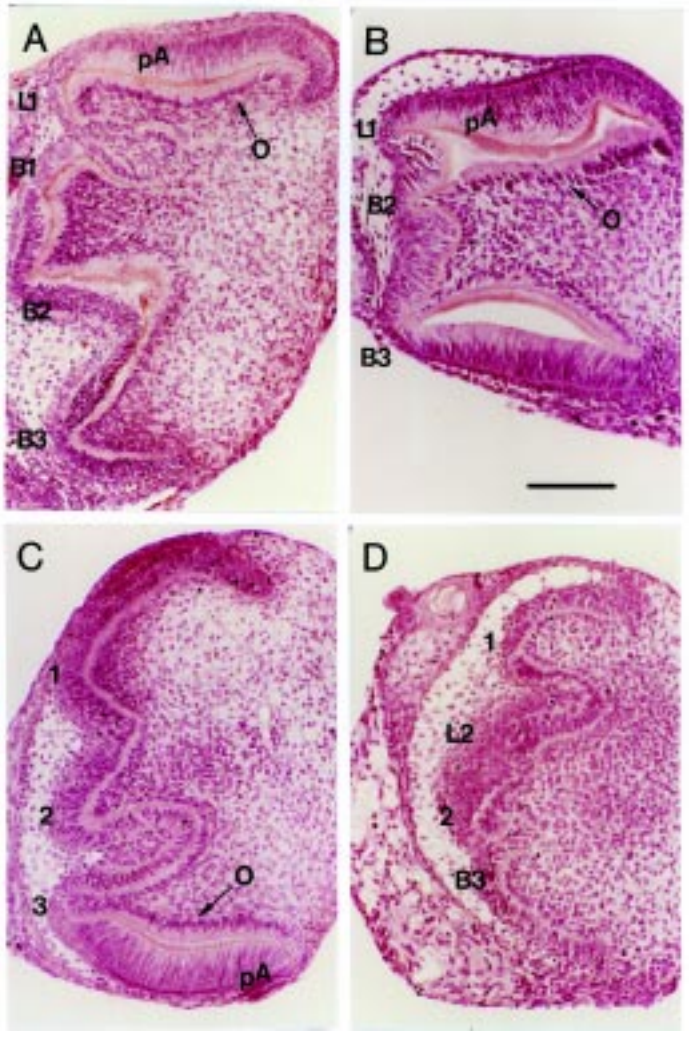

Fig. 5. Histological sections documenting intact molars M_1 (A), M^1 (C) and heterotopic tissue recombinations M^1ep/M_1mes (B) and M_1ep/M^1mes (D) corresponding respectively to the 3D reconstructions on Figure 3D, 4E and 3E, 4F. **(A)** *This sagittal section reveals cusps L1, B1, B2 and B3. Functional odontoblasts (O) and polarizing ameloblasts (pA) exist in cusps L1, B1 and B2.* **(C)** *This sagittal section shows cusps 1, 2 and 3. Functional odontoblasts (O) and polarizing ameloblasts (pA) are seen in cusp 3.* **(B)** *The oblique section reveals functional odontoblasts (O) and polarizing ameloblasts (pA) in cusps L1, B2 and B3.* **(D)**. *This rather sagittal section shows cusps 1, L2, 2 and B3. No functional odontoblasts are seen. The chronology of cytodifferentiation cannot be assessed from single sections. Bar, 100 μm.*

mycin (0.1 mg/ml, Gibco) and Difco agar (0.5%) The teeth were incubated and grown at 37°C in a humidified atmosphere of 5% CO_2 in air and the medium was changed every two days.

Histology

Teeth and recombinants were fixed in Bouin-Hollande fluid and embedded in paraffin wax. Five μm serial sections were stained with Mallory's stain.

3D reconstructions

Drawings of the contours of intact and recombined teeth were made at 5 μm intervals from serial histological sections using a Zeiss microscope equipped with a drawing chamber. The digitalization of the serial drawings was achieved using a Hamamatsu C2400 camera connected to a digital imaging system. The digitalization of the serial drawings and correlation of successive images (Olivo *et al.,* 1993) have been previously described (Lesot *et al.,* 1996). Software packages allowing image acquisition and treatment were developed and adapted to this work. Three-dimensional images were generated using a volume rendering program (Sun Voxel, Sun Microsystems).

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