Cell Differentiation and Matrix Organization in Engineered Teeth

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ABSTRACT

Embryonic dental cells were used to check a series of criteria to be achieved for tooth engineering. Implantation of cultured cell-cell re-associations led to crown morphogenesis, epithelial histogenesis, organ vascularization, and root and periodontium development. The present work aimed to investigate the organization of predentin/dentin, enamel, and cementum which formed and mineralized after implantation. These implants were processed for histology, transmission electron microscopy, x-ray microanalysis, and electron diffraction. After two weeks of implantation, the re-associations showed gradients of differentiating odontoblasts. There were ciliated, polarized, and extended cell processes in predentin/dentin. Ameloblasts became functional. Enamel crystals showed a typical oriented arrangement in the inner and outer enamel. In the developing root, odontoblasts differentiated, cementogenesis occurred, and periodontal ligament fibroblasts interacted with the root surface and newly formed bone. The implantation of cultured dental cell re-associations allows for reproduction of complete functional differentiation at the cell, matrix, and mineral levels.

KEY WORDS: tooth, tissue engineering, dentin, enamel, cementum.

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INTRODUCTION

Several strategies have been developed to engineer dental constituents (Flores *et al*., 2008; Honda *et al*., 2009) or the whole tooth, either using artificial scaffolds or matrices, or without such supporting elements (Young *et al*., 2002; Ohazama *et al*., 2004; Hu *et al*., 2005a, 2006a; Sonoyama *et al*., 2006; Nakao *et al*., 2007; Honda *et al*., 2008; Arany *et al*., 2009; Nakagawa *et al*., 2009; Kim *et al*., 2010).

Cultured re-associations between dissociated single dental epithelial and ecto-mesenchymal cells allowed for the development of multicusp teeth with a correct epithelial histogenesis, and odontoblast differentiation. The further implantation of these re-associations under the skin of adult mice allowed for the initiation of root formation and revascularization of the implants (Hu *et al*., 2006a; Nait Lechguer *et al*., 2008). Vascularization is involved in tooth growth, in the maintenance of odontoblasts' and ameloblasts' functional differentiation, and in the mineralization of dental matrices.

The quality and organization of the dental matrices (dentin, enamel, and cementum) are important parameters in determining the functionality of the tooth. The purpose of this study was to investigate the organization of the different dental matrices that formed and mineralized after the implantation of cultured cell-cell re-associations (referred to hereafter as re-associations). To have roots engaged in the development and initiation of cementogenesis, most of the analyses were performed after 2 wks of implantation (Hu *et al*., 2006a). This was analyzed by means of histology, transmission electron microscopy, x-ray microanalysis, and electron diffraction.

MATERIALS & METHODS

First lower molars were dissected from ICR mouse embryos (obtained from Charles River Laboratories, l'Arbresle, France) at Embryonic Day (ED) 14. All procedures were in compliance with the recommendations of the European Economic Community (86/609/CEE) on use and care of laboratory animals.

In vitro Culture

Separation of dental mesenchyme from enamel organs and further dissociation of each tissue into single cells were performed as previously described (Appendix 1 in Nait Lechguer *et al*., 2008). Cell-cell re-associations were performed in the absence of carriers and cultured on a semi-solid medium for 8 days, according to Hu *et al.* (2005b).

In vivo Implantation

After *in vitro* culture, dental epithelial and mesenchymal cell re-associations were implanted between skin and muscles behind the ears in 8-week-old ICR mice. The implantations were maintained up to 2 wks. Samples analyzed in this study were taken from a previous collection (Nait Lechguer *et al*., 2008; refer to this study for the number of cultured and implanted molars and reassociations).

Immunofluorescence and Histology

Implants were embedded in Tissue-Tek® (Agar Scientific, Saclay, France). Immunostainings (Appendix 2) were performed on serial frontal frozen sections (7 μm) with polyclonal rabbit anti-mouse Collagen-IV (1/400) (Abcam®, TebuBio, Paris, France) and rabbit anti-pan-cytokeratin (1/200) (Santa Cruz Biotechnology, Le-Perray-en-Yvelines, France). Sections were incubated for 2 hrs at room temperature with the primary antibodies, and for 1 hr with chicken anti-rabbit secondary antibodies conjugated to either Alexa 488 (1/200) or Alexa 594 (1/500) (Molecular Probes, Invitrogen SARL, Cergy Pontoise, France). Negative controls were performed with corresponding sera instead of the primary antibody.

For histology, samples were fixed in Bouin-Hollande and embedded in paraffin, and 5-μm serial sections were stained with Mallory. In some instances, implanted samples were demineralized in 4.3% EDTA.

Transmission Electron Microscopy

Specimens were fixed for 2 hrs with 2% glutaraldehyde-0.1 M cacodylate buffer (pH 7.2), post-fixed with 2% osmium tetroxide-0.2 M cacodylate buffer, dehydrated in ethanol, and embedded in Epon. Ultra-thin sections were contrasted with uranyl acetate and lead citrate.

X-ray Microanalysis and Electron Diffraction Analysis

Stained ultra-thin sections were analyzed for mineral deposit. X-ray microanalysis and electron diffraction were performed with an EDX Si-Li 30 mm2 Noran detector-Voyager III analyzer on a Jeol 1200X TEM (JEOL Ltd., Tokyo, Japan). For EDX analysis, high voltage was 80 kV, and the live time for each measurement was 100 sec. Diffraction patterns were realized on the same sections, analyzed with the ProcessDiffraction program (Lábár, 2005), and compared with the diffraction spectra of hydroxyapatite (Couble *et al*., 2000).

RESULTS

The status of re-associations before and after implantation is described in Appendix 1.

Odontoblasts and Dentinogenesis in the Crown

In re-associations implanted for 2 wks, odontoblasts were elongated and showed a cytological polarization, as evidenced by the position of the nucleus, opposite the secretory pole, and that of golgi and endoplasmic reticulum localized in between (Figs. 1B, 2A). Odontoblasts also showed functional differentiation: development of the rough endoplasmic reticulum (Fig. 2E), presence of secretory granules (Fig. 2A), and polarized secretion of predentin/dentin (Figs. 1A, 1B). Odontoblasts in the re-associations were ciliated (Fig. 2F). The mineralization of predentin occurred normally (Figs. 1A-1C, 2B). Numerous odontoblast cell processes were observed in predentin (Fig. 2D) and extended in dentinal tubules (Fig. 1C). Gradients of odontoblast differentiation were maintained in re-associations (Appendix 2, Fig. B) as *ex vivo*, with the most differentiated cells at the tips of the forming cusps (Appendix 1, Fig. B).

Dentin (Figs. 1A-1C, 2B) showed well-banded tactoid and irregularly arranged collagen fibers (Figs. 2C, 2D). X-ray microanalysis revealed the presence of a high amount of calcium and phosphorus (Fig. 4B), as also in the root (Figs. 3F-3G). The Ca/P ratio varied from 1.6 to 1.8, depending on the analyzed area. Electron diffraction (Figs. 4C, 4G) showed the crystalline nature of hydroxyapatite.

Ameloblasts and Amelogenesis

Predentin/dentin allowed for the induction of ameloblast cytological and functional differentiation (Figs. 1D-1F, 3A). This was not yet initiated *in vitro*, before implantation (Appendix 1). After 2 wks of implantation, ameloblasts in re-associations were elongated and polarized (Figs. 1D-1F, 3A). Cells of the stratum intermedium (SI), in contact with ameloblasts, sometimes also interacted with BV (Fig. 1F). Ameloblasts secreted enamel (Figs. 1D-1F) with typical crystal organization (Figs. 1E, 3C). Vesicles containing stippled material were present at the secretory pole of ameloblasts (Figs. 3A, 3B). In the same region, the presence of many coated vesicles (Fig. 3B) suggested a locally active re-internalization process.

A decussating pattern of enamel rods was observed next to the dentin enamel junction, while rods were running parallel in the external half of enamel thickness (Fig. 1E). Besides typical patterns of rods and inter-rods (Fig. 3C), the organization of enamel was different near the dentin-enamel junction (Fig. 3E), as observed *ex vivo* (Skobe and Stern, 1978). The enamel was mineralized (Figs. 1A, 1C, 3D, 3E), and x-ray microanalysis showed a Ca/P ratio varying from 1.5 to 1.76 (Fig. 4E). The presence of hydroxyapatite crystals in enamel was shown by electron diffraction (Figs. 4F, 4G).

Cementogenesis

Next to the crown-root junction in re-associations implanted for 2 wks, elongated mesenchymal cells were in contact with the acellular cementum on the root surface (Figs. 1G, 1I). These cells, corresponding to the principal periodontal ligament fibroblasts, extended toward newly formed bone (Fig. 1A). BV came in close contact with functional odontoblasts in the root (Fig. 1H), which extended quite far from the crown-root boundary in re-associations (Fig. 1A). The same was observed in implanted molars (Appendix 2, Fig. A).

In implanted re-associations and molars, there was a discontinuity between the enamel organ and Hertwig's epithelial root sheath (HERS) cells in the root portion, when cementogenesis had already started (Appendix 2). In implanted re-associations, cementoblasts were functional and deposited cementum (Figs. 1H, 1I). TEM showed that the collagen fibers, deposited at initial stages of cementogenesis, formed bundles with a complex spatial arrangement (Figs. 3F, 3G). Some were perpendicular to the mineralized root dentin, while others were parallel to it (Fig. 3G).

DISCUSSION

The organization of predentin/dentin, enamel, and cementum is critical for tooth functionality. In the context of tooth engineering, however, very few investigations have been conducted relative to the organization of these different dental matrices, and these were performed only by means of histology (Hu *et al*., 2006a; Honda *et al*., 2008). For the first time, we investigated this at the ultrastructural level and took into account crystal nature and organization.

Odontoblasts and Dentinogenesis in the Crown

In cultured cell-cell re-associations, odontoblast functional differentiation was only beginning after 8 days, just before implantation (Hu *et al*., 2006a). The kinetics of odontoblast differentiation in early stages of implanted re-associations has been previously illustrated in detail (Nait Lechguer *et al*., 2008). After 2 wks of implantation, odontoblasts were functional and accumulated predentin and dentin with characteristic organization as seen by TEM. X-ray microanalysis and electron diffraction further documented an apatite type of mineralization.

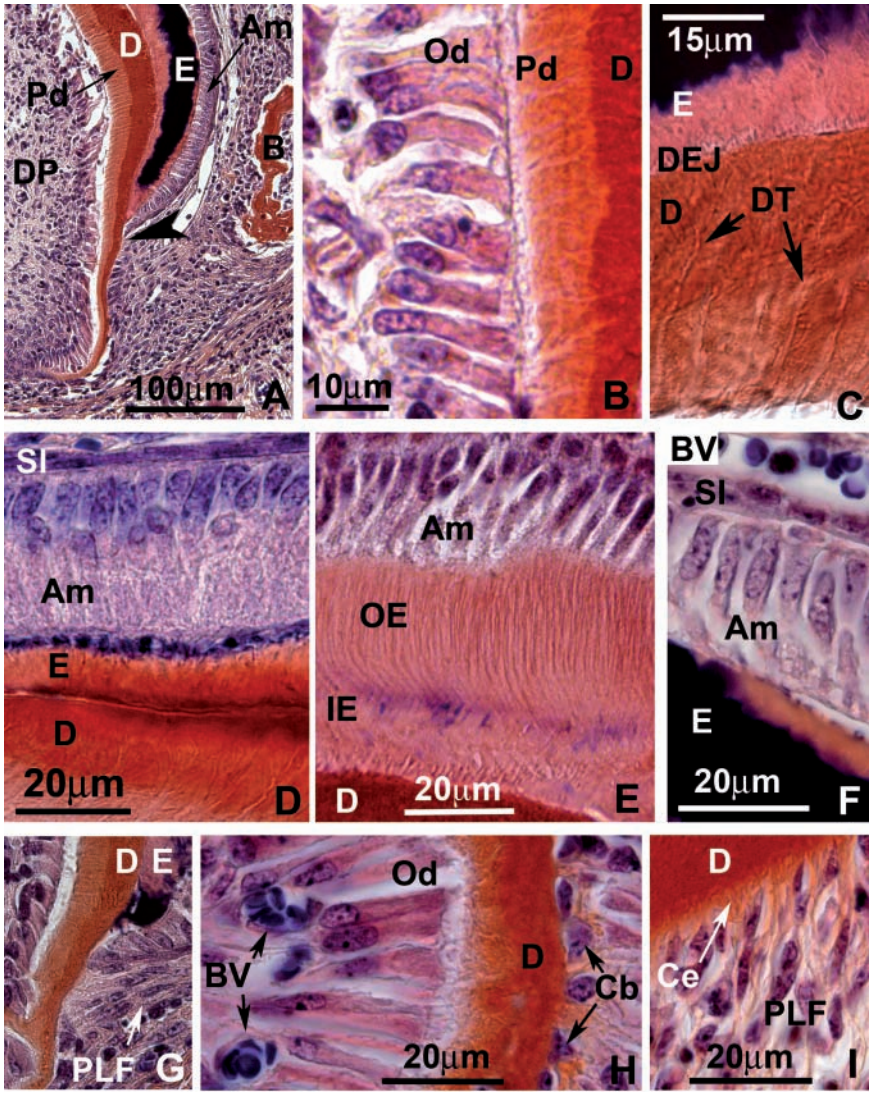


Figure 1. Histology of dental epithelial and mesenchymal cell-cell re-associations cultured for 8 days and implanted for 2 wks. **(A)** The crown was well developed, and both odontoblasts and ameloblasts (Am) became functional to secrete predentin/dentin (Pd/D) and enamel (E). An arrowhead indicates the sharp crown-root junction. **(B)** Elongated and polarized odontoblasts (Od) secreted predentin and dentin. **(C)** Dentinal tubules (DT) extended toward the dentin-enamel junction (DEJ). **(D)** At an early stage of enamel secretion, ameloblasts formed a monolayer of elongated and polarized cells in contact with the stratum intermedium (SI). **(E)** At a later stage, crystals showed decussation in the inner part of the enamel (IE), while running parallel in the outer enamel (OE). **(F)** Blood vessels (BV) could be found in direct contact with the stratum intermedium (SI). **(G)** Below the crown-root junction, periodontal ligament fibroblasts (PLF) were already attached to the root and extended until reaching newly formed bone (B), as shown in (A). **(H)** In the root, blood vessels were observed next to functional odontoblasts. In contact with the external surface of dentin, cementoblasts (Cb) were embedded in extracellular matrix. **(I)** Periodontal ligament fibroblasts attached to the cementum (Ce).

After implantation, blood capillaries came in close association with functional odontoblasts in the root as in the crown. The *in vivo* relationship between capillaries and functional odontoblasts facing either enamel or cementum has been investigated during rat incisor development (Oshima and Yoshida, 1992). Depending on odontoblast activity at the late stage of dentinogenesis, the ultrastructure (fenestration or not) and position of the capillaries (underlying or within the odontoblast layer) varied (Oshima and Yoshida, 1992). These authors correlated these chronological variations with the formation of gap junctions between odontoblasts. These junctions make the odontoblast layer impermeable to ion fluxes except for an inter-odontoblastic route.

As *in vivo*, the odontoblasts in implanted re-associations were ciliated. The cilium in odontoblasts might be involved in dentin formation and architecture, as well as in pain transmission

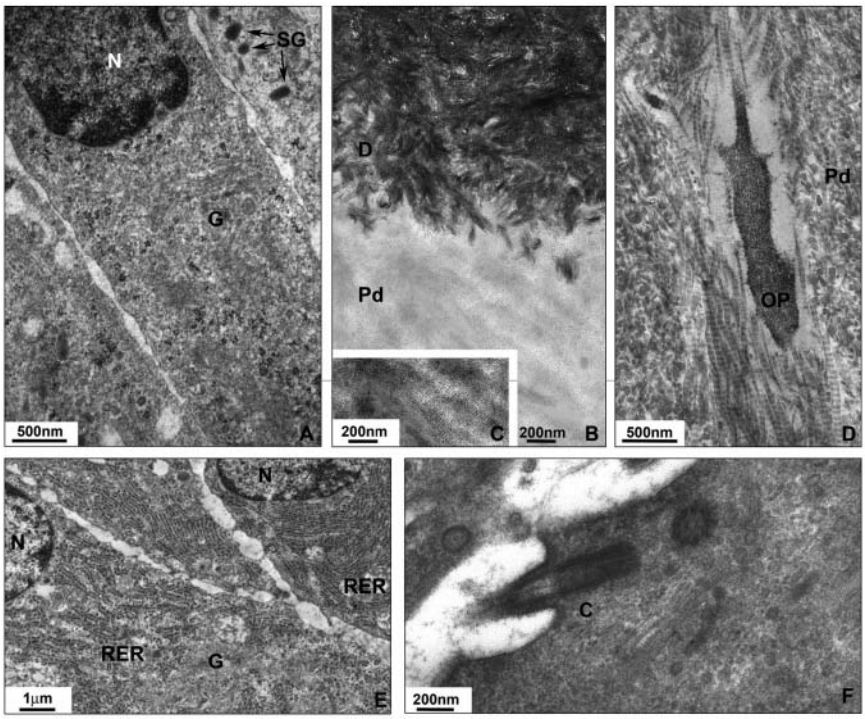


Figure 2. Transmission electron microscopy of odontoblasts and dentinogenesis in dental epithelial and mesenchymal cell-cell re-associations cultured for 8 days and implanted for 2 wks. **(A)** Elongated odontoblast showing a polarized position of the nucleus (N) and a well-formed golgi apparatus (G). The presence of secretory granules (SG) further illustrated the functional differentiation of odontoblasts. **(B)** Junction between predentin (Pd) and mineralized dentin (D). **(C)** Increasing the contrast in predentin better showed the typical periodic striation of collagen fibers. **(D)** Odontoblast process (OP) surrounded by collagen fibers from predentin. **(E)** Odontoblasts with developed rough endoplasmic reticulum (RER). **(F)** In odontoblasts, the presence of a cilium (C) was observed as *ex vivo*.

(Thivichon-Prince *et al*., 2009). Odontoblast cilia and nerve fibers show intimate associations *in vivo* at late stages. At early stages of odontoblast differentiation, which still exist in the root, the cilium might also be involved in cell polarization. Indeed, this was impaired in knock-out mice for Ofd1, a gene encoding for a centrosomal protein required for primary cilia formation (Ferrante *et al*., 2006). In implanted re-associations, the presence of functional primary cilia in odontoblasts would thus be critical for predentin/dentin deposition.

Ameloblasts and Amelogenesis

Contact with predentin/dentin induces ameloblast differentiation. This is mediated by BMP-2, and expressed by odontoblasts and cells of the SI (Nadiri *et al*., 2004, 2006), which might intervene at different times and thus affect different steps of ameloblast differentiation (Wakita and Hinrichsen, 1980). The SI/ameloblasts might also form a functional unit during amelogenesis, and, recently, it was suggested that the regulation of desmosomes between the two cell types could play a critical role in enamel mineralization (Barron *et al*., 2008). In cell-cell re-associations, ameloblast functional differentiation had started before 7 days of implantation, and at this stage, the enamel organ was fully vascularized (Nait Lechguer *et al*., 2008). Ameloblasts secrete enamel proteins and transport the calcium ions involved in apatite enamel crystal mineralization, as documented by x-ray microanalysis. After 2 wks of implantation, the enamel was mineralized. Vesicles containing stippled material were present at the secretory pole of ameloblasts. In this area, where mineralization was initiated, there was a rapid degradation of the organic components of the enamel (Simmer and Hu, 2002), which then might be cleared by endocytosis (Sasaki, 1984; Tye *et al*., 2009). Such a possibility has been investigated by Nanci and Warshawsky (1984). Although these authors suggested that it should correlate with the quality of fixation, the organic fraction of the enamel must be removed anyway. During the different stages of their differentiation, the shapes of ameloblasts change (Smith, 1998). After 2 wks of implantation in re-associations, most ameloblasts already appeared as short cells with few rough endoplasmic reticulum cisternae, suggesting that they had already reached a maturation phase. This reflected what happens during amelogenesis in mouse molars *ex vivo*. Ameloblast cytological and functional differentiation in these experimental conditions thus appeared normal, although initially cell positional information was lost in the re-associations (Hu *et al*., 2005b). This was further emphasized when we analyzed the organization of enamel crystals. In the implanted re-associations, the organization of rod and inter-rod enamel and the decussation of crystals were similar to what has been described *ex vivo* (Skobe and Stern, 1978). Electron diffraction confirmed the strong crystal orientation.

Root Development

The implantation of cultured re-associations allowed for the initiation of root development, which does not occur *in vitro* (Hu *et al*., 2006a). Odontoblasts differentiated and deposited predentin/dentin while root development progressed (Appendix 2). BV were often detected at the extremity of the root, where they entered the dental papilla (Appendix 2). Only a slight delay in vascularization has been reported in re-associations, compared with molars, and in both cases BV originated from the host, exclusively (Nait Lechguer *et al*., 2008). This step is very important for the further secretion and mineralization of the dental matrices analyzed in this study.

Cementoblasts and Cementogenesis

Although its origin remains controversial (Diekwisch, 2001, for review; Huang *et al*., 2009; Yamamoto and Takahashi, 2009), cementum was deposited in implanted re-associations. The most probable origin of cementoblasts would be the dental follicle. Their differentiation becomes possible when HERS cells start to form discontinuities. This allows for direct interaction between root dentin and the follicle cells and their induction to differentiate into cementoblasts (Diekwisch, 2001). In implanted re-associations, cementoblasts secreted collagen, which formed fibers in contact with the mineralized root dentin. The complex 3D organization of these collagen fibrils has previously been described *ex vivo* in different species, including the mouse (Bosshardt and Schroeder, 1992; MacNeil and Thomas, 1993; Diekwisch, 2001).

After two weeks of implantation, elongated cells were in contact with the root surface and extended toward newly formed bone. These cells organized as periodontal ligament fibroblasts (Hu *et al*., 2006a). At this stage, their presence along the root was limited to its upper part, since cementogenesis was still in progress in the more apical region. Thus, in implanted re-associations, the geometry in the organization of the tooth attachment apparatus was also maintained.

The implantation of cultured dental cell-cell re-associations had previously been shown to allow for the development of vascularized multicusp teeth with complete epithelial histogenesis and the initiation of root, periodontium, and bone development (Hu *et al*., 2005a, 2006a; Nait Lechguer *et al*., 2008). In our experimental approach, cell-cell re-associations were performed without any carrier. For the first time, the dental matrices deposited in the root and crown were analyzed at the ultrastructural level, and physical methods were used to show the nature and orientation of the crystals. The results from the present study further showed that predentin/dentin, enamel, and cementum were secreted, mineralized, and organized as in physiological conditions. These criteria will have to be satisfied in attempts to replace one cell compartment with non-dental cells, either with the same experimental strategy or with the strategy adjusted to new constraints, possibly linked to the new cell sources (Hu *et al*., 2006b).

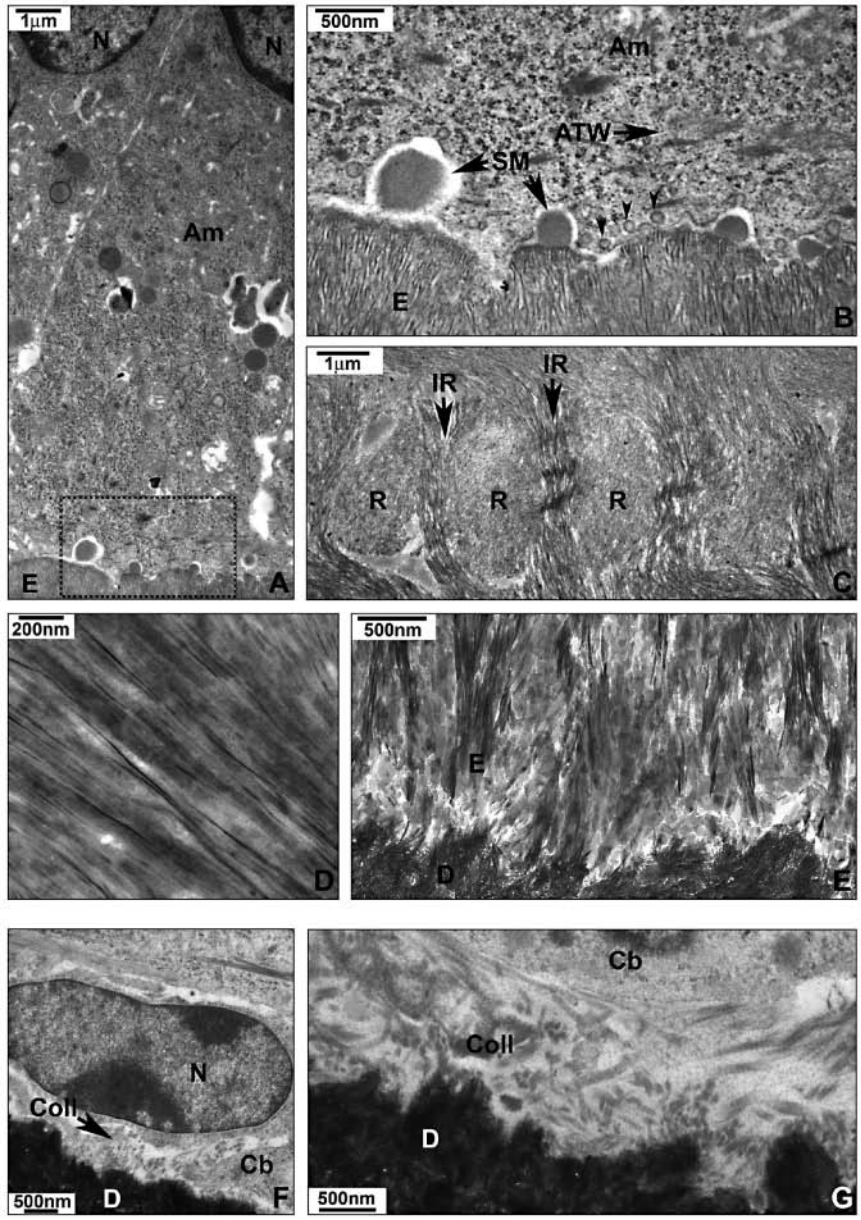
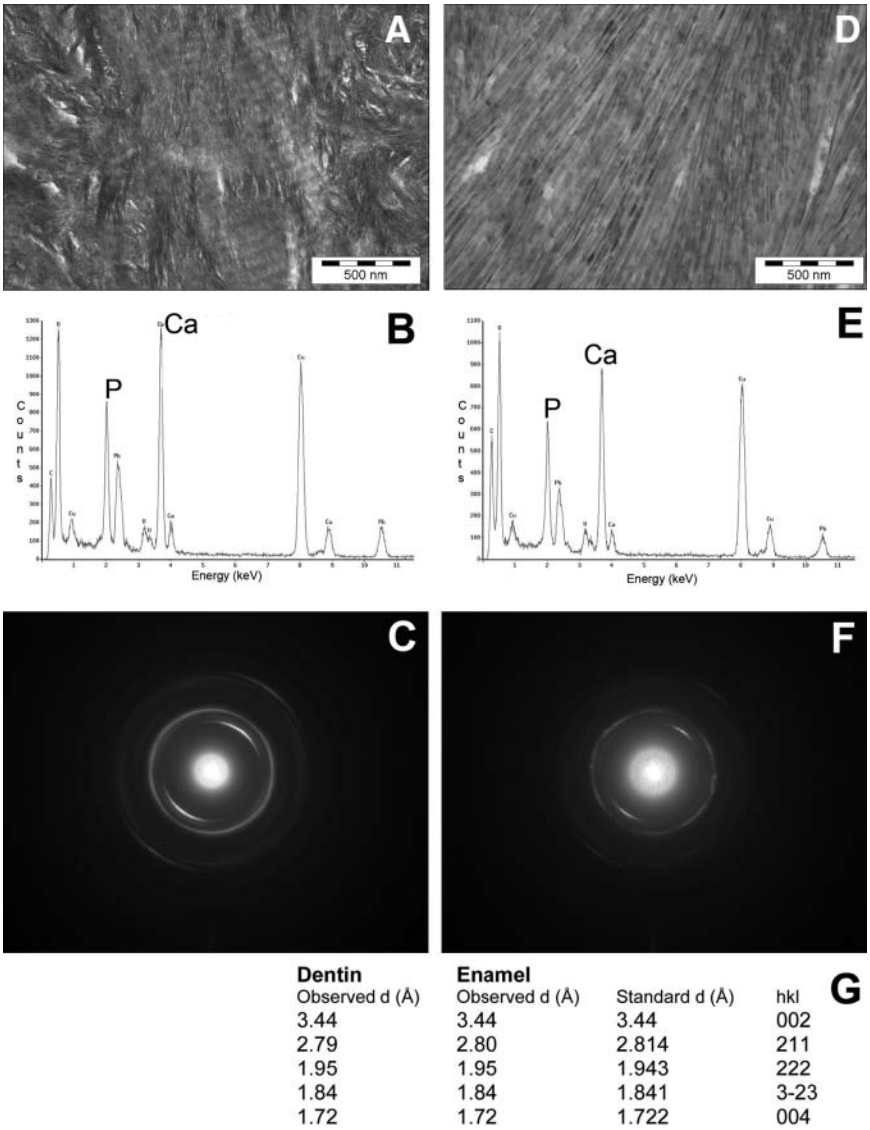


Figure 3. Transmission electron microscopy of ameloblasts-enamel **(A-E)** and of cementoblasts-cementum **(F,G)** in dental epithelial and mesenchymal cell-cell re-associations cultured for 8 days and implanted for 2 wks. (A) Ameloblasts (Am) were elongated, and their nuclei (N) were distant from the secretory pole, in contact with the enamel (E). (B) corresponds to the box in (A), showing the secretory pole of ameloblasts. Below the apical terminal web (ATW), intense activity was observed, with the secretion of stippled material (SM) and the presence of numerous coated vesicles (arrowheads) suggesting a simultaneous resorbing activity. (C) The enamel showed a characteristic organization of rods (R) and inter-rods (IR). (D-E) High magnifications of enamel crystals in the decussation zone (D) and near the dentin-enamel junction (E). (F-G) Cementoblasts (Cb) secrete collagen (Coll) in contact with root dentin (D).



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Dentin	Enamel		
Observed d (Å)	Observed d (Å)	Standard d (Å)	hkl
3.44	3.44	3.44	002
2.79	2.80	2.814	211
1.95	1.95	1.943	222
1.84	1.84	1.841	3-23
1.72	1.72	1.722	004

Figure 4. X-ray microanalysis **(B, E)** and electron diffraction **(C, F, G)** were performed for mineralized dentin **(A)** and enamel **(D)** from samples prepared for TEM microscopy. After x-ray microanalysis, calcium (Ca) and phosphorus (P) peaks were observed at 2 keV and 3.7 keV, respectively (B, E). Parasite peaks also appeared on the spectrum accounting for Pb and U due to the staining and Cu to the grid support. Electron diffraction patterns (C, F) and d-spacings (G) indicate a crystalline HAP structure for the two mineralized matrices. Strong crystal orientation in both samples is visible from transmission electron microscopy (A, D) and highlighted by reinforced diffraction intensities for 002 reflections (C, F). d, interplanar distances; hkl, Miller indices.

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