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Original Article

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

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ABSTRACT First lower E-14 and E-16 mouse molars and E-13 lower incisors were cultured *in vitro* and either sequentially or continuously labelled with BrdU (5-bromo-2'-deoxyuridine). The behaviour of the non-cycling inner dental epithelial cells emerging from the enamel knot area of the molars was analysed by 3D (three dimensional) reconstructions of serial sections. These cells, as well as slow cycling cells underwent a coordinated temporo-spatial patterning leading to their patchy segregation at the tips of the forming cusps. In incisors (*in vitro* and *in vivo*), non-cycling cells were also present in the inner dental epithelium of the enamel knot area. However, these cells were not redistributed during incisor morphogenesis. These non-dividing inner dental epithelium cells of the enamel knot area which are either redistributed or not according to the tooth type specific morphogenesis might represent the organizers of morphogenetic units (OMU), the cusps.

KEY WORDS: *mouse molar, incisor, enamel knot, inner dental epithelium, cell proliferation kinetics, enamel free area, cuspidogenesis*

Introduction

Mammalian teeth develop from oral ectoderm and neural crest derived ecto-mesenchyme. The progressive and continuous steps of odontogenesis have been classically subdivided into several successive stages: dental lamina, bud, cap and bell-stages. All these developmental processes implying cell migration, cell proliferation, apoptosis, histo-morphogenesis and cytodifferentiation are controlled by homotypical and heterotypical cell-cell and cell-matrix interactions. There has been rapid progress in the identification of signalling networks regulating dental development (for reviews see Ruch, 1995; Maas and Bei, 1997; Stock *et al.*, 1997; Thesleff and Sharpe, 1997; Dassule and McMahon, 1998).

The histogenesis of the enamel organ leading to the appearance of the inner and outer dental epithelium, the stellate reticulum and the stratum intermedium is initiated during the transition from bud to cap stage. The mouse inner dental epithelium (ide) contains progenitor cells of functional ameloblasts and the ameloblasts of enamel free areas (efa), regions of the dentin without an enamel cap at the cusp tip in rodent molars (Addison and Appleton, 1921). The histogenesis of the ide is accompanied by the transitory presence of the enamel knot (ek), a dynamic histological structure composed of apoptotic cells and living replacement cells. (see Lesot *et al.*, 1996, 1999 in this issue; Jernvall *et al.*, 1998). Tooth specific crown morphogenesis, giving rise to one or several cusps, occurs during the bell-stage (for morphological data see Lesot *et al.*, 1996).

The cell proliferation kinetics of first lower mouse molar and incisor have been partially investigated during *in vivo* and *in vitro* mouse odontogenesis (Ahmad and Ruch, 1987, 1988). *In vivo*, the average duration of the cell cycle (Tc) established by the percentage labelled mitoses technique (plm), is 13.5 h for the preameloblasts. *In vitro*, a significant lengthening of Tc exists and the average duration of Tc for preameloblasts is around 19 h. However, plm values tend to be influenced by the cells that are quickest cycling and the presence of slower cycling cells cannot be excluded. In fact, long pulse labelling (6 h) with 3H thymidine or ^{3}H BrdU of cultured mouse molars demonstrated the presence of unlabelled preameloblasts (Ruch *et al.*, 1978).

To get more information on the cell proliferation kinetics of the ide cells, we have sequentially labelled with BrdU (8 h pulses), E-14 and

Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; ek, enamel knot; efa, enamel free area; ide, inner dental epithelium; plm, percentage labelled mitoses; 3D, three dimensional; SHH, Sonic hedgehog; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; OMU, organizers of morphogenetic units.

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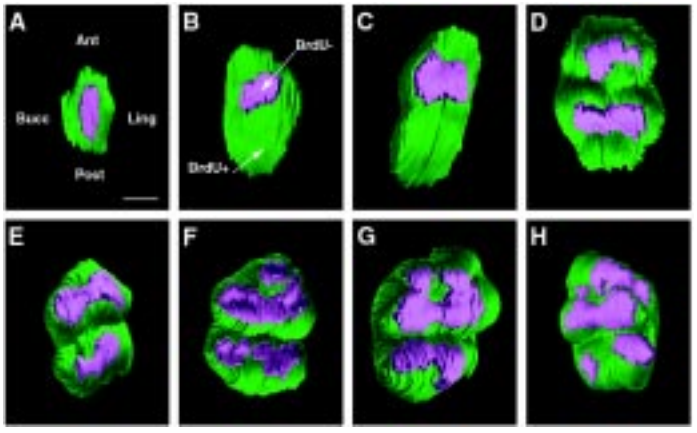

Fig. 1. Computer assisted 3-D reconstructions of E-14 first left and right lower molars, pulse labelled *in vitro* with BrdU for 8 h after 2 (A), 12 (B), 24 (C), 36 (D), 48 (E), 60 (F), 72 (G) or 84 h (H) of culture. *For each 12 h interval, the left and right handed lower molars from the same embryo were compared. The 5-bromo-2'-deoxyuridine negative (BrdU-) groups of inner dental epithelial cells are represented in pink. The 5-bromo-2'-deoxyuridine positive (BrdU+) domains are stained in green. The initial negative area corresponded partially to the ek* **(A)**. *Twelve hours* **(B)** *and 24 h* **(C)** *later, a negative area is present in the anterior part of the tooth. After 36 h* **(D)**, *two distinct anterior and posterior negative areas existed. Later on, progressively, negative patches corresponded to the tips of forming cusps* **(E,F,G** *and* **H)**. *ant, anterior; post, posterior; bucc, buccal; ling, lingual. Bar, 100 μm.*

E-16 lower mouse molars cultured i*n vitro* and have followed the distribution of non-labelled cells by means of computer assisted 3D (three dimensional) reconstructions. Continuous labelling with BrdU was also performed as well as pulse labelling *in vivo*. These investigations were completed by pulse labelling of lower incisors *in vivo* and *in vitro* and continuous labelling *in vitro*. Our results demonstrated the presence of both non-cycling and slowly cycling preameloblasts which originated from the ek area. Their temporo-spatial behaviour correlated with the tooth class specific cusp pattern: the initial ensemble of non-cycling cells remained coherent in the incisor whereas it was subdivided in the molar, in as many independent groups of cells as cusps developed.

Results

Sequential 8 h pulse labelling of E-14 and E-16 molars with BrdU

E-14 molars were incubated *in vitro* with BrdU at 12 h intervals from 2 h to 84 h of culture. The temporo-spatial distribution of labelled versus unlabelled cells of the ide (preameloblasts) is documented in Figures 1 and 2.

After the first pulse, a group of non-dividing cells was located in the centre of the ide with an antero-posterior orientation (Fig. 1A) and corresponded to the ek. Numerous apoptotic bodies were observed on corresponding histological sections. Twelve hours later, the group of non-dividing cells assumed a more anterior and transversal localisation (Fig. 1B) and fewer apoptotic bodies were observed on corresponding histological sections. After 24 h the E-14 molars elongated and non-dividing cells, not showing apoptosis, were located in the anterior part of the tooth assuming a bilobular distribution (Fig. 1C). Twelve hours later, in the contralateral tooth of the same embryo, two groups of non-dividing cells were located respectively in the anterior and posterior parts of the tooth (Fig. 1D). During the subsequent pulses anterior and posterior non-dividing cells progressively subdivided into distinct groups corresponding to the forming cusps (Fig. 1E,F,G,H, and 5A,B,C).

After 2 h in culture, E-16 molars were also pulsed with BrdU at 12 h intervals. After the first pulse four groups of non-cycling cells were present (Fig. 2A). This pattern partially reflected the situation observed for E-14 teeth after 48 h of culture (compare Fig. 1E with Fig. 2A). After the next two pulses, again left and right E-16 molars from the same embryo were compared. The frequency of non-dividing cells increased both in the anterior and posterior parts of the teeth (Fig. 2B and C). This pattern was comparable in some respects with the data provided by E-14 teeth pulsed after 60 and 72 h *in vitro* (compare Fig. 2B and C with Fig. 1F and G). The subsequent pulses (after 36 to 84 h *in vitro*) revealed a progressive individualisation of the non-cycling cells into 7 groups, each group corresponding to one of the 7 forming cusps (Fig. 2D to 2H). In fact, these non-labelled cells were restricted to the tip of the cusps at the level of invaginations of the ide corresponding to the future enamel free areas (Figs. 4B,C and 5H,I).

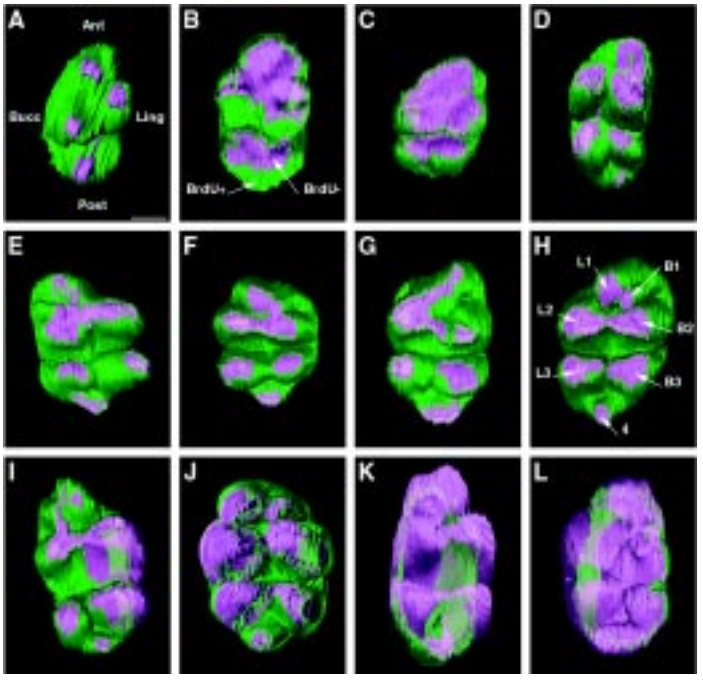

Fig. 2. Computer assisted 3-D reconstructions of E-16 first left and right molars, pulse labelled *in vitro* with BrdU for 8h, after 2 (A), 12 (B), 24 (C), 36 (D), 48 (E), 60 (F), 72 (G), 84 (H), 96 (I), 108 (J), 120 (K) and 132 (L) of culture. *For each 12 h interval, the left and right handed lower molars from the same embryo were compared. The 5-bromo-2'-deoxyuridine negative (BrdU-) domains of the inner dental epithelium are stained in pink, the 5-bromo-2'-deoxyuridine positive (BrdU+) areas are stained in green. Initially, four distinct negative domains existed* **(A)**. *After 12 h* **(B)** *and 24 h* **(C)**, *two distinct large anterior and posterior negative domains were observed. Afterwards* **(D,E,F,G** *and* **H)** *distinct negative areas corresponded to the tips of the forming cusps. The nomenclature of the cusps (Fig. H) is that of Gaunt (1955). Later on, after 36 h, post-mitotic ameloblasts emerged sequentially* **(I,J,K** *and* **L)**. *The terminal differentiation of ameloblasts was initiated in cusps L2, L3 and then extended to all of the other cusps. ant, anterior; post, posterior; bucc, buccal; ling, lingual. Bar, 100 μm.*

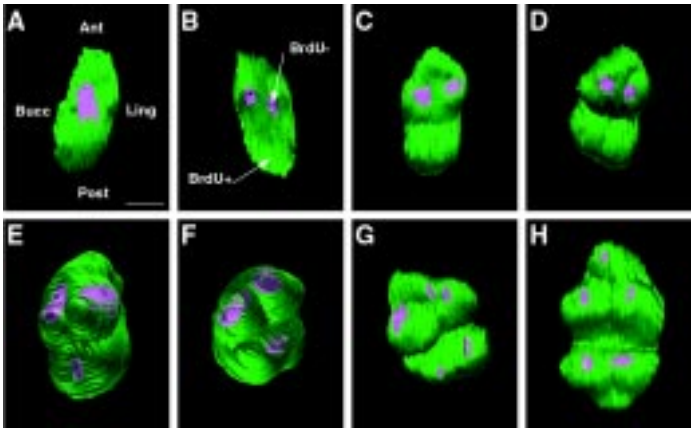

Fig. 3. Three-D representation of E-14 (A,B,C and D) and E-16 (E,F,G and H) molars cultured *in vitro* in continuous presence of BrdU for 12 (A,E), 24 (B,F), 48 (C,G) or 72 h (D,H). *The 12-24 h and 48-72 h labelling used left and right handed molars from the same embryo. The 5-bromo-2'-deoxyuridine negative (BrdU-) domains of the inner dental epithelium are stained in pink, the 5-bromo-2'-deoxyuridine positive (BrdU+) areas are stained in green.* **(A)** *For E-14 molars the initial 12 h labelling revealed the presence of one negative area corresponding to the ek (compare with Fig. 1A). After 24 h these negative cells segregated into two distinct domains* **(B)**. **(C,D)** *Later on no further alterations were observed. The development of the teeth was delayed (compare with Fig. 1). For E-16 molars the initial labelling* **(E)** *revealed two negative anterior domains (compare with D). Subsequently these negative cells segregated into 3* **(F)**, *then 5* **(G,H)** *distinct negative areas corresponding to the tips of forming cusps. ant, anterior; post, posterior; bucc, buccal; ling, lingual. Bar, 100 μm.*

Teeth pulsed with BrdU after 96 to 132 h *in vitro*, showed the sequential emergence of post-mitotic ameloblasts which overtly differentiated (Fig. 2I,J,K,L). This behaviour started on the lingual side (cusps L2, L3) and then extended to the lateral cusps (B2, B3) and finally to the cusps L1, B1 and 4.

Continuous labelling of E-14 and E-16 molars for 12, 24, 48 or 72 h with BrdU

The temporo-spatial distribution of non-cycling cells is illustrated in Figure 3. Continuous labelling of E-14 molars resulted in growth limitation and inhibited cusp formation. The localisation of non-labelled cells after 12 h incubation with BrdU was similar to that observed after 8 h labelling (compare Fig. 1A and Fig. 3A). After longer continuous labelling (24, 48 and 72 h) the initial non-cycling cells segregated into 2 distinct subpopulations corresponding to the 2 forming cusps (Fig. 3B,C and D).

Continuous labelling of E-16 teeth demonstrated after 12 h the presence of three groups of non-cycling cells (Fig. 3E, compare with Fig. 2A). During longer (24, 48 and 72 h) continuous labelling the initial non-cycling cells segregated into five distinct subpopulations corresponding to five individual cusps (Fig. 3F,G and H). Virtual sections in the reconstructions (Fig. 4B and C) as well as histological sections (Fig. 5G) indicated that some of these cells corresponded to the invaginating zone of the ide which will lead to the enamel free area.

BrdU labelling of E-13 in vitro cultured incisors

Sequential 8 h pulse labelling at 12 h intervals, as well as continuous labelling, followed by careful analysis of the serial sections, revealed the presence of one coherent group of non-cycling cells at the tip of the teeth (Fig. 6A,B). E-13 incisors, pulse labelled after 48 h demonstrated the presence of some BrdU positive cells inside the formerly negative area (Fig. 6C).

BrdU labelling in vivo

In order to mimic the 8 h pulse labelling *in vitro*, BrdU was administrated to E-13, -15 and -17 pregnant mice through three intraperitoneal injections at 2 h intervals and the embryos were removed 4 h after the last injection.

The distribution pattern of non-cycling preameloblasts in E-17 molar is illustrated in Figure 5A. The comparison with the *in vitro* labelling (Fig. 2E,F or G) revealed a very similar pattern.

In E-13 incisors, a single group of non cycling cells was located at the tip of the teeth (Fig. 6E). In E-15 incisors, some BrdU positive cells were seen inside the previously completely negative area (Fig. 6F).

Complementary histological observation

The careful analysis of the serial histological sections stained with the specific anti-BrdU antibody demonstrated the presence of a few cycling ide cells localised within the globally BrdU negative areas of the molar (Fig. 5D,E,F). Such cells were not observed in the initially BrdU negative ide compartment of the incisor.

Discussion

The pyrimidine analogue, BrdU, is incorporated as bromouracil in place of thymine in replicating DNA. The effects of this substitution have been analysed in a number of cells, tissues and organs

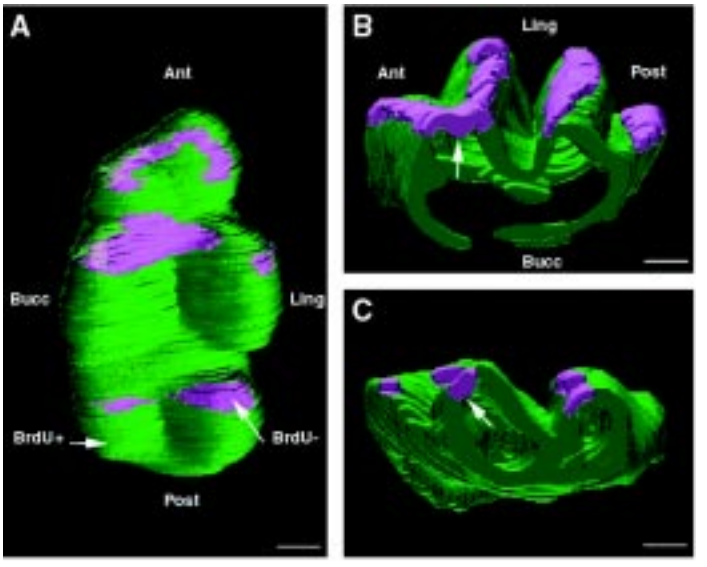

Fig. 4. Computer assisted 3D reconstruction of a E-17 lower mouse molar labelled *in vivo* with BrdU (A) and virtual computer assisted sagittal sections of E-16 molars (B and C) corresponding respectively to the teeth of Figures 2G and 3H. (A) *The in vivo incorporation of BrdU revealed the existence of negative areas (BrdU-) of the inner dental epithelium corresponding to the tips of forming cusps (compare with Fig. 2H) Bar, 100 μm.* **(B,C)** *The unlabelled cells corresponded to the invaginations of the ide preceding appearance of the enamel free areas (arrows). ant, anterior; post, posterior; bucc, buccal; ling, lingual; BrdU, 5-bromo-2'-deoxyuridine. Bar, 80 μm.*

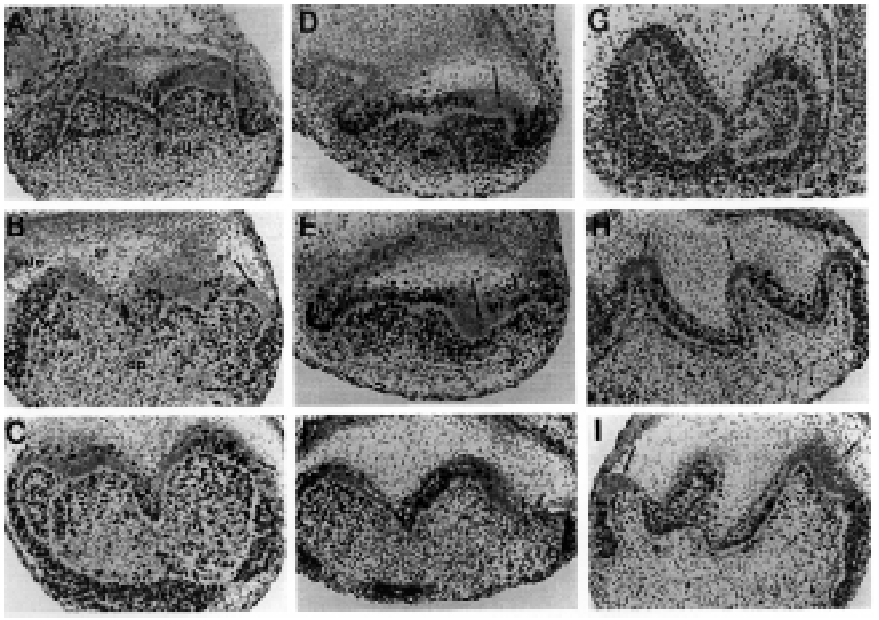

Fig. 5. Histological sections of E-14 (A,B,C,D,E) and E-16 (F,G,H,I) first molars, either pulse labelled *in vitro* with BrdU for 8 h after 12 (E,F), 48 (A), 60 (B), 72 h (C,H,I) or cultured *in vitro* in continuous presence of BrdU for 12 (D) or 72 h(G). (A,B,C) *Distinct 5-bromo-2'-deoxyuridine negative (BrdU-) and 5-bromo-2'-deoxyuridine positive (BrdU+) zones were observed in the inner dental epithelium (ide).* **(D,E,F)** *For E-14 molars, the initial continuous labelling stage* **(D)** *as well as the initial pulse labelling stage* **(E)** *revealed the presence of isolated cycling cells entrapped within the BrdU negative areas (arrows). The same observation was made for E-16 molars pulse labelled for 8 h after 12 h of culture* **(F). (G,H,I)** *For E-16 molars cultivated in vitro in the continuous presence of BrdU for 72 h* **(G)**, *as well as for molars pulse labelled for 8 h after 72 h of culture* **(H,I)**, *BrdU negative zones were found at the tip of the cusps (arrows). In each case, these zones corresponded to the invagination of the ide preceding appearance of the enamel free areas (compare Fig. 5G,H,I with Fig. 4B,C). dp, dental papilla; ode, outer dental epithelium; si, stratum intermedium; sr, stellate reticulum. Bar, 70 μm.*

in vitro and have been found to block terminal differentiation without alteration in cell division or basic cell function (Wilt and Anderson, 1972). However *in vivo*, BrdU has been shown to cause neural tube defects, dwarfism and polydactily (see Ruch *et al.*, 1978; Bannigan and Langman, 1979; and references therein). Bannigan and Langman also demonstrated that BrdU (500 mg/Kg) administrated to pregnant mice increased the duration of the S-phase and mitosis of embryonic neuroepithelial cells.

In this study, we have performed organotypic cultures of first mouse molars and incisors and immunohistochemical analysis with BrdU labelling in order to determine the temporo-spatial distribution of cycling and non-cycling cells of the ide.

The isolated teeth incubated *in vitro* for 8 h in the presence of 3 mg/ml medium of BrdU and immediately fixed after the pulses as well as the embryonic teeth removed 4 h after 3 intraperitoneal injections (at 2 h intervals) of 6 mg/100 gr of BrdU, demonstrated no obvious histological alterations.

The preameloblasts divide asynchronously and the average duration of the cell cycle (Tc) and of DNA replication (Ts) is 19 h and 10 h respectively (Ahmad and Ruch, 1988). After 8 h pulse labelling with BrdU, about 95% of the faster cycling preameloblasts should be labelled and 100% after 12 h. In these conditions, unlabelled cells correspond to a variable fraction on the basis of the Tc of slower cycling cells and of all of the non-cycling cells whose existence should be revealed by continuous labelling. The distribution patterns of BrdU negative cells after pulse labelling are similar after both *in vivo* and *in vitro* BrdU administration and together with the results of continuous labelling strongly suggest 1) the presence

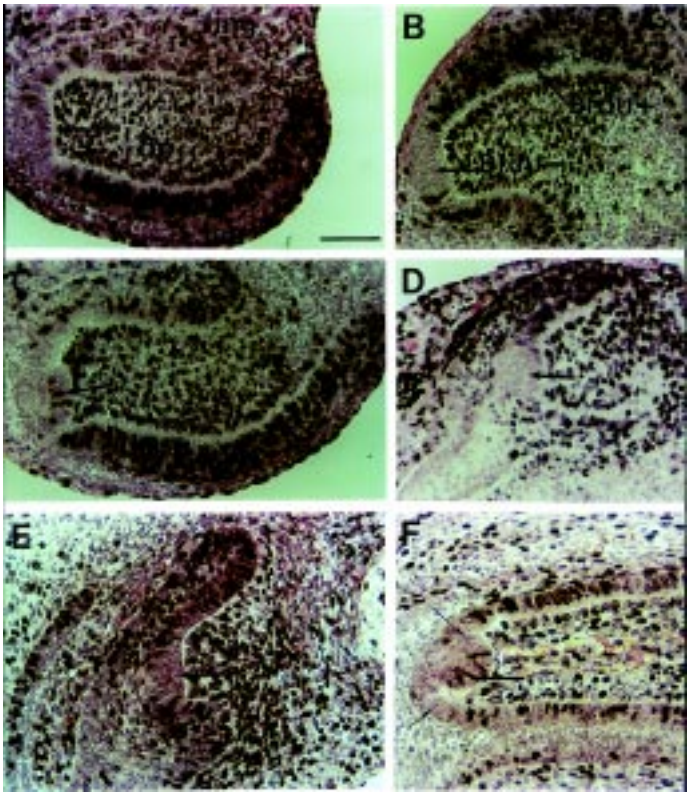

Fig. 6. Histological sections of E-13 incisors cultured *in vitro* in presence of BrdU (A,B,C,D) and of E-13 (E) and E-15 (F) *in vivo* labelled incisors. (A,B,C) *Eight hour pulse labelling respectively after 24, 36 and 48 h. One distinct 5-bromo-2'-deoxyuridine negative (BrdU-) zone of the inner dental epithelium existed at the tip of the incisors (arrow). Note that the lingual part of the inner dental epithelium (ide) was less labelled when compared to the labial one.* **C** *documents the presence of some BrdU positive cells in the future enamel free area (arrow).* **(D)** *Incisor cultured for 48 h in continuous presence of BrdU. This slightly more lateral section illustrates the apical BrdU negative zone (arrow).* **(E)** *In vivo labelling of E-13 incisor revealed an apical BrdU negative zone (arrow).* **(F)** *In E-15 in vivo labelled incisor some positive cells existed in the developing enamel free area (arrow). Negative cells circumscribe this invaginating zone (small arrows). lab, labial; ling, lingual; dp, dental papilla. Bar, 70 μm.*

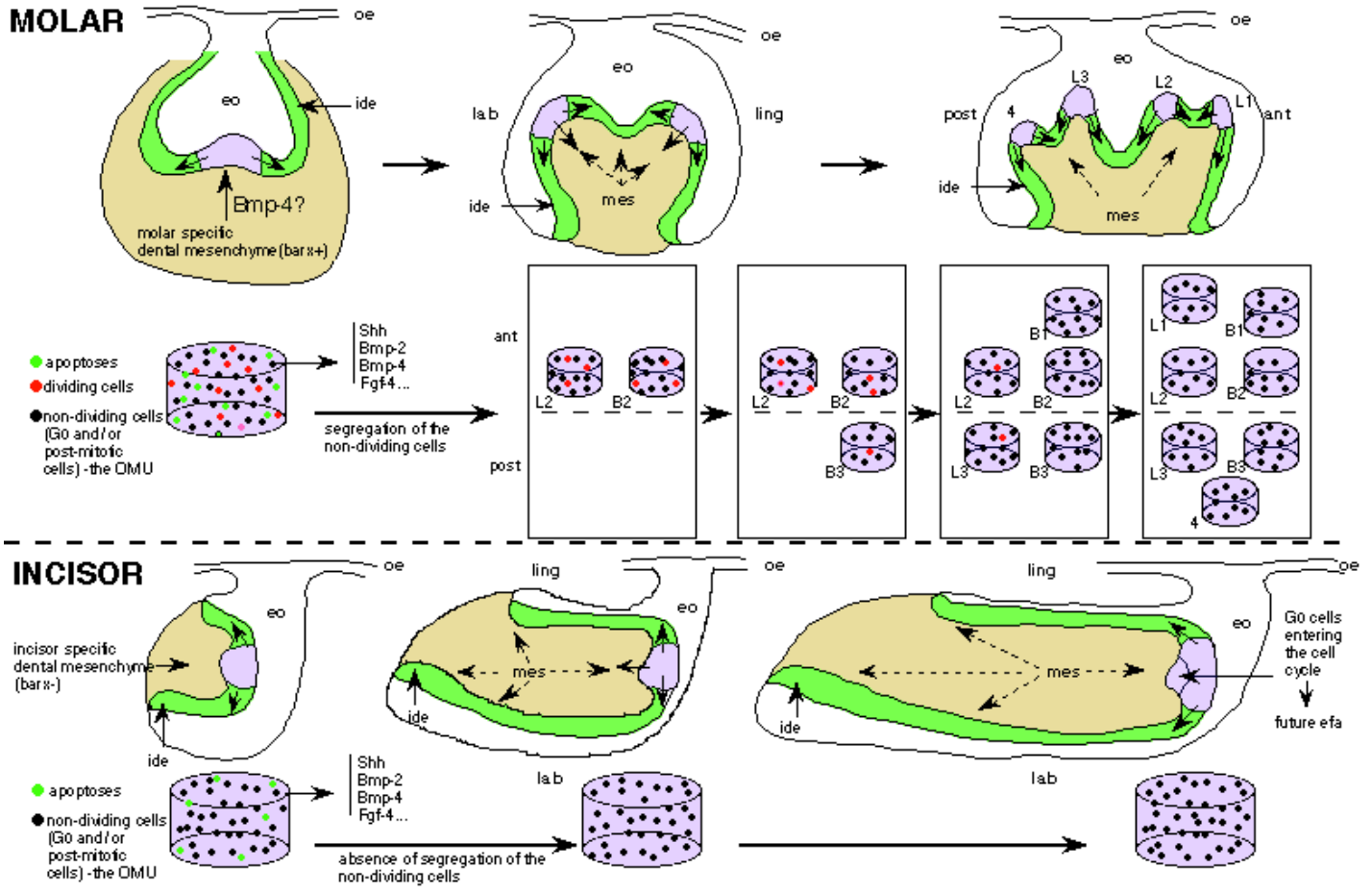

Fig. 7. The organizers of morphogenetic units (OMU): a working hypothesis. *Specific molar or incisor bud-cap staged mesenchyme (Barx-1 positive or negative; Tissier-seta et al., 1995) control individual tooth morphogenesis through induction of a tooth specific assembling of non-cycling inner dental epithelial (ide) cells (BMP-4 might be involved -arrow), (Jernvall et al., 1998). In the molar intercalated cycling cells exist. The non dividing cells (G0 and/or post mitotic cells), the OMU, express signaling molecules including Shh, Bmp-2 and -4, Fgf-4 (arrows), (Keränen et al., 1998). In the molar the OMU sequentially segregate into distinct aggregates each one organizing one cusp. Seven cusps (L1, L2, L3, B1, B2, B3 and 4) develop in the lower first mouse molar. The possible filiation of OMU rather than the chronology of cusp formation is depicted. In the incisor the OMU do not segregate and one cusp develop. G0 cells give rise to the ameloblasts of the enamel free areas (efa). The initial prepattern of the OMU and intercalated dividing cells is refined through apoptosis to a tooth specific pattern. During the bell stage the dental mesenchyme plays a more permissive albeit necessary role (broken arrows). oe, oral epithelium; eo, enamel organ; ling, lingual; lab, labial; ant, anterior; post, posterior.*

of fast, slow and non-cycling cells in the molar and incisor, 2) the progressive cusp specific patterning of the non-cycling cells in the molar and 3) the absence of segregation of non-cycling cells in the incisor.

The initial pulse labelling of E-14 molars revealed one negative cell population corresponding roughly to the ek. This observation agrees with data from short labelling experiments (Jernvall *et al.*, 1998). After subsequent sequential pulse labelling of E-14 and E-16 molars, the negative cells appear to be localised at the tip of the progressively forming cusps. Overlapping of the distribution pattern of negative cells of E-14 teeth cultured for two days or more before pulse and E-16 molars cultured for short periods, was seen even if the 7th cusp of E-14 molars cultured for 92 h had not yet developed. It is well known that all the embryos of one litter are not at the same developmental stage but the sequential comparison at 12 h intervals of first left and right lower molars from the same embryo allowed for comparison of changes occurring during these intervals and demonstrated fast dramatic modifications. These were particularly obvious for E-14 molars during 24-36 h *in vitro* and for E-16 molars during the initial 36 h of culture. The *in vivo* labelling demonstrated that the presence of BrdU negative cells at the tips of the cusp was not a culture artefact.

Continuous exposure of cultured teeth to 3 mg/ml of culture medium delayed the cusp formation. This effect was more pronounced for E-14 molars than for E-16 teeth and has been observed previously (Ruch *et al.*, 1978). Our E-14 data demonstrated the existence of living, non-dividing cells in the ek and their segregation into two distinct populations corresponding to the anterior cusps. For E-16 molars, the continuous labelling revealed the progressive segregation of three groups of non-dividing cells into five distinct groups corresponding to the five developing cusps. Globally, we have demonstrated the presence of non-cycling cells in the ek area of the molars and their progressive subarrangement into individual groups corresponding to the tips of forming cusps. Obviously the

surface areas of the unlabelled cells, identified after continuous labelling, are much smaller than the areas of negative cells after pulse labelling, strongly suggesting the presence of slower cycling cells as well as of non-cycling cells. Further investigations are required to establish if these latter cells are post-mitotic or correspond to transitory G0 cells.

How can we explain the temporo-spatial segregation of the unlabelled cells in the molar? Careful histological analysis of the negative areas revealed the presence in the molar of some cycling cells entrapped within these zones. The faster division of these cells may lead to the subdivision of the negative domains, and their patterning on the basis of the cusp formation. Such a behaviour probably is not so surprising: dissipation of coherent clonal growth has been documented under several circumstances (see Gardner and Cockroft, 1998 and references therein).

On the other hand, and most interestingly in the incisor the initial ensemble of non-cycling cells kept its cohesiveness. This group of cells included post-mitotic cells and G0 cells, the latter being precursors of the ameloblasts of the enamel free area.

Jernvall *et al.* (1994, 1998), Vaahtokari *et al.* (1996a,b), Keranen *et al.* (1998), suggested that the ek expressing SHH, BMP-2, 4, 7, FGF-4 represents a signalling center for tooth morphogenesis causing unequal growth of the enamel epithelium and inducing the formation of secondary enamel knots located at the tips of forming cusps. The cessation of cell proliferation in the ek and in post-mitotic dental cells more generally has been correlated with up regulation of the cyclin dependant kinase inhibitor p21 (Bloch-Zupan *et al.*, 1998; Jernvall *et al.,* 1998).

Knowing that the dental mesenchyme controls tooth class specific morphogenesis (Kollar and Baird, 1969, 1970a,b; Osman *et al.*, 1977; Schmitt *et al.*, 1999 in this issue) and considering the transcriptional activities of cells of the primary and secondary enamel knots (Jernvall *et al.*, 1998; Keranen *et al.*, 1998), we suggest the following working hypothesis (Fig. 7): the bud-cap staged dental mesenchyme controls the formation of the ek. This structure includes a tooth specific mosaic of prepatterned non-cycling cells (G0 cells and post-mitotic cells), the OMU: organizers of morphogenetic units, and intercalated cycling cells. This prepattern of the future cusps is tuned through apoptoses to fit with the definitive tooth specific cusp pattern. In the molar the dividing intercalated cells contribute to the redistribution of the OMU. In the incisor, the OMU keep their initial location. The OMU regulate cuspidogenesis controlling local cell proliferation, cell adhesion and cell-matrix interactions. These cells could also control the initiation of the gradient of terminal differentiation of odontoblasts and ameloblasts according to the reference point hypothesis (Ruch, 1990).

This working hypothesis is in agreement with Britten's (1998) assumptions of developmental models and can be tested in experimental conditions. It will be of great interest to investigate if OMU also exist in other epithelio-mesenchymal organs.

Materials and Methods

Tissues

Mouse embryos were obtained by mating ICR mice. The morning of the appearance of the vaginal plug was designed as day 0 of embryonic development.

First left and right lower molars and incisors were dissected on days 13, 14 and 16 of gestation (E-13, -14, -16) and cultured *in vitro*. E-13, -15 and 17 mandibles were also isolated.

Organ culture

E-14 and E-16 molars and E-13 incisors were cultured 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), L-Glutamine (2 mM), foetal calf serum (20%), kanamycin (0.1 mg/ml) and Difco agar (0.5%) The teeth (3 per dish and for each culture period) were incubated and grown with diluted BrdU (1/1000) at 37°C in a humidified atmosphere of 5% CO_2 in air and the medium was changed every 2 days.

Cell proliferation assays

Cell proliferation was investigated by mapping the distribution of S-phase cells after incorporation of the thymidine analogue 5-bromo-2-deoxy-uridine (BrdU, cell proliferation kit; Amersham Life Science).

In vitro, sequential 8h pulses or continuous labelling were performed by adding 0.4 ml of BrdU at a concentration of 3 μg/ml to the cultured teeth.

Sequential 8h pulses

E-14 and E-16 left and right lower molars were labelled for 8 h, 2, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120 and 132 h after the onset of the culture. For each 12 h interval, the left and right molars of the same embryo were used.

Such a protocol allowed for direct comparison at 12 h intervals of left and right lower molars from the same embryo. E-13 incisors were also sequentially labelled for 8 h.

Continuous in vitro labelling

Two hours after the onset of the culture, E-14 and E-16 molars and E13 incisors were cultured in the continuous presence of BrdU for 12, 24, 48 or 72 h.

In vivo BrdU incorporation

Two ml/100 gr containing 3 μg/ml of BrdU were intraperitoneally administrated to ether anaesthetised E-13, -15 and -17 pregnant mice at 8 h, 10 h and 12 h. The mice were killed at 16 h and the embryos were fixed.

Immunohistochemistry

All the cultured teeth and E-13, -15 and 17 mandibles were fixed in Bouin-Hollande's solution and embedded in paraffin. Serial 5 mm thick sections were performed. BrdU incorporated into DNA was located on the de-waxed sections with a specific mouse monoclonal antibody and immunoperoxidase labelling following the manufacturer's instructions, (Amersham). After immunostaining, sections were counterstained with eosin.

3D reconstructions

For the 3 teeth of each *in vitro* experimental group, as well as for the first lower E-17 molars, serial drawings of the mesenchyme and the inner dental epithelium were made at magnification x250, using a Zeiss microscope, equipped with a drawing chamber. The inner dental epithelium was subdivided into different zones corresponding to the positive and negative cells as shown in Figures 1B, 2B, 3B, 4A and 5. The serial drawings were digitalized by 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 previously been 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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