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Review

Developmental Biology of Cementum

THOMAS G.H. DIEKWISCH*

Allan G. Brodie Laboratory for Craniofacial Genetics, University of Illinois at Chicago, USA

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Tooth cementum is a bone-like mineralized tissue secreted by cementoblasts on the surface of root dentin or, in some animals, crown enamel. Cementum formation begins when both epithelial cells of Hertwig's root sheath (HERS) and mesenchymal cells of the dental follicle are in proximity to the developing root surface. In recent decades, numerous authors have generated much information on the role of the tissues involved in root formation, but the contribution of epithelial and mesenchymal components toward cementogenesis remains an area of debate. The purpose of this review is to carefully analyze the existing literature on epithelial and mesenchymal tissues involved in cementogenesis and to discuss this information in the light of a series of specimens which we have prepared for this review paper. Based on our literature review and careful analysis of the preparations presented, we report the following conclusions: (i) HERS becomes disintegrated prior to any cementum deposition, (ii) mesenchymal cells from the dental follicle penetrate the HERS bilayer and deposit initial cementum matrix while immediately adjacent epithelial cells are separated from the root surface by a basal lamina and do not secrete any cementum matrix, (iii) in contrast to rodents, in humans, HERS is removed from the root surface prior to cementum deposition, (iv) both amelogenin mRNAs and proteins are absent from the root surface and from the cervical-most ameloblasts, and (v) cementum protein extracts do not cross-react with amelogenin antibodies on Western blots. Overall, our studies confirm the classical theory of cementum as a dental follicle derived connective tissue that forms subsequent to HERS disintegration.

Origins of cementum - a scientific "whodunit"

Mammalian teeth are compound organs featuring three vastly different mineralized tissues attached to each other: enamel, dentin, and cementum. Such a close association of three different biominerals is found nowhere else in the mammalian body and rarely even within the animal kingdom. The formation of the dental biominerals in higher vertebrates does not occur at random or in bulk as frequently observed in invertebrates (Lowenstam, 1981) but is rather tightly controlled by organic matrices secreted by highly specialized cells (Slavkin and Diekwisch, 1996, 1997). In enamel and dentin, the deposition of biopolymer matrices and the secretion of mineral are accomplished by a unique layer of densely packed cells directly adjacent to the corresponding mineral layer and thus leaving no question about the cellular origin of these tissues. In contrast to enamel and dentin, the development of the third mineralized tissue, cementum remains enigmatic. In many mammals, both epithelial cells of Hertwig's root sheath and mesenchymal cells of the dental follicle are in proximity to the developing root surface when cementum is formed. The contributions of each tissue, epithelium and mesenchyme, are not clearly defined and have been the basis of numerous debates (Ten Cate, 1996a,b; Hammarstrom *et al.,* 1996).

The classical theory suggests that mesenchymal cells of the dental follicle become cementoblasts and secrete cementum after

Abbreviations used in this paper: HERS, Hertwig´s epithelial root sheath;

***Address correspondence to:** Dr. Thomas G.H. Diekwisch (Director). Allan G. Brodie Laboratory for Craniofacial Genetics, UIC College of Dentistry MC 841, 801 South Paulina, Chicago Il 60612. Fax: +1-312-996-7138. e-mail: tomdkw@uic.edu

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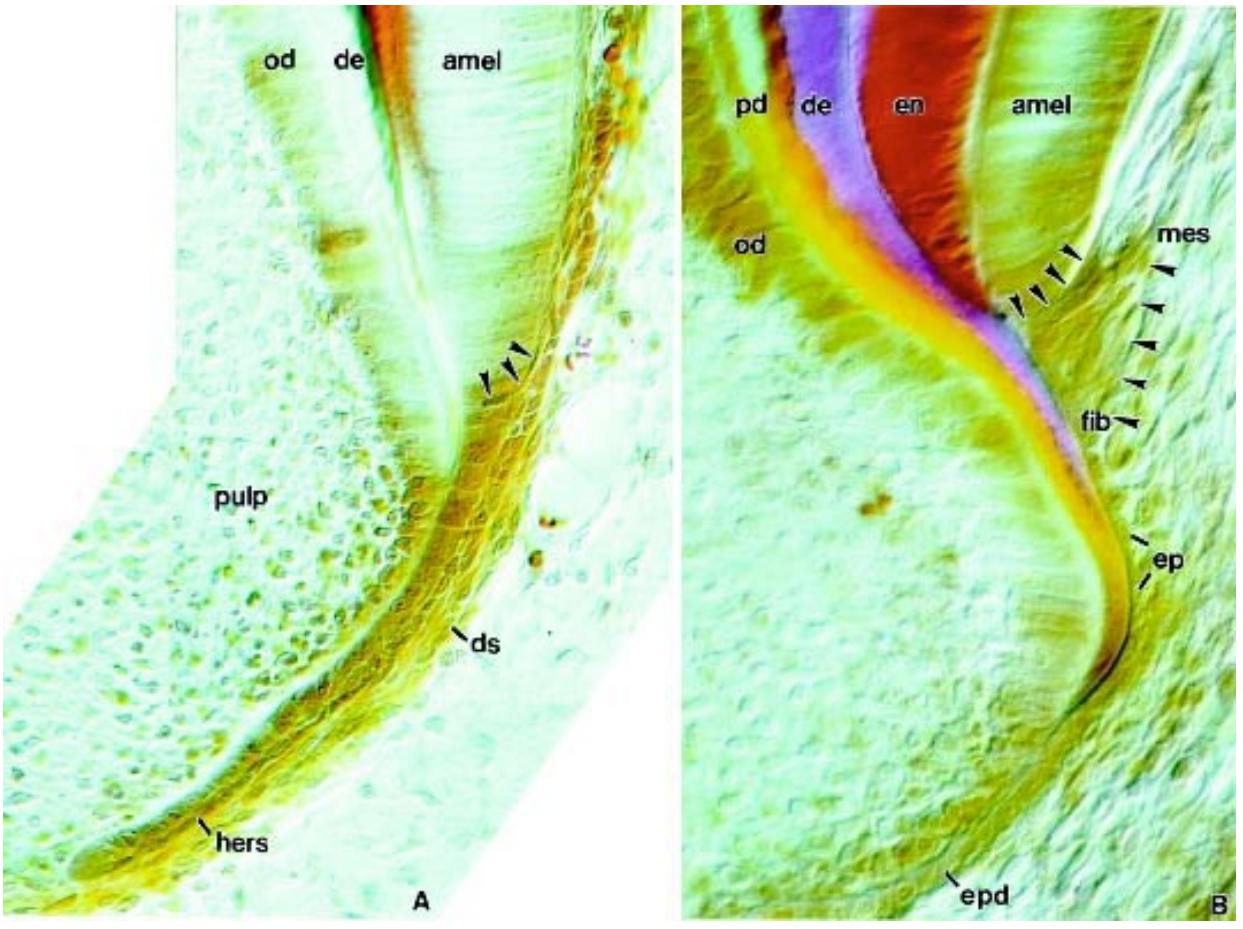

Fig. 1. Hertwig's epithelial root sheath (HERS) continuity at two different stages of rat first mandibular molar tooth formation. (A) *A preparation of a 6 days postnatal mouse molar stained with AZAN. In the upper third of the image the cervical margin of the developing tooth crown was visible, including pulp (pulp), odontoblasts (od), dentin (de, predentin appears white, dentin green), enamel (red color), and ameloblasts (amel). In apical direction HERS (hers) formed a bilayered cell sheath in continuation with the ameloblast cell layer (amel) and outer layers of the enamel organ. Both HERS and the enamel organ were surrounded by mesenchymal cells of the dental sac (ds). HERS did not show any signs of fenestration at this stage. However, single cells (arrowheads) were positioned between ameloblasts (amel) and the beginning of HERS.* **(B)** *A preparation of a 10 days postnatal mouse molar stained with AZAN. Tooth mineralization was significantly advanced compared to (A). Dentin (de) appeared bright blue, predentin (pd) yellow and enamel (en) red. Both odontoblasts (od) and ameloblasts (amel) were in their secretory stage. The bilayered unit of HERS was still distinguishable at the apex (hers) but had lost its continuity with the ameloblast layer (amel). Instead, bundles of mesenchymal cells (mes, between arrowheads) as well as fibrous structures (fib) were occupying the developing root surface. Isolated epithelial cells (ep) were localized between mesenchymal tissues and HERS. Note the clear separation between ameloblast cell layer (amel, upper row of arrowheads) and mesenchymal cells (mes) covering the developing root surface. Both preparations were 5 μm ultrathin ground sections. Magnification, 600x.*

having transmitted the barrier of Hertwig's epithelial root sheath (Paynter and Pudy, 1958; Lester, 1969a,b; Ten Cate, 1969a,b; Furseth, 1986; Provenza, 1988; Schroeder, 1986). Originally, the disintegration of HERS and the penetration with connective tissue cells from the dental sac have been described by von Brunn (1891) who believed that the connective tissues of the dental sac were growing into the folds of the enamel organ. While some authors (Paynter and Pudy, 1958; Orban, 1944) assume that the cells of the dental sac simply come into contact with the root dentin after uncoupling of HERS, others understand this process more actively and describe it as migration or break through (Schour, 1953; Cho and Garant 1988).

A second school of thought proposed that acellular cementum and cementogenesis as a process were originated from epithelial cells (Stahl and Slavkin, 1972; Slavkin, 1976). This theory was based on microscopical studies of the lingual cementum of rodent and rabbit incisors as well as on suggested immunological similarities between enamel and cementum proteins (Slavkin and Boyde, 1974; Schonfeld, 1975; Slavkin, 1976; Schonfeld and Slavkin, 1977; Owens, 1978; Slavkin *et al.*, 1988, 1989). The idea of an epithelial origin of cementum has been questioned by Thomas *et al.* (1986) who demonstrated the absence of any enamel proteins in murine cementum and Luo *et al.* (1991) who documented that HERS cells did not transcribe amelogenin. The more inclusive idea that HERS cells generate a functionally relevant protein layer goes back to Hertwig's concept of the "Schmelzoberhäutchen" (Hertwig, 1874). Hertwig believed that the epithelial cells of the tooth germ were secreting a *cuticula* ("Schmelzoberhäutchen") that would preform the "Gestalt" of the developing tooth including the root (Hertwig, 1874). According to Hertwig, this *cuticula* induced tooth dentin formation (Hertwig, 1874).

Other theories focused on the bone-like molecular characteristics of cementum (Somerman *et al.,* 1993; d'Errico *et al.,* 1997) or on the functional importance of alkaline phosphatase as an essential enzyme to mediate cementogenesis (Beertsen and Everts, 1990; Beertsen and van den Bos, 1990; Groeneveld *et al.,* 1992, 1994; Beertsen *et al.* 1999). A number of implantation and tissue recombination studies (Hoffman, 1960; Ten Cate *et al.,* 1971; Lumsden, 1988; Palmer and Lumsden, 1987) demonstrated that periodontal tissues, including cementum, were tooth related and neural crest derived. Using his extensive collection of large human tissue sections, the Vienna oral biologist Bernhard Gottlieb observed an absence of epithelial tissues wherever new cementum was formed (Gottlieb, 1942). A re-plantation experiment by Heritier (1982) in which cementum was formed on the surface of crown enamel denuded of ameloblasts also provided support for the concept of cementum formation in the absence of epithelial cells (discussed in Ten Cate, 1996a,b). A similar finding, namely that cementum formation was exclusively observed in areas devoid of ameloblasts was reported by Dubielzig (1986) based on observations in equine tooth development and tumors. Further evidence for cementum formation in the absence of ameloblasts was presented by descriptions of amelogenesis imperfecta cases in which cementum was formed in areas where ameloblasts were missing (Weinmann *et al.,* 1945; Listgarten, 1967).

In the present review we will shed light on several key issues in initial cementogenesis. Addressing key questions, we will carefully review the existing literature and provide current data in order to elucidate key aspects of cementogenesis and to determine which cells give rise to and contribute to mammalian cementum. We have used a number of strategies to document the origins of cementum, including ultrathin ground sections to preserve tissue integrity, high resolution 3D imaging to reveal spatial correlations of migratory cells, electron microscopy to analyze early stages of cementum deposition, *in situ* hybridization to detect amelogenin transcripts along the developing root surface, and immunohistochemistry to detect marker epitopes in cells and matrices. We also investigated a number of species, including pig, mouse and human. The species and techniques included in this review were carefully chosen to provide a multifaceted analysis of mammalian cementogenesis and ultimately to generate an integral understanding of the origins of cementum.

Loss of ameloblast continuity and insertion of mesenchymal cells from the dental follicle proper

One of the first events involved in cementogenesis is the disruption of HERS continuity by adjacent cells. The formation of cell processes during initial cementoblast differentiation and their extension toward the root surface has been described in a detailed study by Cho and Garant (1988). Cho and Garant (1988) also reported on the removal of the outer HERS basal lamina prior to cementogenesis and on the penetration of the inner HERS basal lamina by the invading cells from the dental follicle proper. They conclude that the "… fact that the loss of the external basal lamina adjacent to the cells of the dental follicle proper and the subsequent separation of the epithelial cells of the root sheath are events concurrent with the onset of precementoblast differentiation suggests that, whatever instructive and/or permissive role is to be attributed to the epithelial cells, it must be a more transient and subtle process than that played by the inner enamel epithelium cells or the inner root sheath cells in odontoblast differentiation" (Cho and Garant, 1988). Thus, Cho and

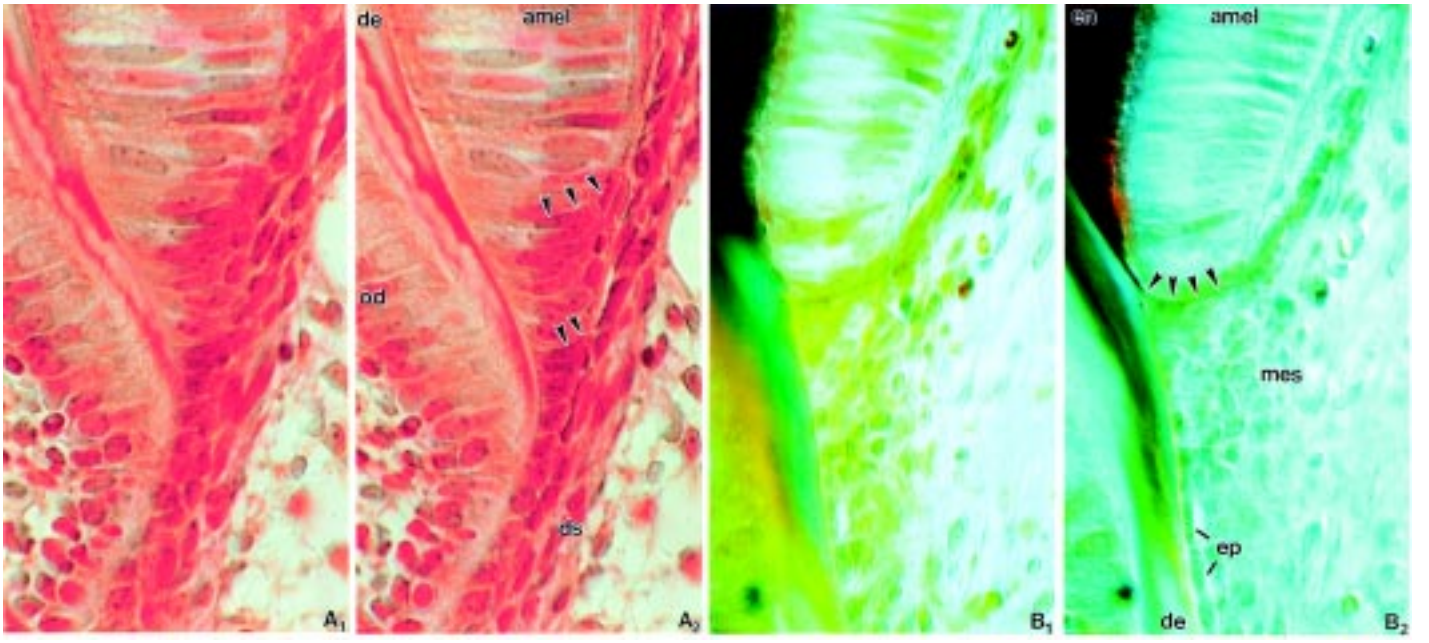

Fig. 2. Stereomicrographs of 5 μm ground sections of the developing mouse first mandibular molar tooth cervix. *Stereopair* **(A_1/A_2)** *was a preparation of a 6 days postnatal rat molar stained with Movat's Pentachrome. Ameloblasts (amel), odontoblasts (od), and dentin (de) were labeled for orientation purposes. Note how tubular cells from adjacent to the ameloblast cell layer (arrowheads) were interrupting the continuity of the ameloblast cell layer (amel). Stereopair* **(B_1/B_2)** *was a preparation of a 10 days postnatal mouse molar stained with Masson Goldner. Oblique lighting was used to enhance detail on this high resolution stereo micrograph. The enamel layer (en) and the ameloblast cell layer (amel) clearly demarked the cervical margin of the developing tooth crown. A membrane (arrowheads) separated the ameloblast cell layer (amel) from the mesenchymal cells (mes) occupying the developing root surface. There were only a few epithelial cells (ep) in immediate proximity to the root dentin surface (de). Magnification, 1200x.*

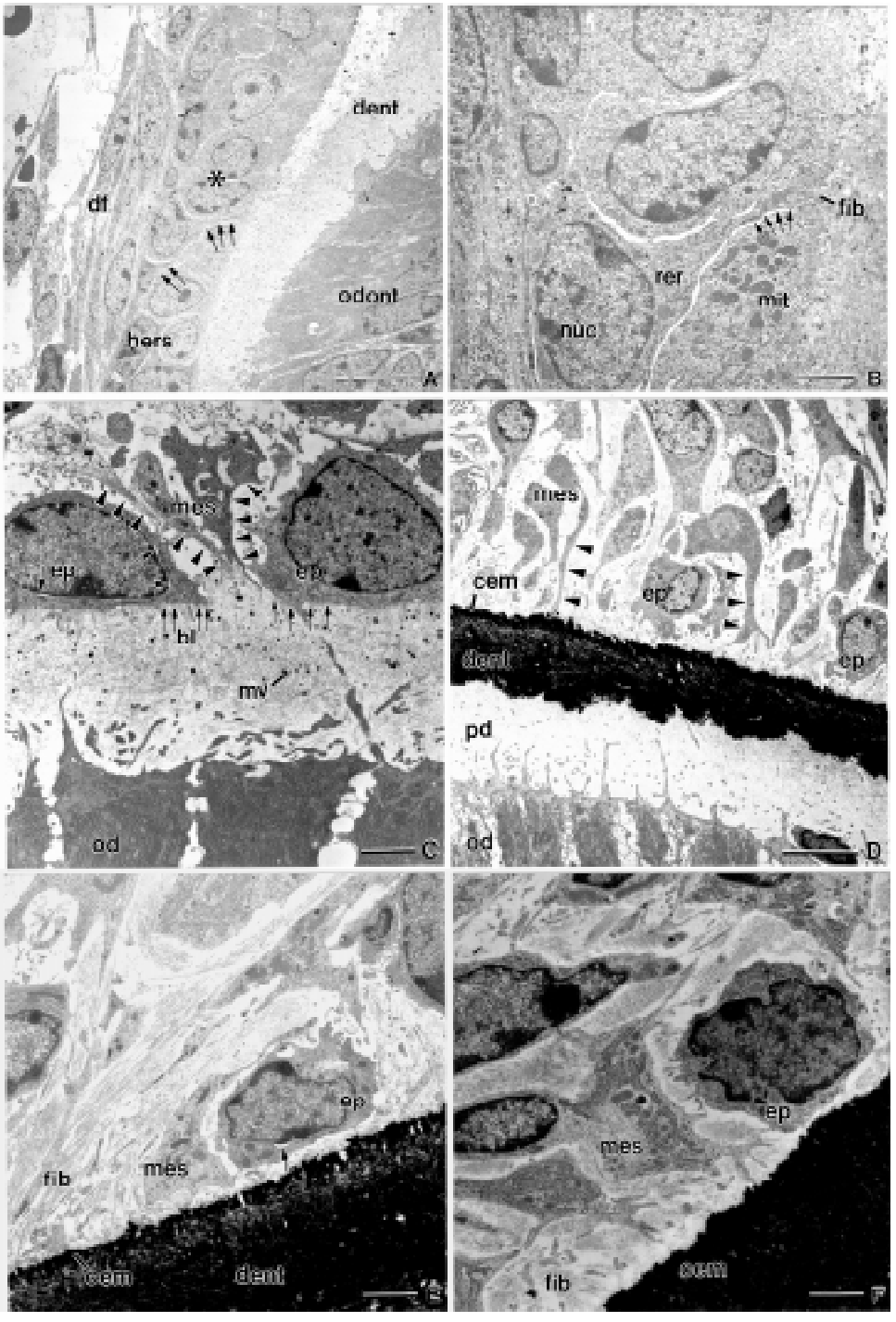

Fig. 3. Transmission electron micrographs illustrating the spatial relationship of epithelial and mesenchymal cells adjacent to the developing root surface. (A,B) *Electron micrographs of 6 days postnatal mouse molars demonstrating cell processes extending toward the root surface (arrows). Arrows indicate cell processes disrupting the continuity of epithelial cells of HERS (hers). (A) provides an overview, covering root odontoblasts (odont), dentin (dent), HERS (hers) and dental follicle (df). (B) is a higher magnification of an area in the center of 3A (asterix). Nucleus (nuc), rough endoplasmic reticulum (rer) and mitochondria (mit) are labeled. In the adjacent dentin surface, collagen fibrils (fib) were distinguished.* **(C,D)** *Electron micrographs of postnatal mouse molars illustrating cell processes (arrowheads) and mesenchymal cells (mes) alternating with epithelial cells (ep). (C) was from 8 days postnatal mouse molars. Cell processes of three mesenchymal cells (mes, arrowheads) were inserting between epithelial cells (ep) and gaining access to the developing root surface. The left of the two epithelial cells featured three crescent-shaped spaces indicative of apoptosis (arrows). A basal lamina (bl) was separating epithelial cells and root dentin but was absent between the mesenchymal cell processes and the dentin surface. (C) illustrates that mesenchymal cells had access to the root surface at a time when the basal lamina between epithelial cells and root predentin was still intact and no cementum had yet been secreted. Note the matrix vesicles (mv) in the developing root dentin. (D) was from 10 days postnatal mouse molars. Here isolated epithelial cells were found between mesenchymal cells (mes) and their processes (arrowheads). This stage was characterized by the presence of an initial cementum layer (cem). Root odontoblasts (od), predentin (pd), and dentin (dent) were labeled for orientation purposes.* **(E,F)** *Twelve days postnatal mouse molars. A distinct cementum layer (cem) covered the root dentin (dent). Singular epithelial cells (ep) were embedded in collagen fibers (fib) and mesenchymal cells (mes). The mesenchymal cells extended numerous cell processes to the root surface while the epithelial cells appeared encapsulated. The epithelial cell in (E) featured a distinct crescent-shaped space indicative of apoptosis (arrow). Bars: 3µm (A), 1µm (B), 2µm (C), 5µm (D), 2µm (E), and 2µm (F).*

Garant believe that since the disruption of HERS appears to be key event during early cementogenesis it is not likely that HERS plays a major role during cementogensis.

In order to further investigate the origins of cells occupying the developing root surface we have used ultrathin ground sections, high-resolution 3D imaging, and electron microscopy (Figs. 1-3). Together, our preparations demonstrate that cells in immediate proximity to HERS extend processes between HERS cells and gain access to the developing root surface prior to any cementum deposition. Our micrographs provide strong support for the before-mentioned quotation by Cho and Garant (1988) in that the developmental dynamics of early root formation appear to be focused on HERS disintegration and dental follicle cell migration rather than on any secretary role of HERS to fabricate cementum.

Analysis of earlier stages of root formation than those published by Cho and Garant (1988)(Figs. 3 a,b,e,f) yielded a most surprising finding: based on the nucleus: cytoplasm ratio the earliest cells disrupting the inner layer of HERS were clearly epithelial cells. The exact layer of origin of the process-forming cells was not obvious since the cells of the condensed enamel organ in this area were quite

densely packed. In our electron micrographs, the process-forming epithelial cells were part of the outer layer of HERS that appeared to be continuous with the outer enamel epithelium. Our findings open up a number of questions about the functions and mechanisms of this epithelial insertion. One might suggest that the process-forming epithelial cells function to disrupt Hertwig's root sheath in certain locations to provide space for mesenchymal cells to penetrate the epithelial barrier.

Initial cementum matrix deposition by mesenchymal cells in proximity to non-secretory epithelial cells

In absence of direct marker proteins for cementum and cementoblasts, ultrastructural evidence is of great importance to establish the sequence of events involved in cementogenesis. One of the important issues for understanding cementogenesis is the time of persistence of the inner HERS basal lamina since this basal lamina prevents matrix deposition directly on the dentin surface. In other words: cells that are protected from the dentin surface by a basal lamina are most likely not active as cementoblasts. A second important question is which cell type, epithelial or mesenchymal, contributes to initial matrix deposition. Several authors have reported either on the disintegration of the basal lamina prior to cementum deposition and/or on the early cementum deposition by mesenchymal cells (Lester, 1969a,b; Owens, 1978; Cho and Garant, 1988).

We have performed a number of electron microscopic studies to ask the questions (i) whether epithelial or mesenchymal cells first deposit cementum matrix and (ii) whether HERS inner basement membrane is persistent even after initial cementum deposition by mesenchymal cells. Our electron micrographs of developing mouse molar root surfaces revealed that initial cementum matrix deposition exclusively occurred in areas in which mesenchymal cells had access to the root surface, while adjacent epithelial cells were separated from the root surface by a basal lamina and did not deposit any cementum matrix. This finding suggests that the first cementoblasts that appear during root development are mesenchymal cells of the dental follicle and confirms previous studies on the mesenchymal origin of cementum forming cells (Lester, 1969a,b; Owens, 1978; Cho and Garant, 1988). Our micrographs demonstrated that mesenchymal cells were in direct contact with the initial cementum matrix and were not separated by a basal lamina from the dentin surface. These mesenchymal cells featured several cellular characteristics indicating their involvement in collagenous matrix deposition on the root surface, including numerous cell organelles characteristic of secretory cells, cell processes extending toward the root surface, and dense collagen fibrils in direct contact with the cell membrane. In contrast, epithelial cells were separated from the root surface by a basal lamina and therefore not directly involved in cementogenesis. The electron micrographs in Fig. 3 are of particular interest since they document cementum matrix deposition by mesenchymal cells next to basal lamina persistence between Hertwig's epithelial cells and dentin in a single electron micrograph. Together, these findings confirm the priority of dental follicle mesenchymal cells as cementoblasts. They also document that HERS cells do not have a detectable secretory role during initial cementum deposition since they are separated from the root surface by a dental lamina.

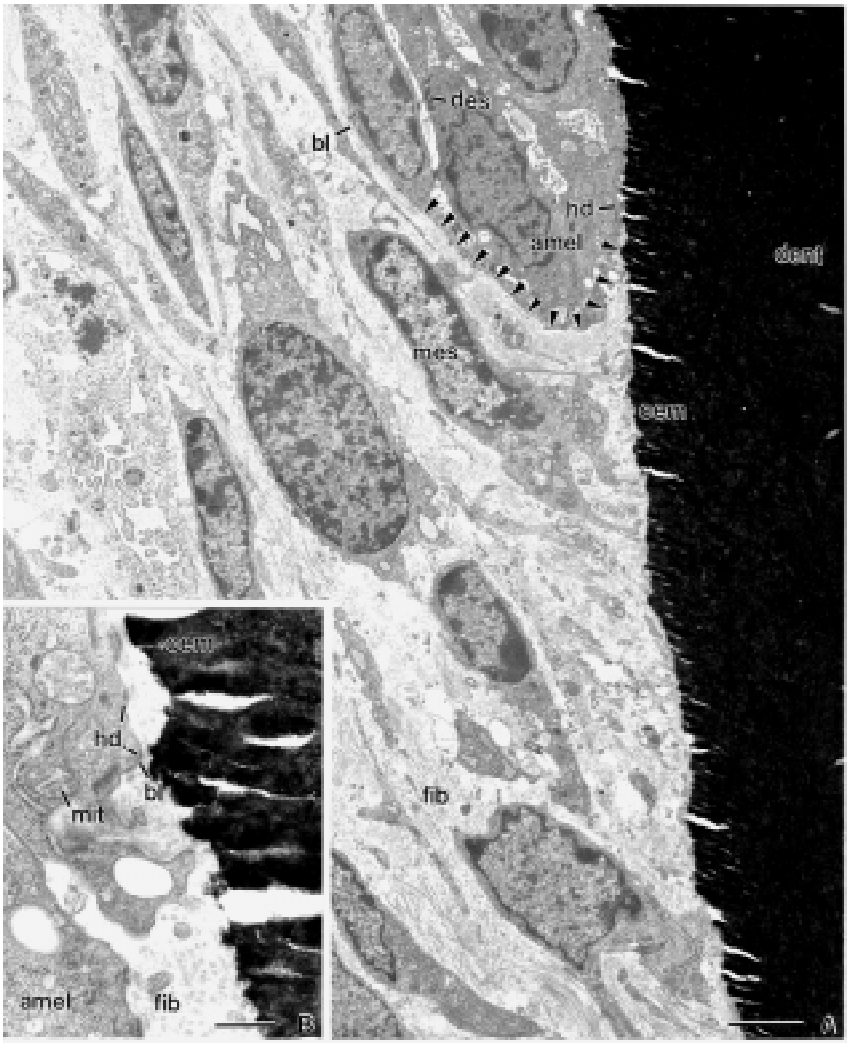

Fig. 4. Transmission electron micrographs illustrating the spatial relationship between epithelial and mesenchymal cells along the cervix of 12 days postnatal mouse molars. (A) *The arrowheads illustrate an intact basal lamina separating the apical tip of the ameloblast cell layer (amel) from the underlying mesenchymal cells (mes) and fibers (fib) of the developing periodontal ligament. Note the numerous hemidesmosomes that connected the ameloblasts with the basal lamina. Distinct desmosomes (des) were located between individual ameloblast cells. At this stage, the root dentin was covered by a distinct cementum (cem) layer.* **(B)** *The insert allows a closer view of the cementum (cem) crystal structure, hemidesmosomes (hd), basal lamina (bl), and mitochondria (mit). Note the fiber bundles (fib) immediately adjacent to the cervical tip of the ameloblast layer (amel). The cervical-most tooth crown surface was covered by cementum (cem) instead of enamel (A,B). This coronal cementum was positioned between the cervical-most ameloblasts and the dentin surface (A). There were no mesenchymal cementoblasts in contact with the coronal cementum. Bars: 1 μm (A), 200 nm (B).*

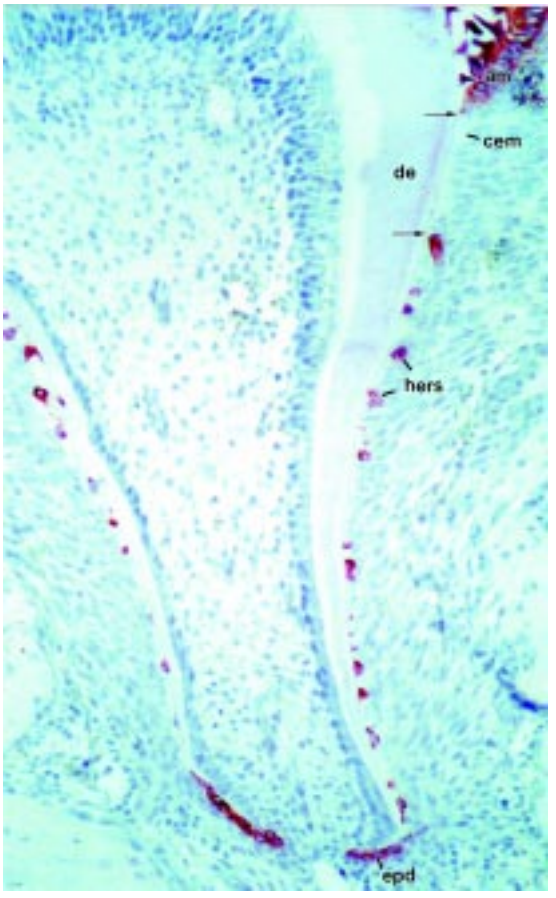

Fig. 5. Labeling of epithelial cells during initial cementogenesis in 20 days postnatal mouse molar roots using an anti-keratin antibody. *In some areas the root surface was covered by a thin layer of initial cementum (cem). The anti-keratin antibody recognized epithelial cells, including crown ameloblasts (am), cells of Hertwig's epithelial root sheath (hers), and cells of the epithelial diaphragm (epd). Note the distances between individual HERS cells (hers) as labeled with the anti-keratin antibody during initial cementogenesis. The distance between the cervical-most HERS cells and the apical margin of the ameloblast cells layer was approximately 0.1 mm (distance between arrows). Magnification, 720x.*

Cementogenesis at the tooth cervix and at the cemento-enamel junction

Another area that might harbor potentially revealing information related to the origin of cementum is the tooth cervix. We have therefore carefully analyzed the tooth cervix of developing mouse teeth using ultrathin ground sections, high resolution 3D-imaging, electron microscopy, and immunohistochemical labeling (Figs. 1,2,4,5). Our analysis revealed two interesting findings that might shed more light on the process of cementogenesis: (i) the absence of HERS cells from the cervical margin of the developing root surface, and (ii) the presence of a cementoid tissue between cervical-most ameloblasts and dentin surface.

Ultrathin ground sections (Figs. 1,2), electron micrographs (Fig. 4), and epithelial marker images (Fig. 5) demonstrate that HERS epithelial cells are absent from the cervical margin of the developing root surface at early stages of root formation allowing for mesenchymal cells to access the root dentin surface. These findings indicate that in rodents, HERS disintegrates at the cervical margin of the root early on during root development. In tandem, the ameloblast layer establishes a distinct basement membrane/basal lamina at its cervical margin that separates the ameloblast layer not only from the surrounding cells of the dental follicle but also from further apical positioned epithelial cells of Hertwig's epithelial root sheath. The reorganization of the dental epithelium during root formation and the establishment of a new basement membrane indicate that the cervical disintegration of HERS is a key event during cementogenesis allowing for the perforation and gradual penetration of HERS by mesenchymal cells.

The disintegration of HERS is an event only found in mammals and does not occur in most reptiles, amphibia, and fishes (McIntosh and Diekwisch, 2000). The cervical disintegration separates the enamel organ epithelium into a coronal portion that includes the ameloblast layer and three other layers of the enamel organ as well as an apical portion that includes HERS and the epithelial diaphragm. While the coronal portion of the enamel organ remains intact as a unit to facilitate enamel maturation and tooth eruption, the apical portion becomes disrupted and develops into the network of HERS. It has been shown that during further root development, HERS cells proliferate only at the apical end of HERS while the entire root surface grows considerably (Kaneko *et al.,* 1999). HERS appears to stretch over the entire root surface and as a result, HERS only covers small portions of the developmentally advanced tooth root. The disproportionate growth rate between the rapidly proliferating root and the stagnating HERS might explain why in further advanced stages of root formation the root surface is only covered by very few epithelial cells. Apoptosis of HERS cells as described in Fig. 3 of the present study and documented elsewhere (Kaneko *et al.,* 1999; Cerri *et al.,* 2000) might be another explanation for the "thinning" of the epithelial net covering the root surface.

The set of electron micrographs of the developing tooth cervix of a 12 days postnatal first mandibular mouse molar contains another highly interesting finding: the presence of coronal cementum-like tissue ("cementoid") between cervical-most ameloblasts and the dentin surface. Proponents of the theory of an epithelial origin of cementum might interpret this finding as a first step of epithelium-induced cementogenesis. Another possible explanation is that the coronal cementoid found in these micrographs was initially formed by mesenchymal cementoblasts and that the ameloblast layer only moved subsequent to cementum deposition on top of the coronal cementum layer. We interpret these data to indicate that the cervical-most ameloblasts do not synthesize amelogenin and thus do not fabricate "true" enamel. We hypothesize that the cervical-most ameloblasts synthesize a mineralized tissue that is essentially enamel without the crystal-shaping properties of amelogenin. Such a mineralized tissue might contain small and randomly oriented densely packed hydroxyapatite crystals as found in acellular cementum as well as in the coronal cementum pictured in our electron micrographs. Our interpretation is supported by a lack of amelogenin expression from the cervical-most portion of the ameloblast layer (Figs. 8 and 9).

Our interpretation of cervical ameloblasts secreting an "amelogenin-less enamel" is supported by several of our earlier works on mechanisms of enamel crystal formation establishing that amelogenins are necessary for long and parallel crystals in higher vertebrates. We have established that inhibition of amelogenin synthesis yields a reduction of enamel hydroxyapatite crystals dimensions (Diekwisch *et al.,* 1993). We have further demonstrated that short and randomly

oriented crystals similar to the ones in "cervical enamel" are found in the primitive enameloid of sharks and rays (Slavkin and Diekwisch, 1996). Lastly, we found that the occurrence of elongated prismatic crystals appears to be associated with the presence of amelogenin in certain shark species (Gurinsky and Diekwisch, 2000). Thus we are proposing that the finding of coronal cementum-like tissue between ameloblasts and root dentin at the tooth cervix is due to the lack of amelogenins in this portion of the enamel layer and not an indication of a separate cementum layer in this area. Our suggestion that epithelial tissues might be capable of cementoid matrix synthesis is supported by histopathological observations by Gottlieb (1926) and Kronfeld (1938a,b) and by immunogold studies of rat molar enamel-free areas (Bosshardt and Nanci, 1997, 1998), the latter ones interpreted differently by the authors however.

Early removal of HERS from the root surface in humans as seen in the Gottlieb collection

In his specimen of human and porcine HERS, Bernhard Gottlieb observed that the "epithelium stops exactly in front of a new layer of cementum, partly uncalcified" and that the "epithelium covers the yet uncalcified part of the root separating it from the connective tissue" (Gottlieb, 1942). Gottlieb interpreted his findings to indicate that the main function of HERS was to separate the root surface from the connective tissue until cementum was formed (Gottlieb, 1942). He believed that the periodontal ligament connective tissue posed a continuous threat to the root surface and that HERS and cementum would go hand in hand to prevent the tooth from ankylosis and root resorption (Gottlieb, 1942). He therefore coined the term "Schutzzement" (protective cementum). Gottlieb, who had one of the finest collections of oral histology slides in his time, was one of the first to call attention to the biological importance of cementum (Kronfeld, 1933). It is likely to assume that Gottlieb's theories on cementogenesis were based on his extensive collection of human tooth sections in which HERS departs from the root surface prior to cementum formation. Gottlieb's concept of the protective role of the periodontium against ankylosis and root resorption is supported by replantation studies demonstrating ankylosis and root resorption following damage or removal of the root surface periodontal ligament (Nyman *et al.,* 1985; Hellsing *et al.,* 1993).

The Gottlieb collection contained a number of specimens in which the apical part of HERS was deflected away from the root surface at a 90^0 angle. These specimens were particularly revealing because they established a dynamic relationship between HERS and the developing dental follicle/periodontal ligament. Ligament fibers mixed with dental follicle cells approached the root surface at a 45^0 angle and inserted on the root surface coronal of the deflected HERS. The descending bundle of dental follicle/periodontal ligament cells inserted between HERS and root surface and deflected the entire apical portion of HERS away from the root surface. In these specimens, cementum was only formed coronal of the inserting dental follicle/periodontal ligament bundle in far distance from the deflected HERS. Based both on the orientation of the dental follicle/periodontal ligament and on the 90^0 deflection of HERS which at earlier stages of root development must have been parallel to the root surface, Gottlieb's slides suggest an apical migration of periodontal ligament tissues and a subsequent insertion of ligament cells between HERS and root surface. We have performed a number of organ culture and vital dye labeling studies to support our hypothesis of apical directed cell migration of the dental follicle and its derivatives, including cementum, ligament, and alveolar bone (Diekwisch *in press*).

The slides from the Gottlieb collection revealed that in human periodontal tissues, HERS cells formed a network of cells parallel to the root surface that was disintegrated prior to cementum formation. Our findings on the absence of HERS during human cementogenesis is supported by Schroeder who reports that "in contrast to previous statements in most current textbooks, Hertwig's root sheath does not cover much of the external surface of newly formed predentine, at least in human premolars. Rather, that surface at the advancing root edge is almost from its beginning accessible to connective tissue cells of the dental follicle proper" (Schroeder, 1992). Our studies of slides of the Gottlieb collection confirm that in opposite to rodents in which initially the entire root surface is covered by HERS, human HERS is fenestrated at an early stage of root formation and contains

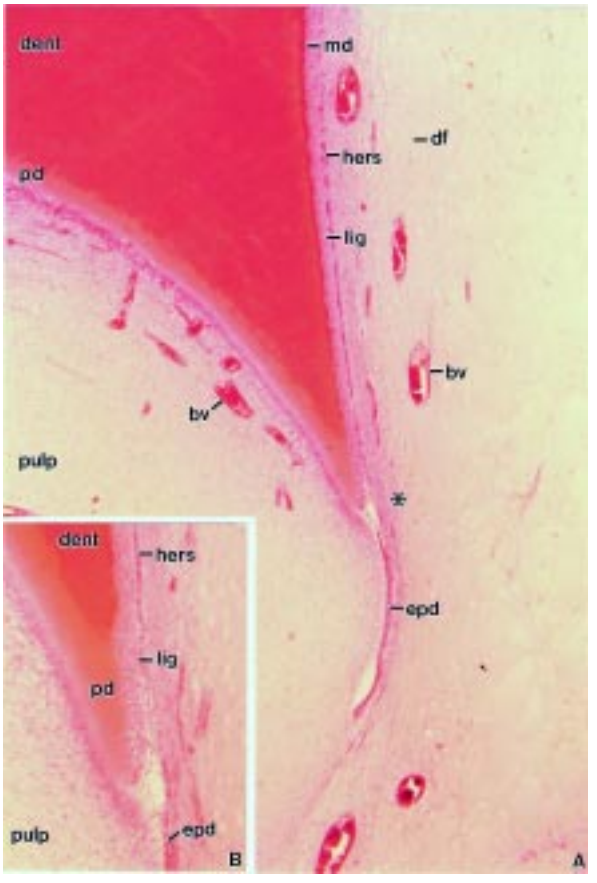

Fig. 6. Section of a developing human tooth germ from the collection of Dr. Bernhard Gottlieb at Baylor College of Dentistry in Dallas/Texas. (A) *The overview illustrates the position of pulp (pulp), dental follicle (df), mineralized dentin (md), periodontal ligament (lig), Hertwig's epithelial root sheath (hers) epithelial diaphragm (epd), predentin (pd), dentin (dent), and several blood vessels (bv), some of which are also featured in the insert (B).* **(B)** *The position of the insert is marked by an asterix (*). Note the position of epithelial and mesenchymal tissues on the predentin surface at the apical tip of the root. At the apical margin of the root dentin the periodontal ligament (lig) was in direct contact with the non-mineralized predentin (pd). Hertwig's root sheath (hers) was separated from the root surface by a periodontal ligament cell layer measuring at least 10 cell layers in thickness. The nude predentin surface (pd) was not covered by mineralized dentin or cementum. At this stage, mesenchymal cells of the ligament (lig) had direct access to the root surface. Due to their spatial separation, HERS cells had lost their opportunity to deposit cementum on the root dentin prior to their departure from the root surface. Magnification, 80x (A), 160x (B).*

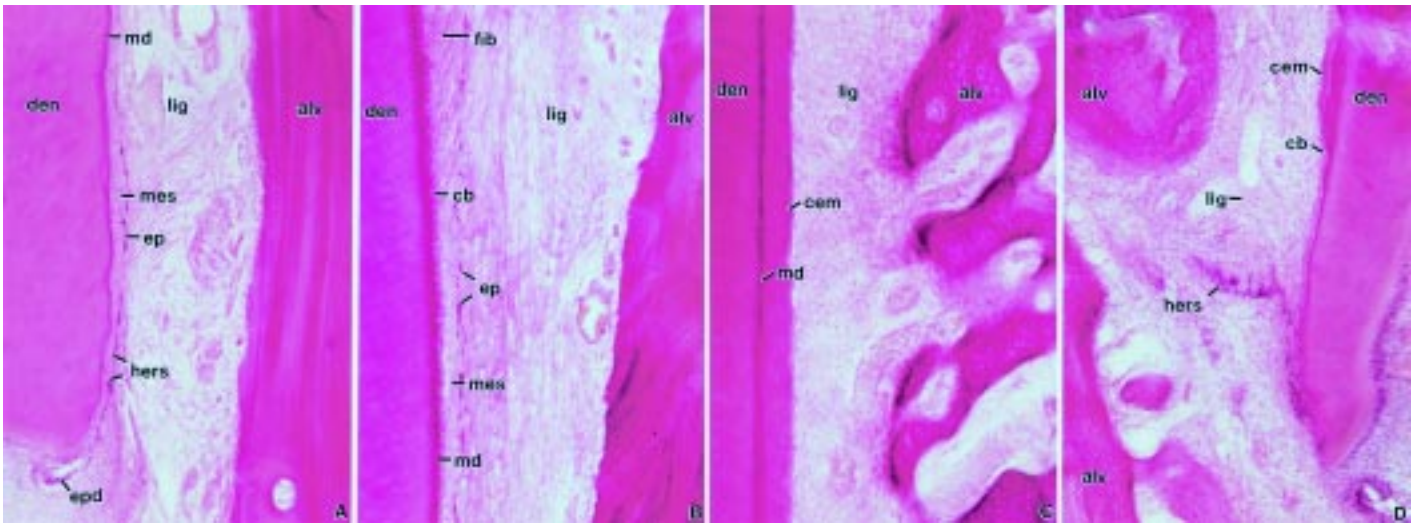

Fig. 7. Sections of the developing human periodontium from the collection of Dr. Bernhard Gottlieb at Baylor College of Dentistry in Dallas/Texas. *For orientation purposes, the position of root dentin (den), periodontal ligament (lig) and alveolar bone (alv) are indicated in all sections.* **(A)** *The localization of HERS (hers) prior to light microscopically-visible cementum deposition in the human. Human HERS formed a thin bilayered line of cells at a distance of five cell layers or more from the root surface prior to any light microscopically visible cementum deposition (A). The root surface was covered by mantle dentin (md). The space between epithelial cells (ep) and root surface was occupied by mesenchymal cells (mes). The epithelial diaphragm (epd) was located at the apical tip of the root.* **(B)** *Micrograph of the developing periodontal ligament in the mid-root area. The space between epithelial cells (hers) and root dentin (den) was occupied by mesenchymal cells (mes). A row of densely stained cementoblasts (cb) reflecting the onset of cementogenesis was lining the root surface. The root surface was covered by mantle dentin (md), staining darker than the adjacent circumpulpal dentin (dent). In the coronal portion of the area, fibers (fib) were inserting at the developing root surface. Note the orientation of inserting fibers (fib) from coronal to apical. Fibers (fib) were typical oblique fibers of the human mid-root area.* **(C)** *is from a further advanced stage of cementogenesis in the human. A layer of acellular cementum (cem) covered the root dentin (den). A dark red line representing mantle dentin (md) separated cementum (cem) and circumpulpal root dentin (dent). Epithelial cells and HERS were not present.* **(D)** *represents the stage of HERS disintegration and initial cementum deposition in the human. Cementoblasts (cb) had covered the developing root surface and deposited an initial layer of cementum (cem). The network of HERS (hers) was deflected away from the root surface and the area of initial cementogenesis at a 90º angle allowing for mesenchymal cells to access the developing root surface. Note the orientation of periodontal ligament fibroblasts toward the root surface from coronal to apical similar to (B). Magnification, 200x.*

wide mesenchymal meshes with thin epithelial frame lines far removed from the root surface and potential sites of cementum deposition. Schroeder is one of the few authors to pay attention to species differences in the presentation of Hertwig's root sheath toward the surrounding mesenchyme (Schroeder, 1992). We are not arguing that cementogenesis in humans and rodents are of completely different nature but rather that rodent molar cementogenesis encompasses a particularly dense series of events that might easily generate misconceptions. To support our argument, we have performed a detailed analysis of the evolution of Hertwig's root sheath throughout vertebrate evolution (Diekwisch, *submitted*) in which we propose that HERS evolved in higher vertebrates to allow for the formation of a sophisticated gomphosis-like periodontal ligament. Our studies of Gottlieb's slides suggest that the number of HERS cells decreases throughout human root development and eventually becomes transformed into a delicate net confirming previous studies by Simpson (1967), and Tertel-Kalweit and Donath (1985). Even in rodents, distances between individual HERS cells increased with advanced root development providing spaces for mesenchymal cells to access the root surface (Wesselink and Beertsen, 1993). Together, our observations in conjunction with the studies quoted above indicate that cell number and density of HERS decrease throughout mammalian root development.

In our examination of the Gottlieb collection, three stages of human cementogenesis were distinguished: (i) HERS and no cementum deposits, (ii) HERS removed from the root surface and initial cementum deposition, and (iii) thick cementum layer with no HERS or few HERS cells present. In opposite to rodent HERS, human HERS had already departed from the root surface prior to any visible signs of cementum deposition. In rodents however, HERS was still in contact with the root surface when initial cementum deposits were light microscopically visible. This indicates that in comparison to rodents, HERS plays a less significant or no role during human cementogenesis.

Role of amelogenins in cementogenesis

The presence or absence of amelogenin or other enamel proteins in acellular and intermediate cementum have been debated for a number of years (reviewed in Hammarstrom *et al.,* 1996; Ten Cate 1996a,b). Infrequently, reports on the presence of amelogenin in cementum appear in the literature (Slavkin *et al.,* 1988; Hamamoto *et al.,* 1996; Fong *et al.,* 2000). Such reports need to be considered with greatest care, particularly since both the study of Luo *et al.* (1991) and our present studys did not detect any amelogenin transcripts in cells of Hertwig's epithelial root sheath. The absence of detectable amounts of amelogenin mRNAs in Hertwig's root sheath epithelium obviously raises the concern of how amelogenins were able to appear at the root surface as some of the above studies suggest. Specificity issues of immunoreactions might be likely explanations. It has been repeatedly demonstrated that a number of polyclonal amelogenin antibodies crossreact with keratins (Aoba *et*

al., 1992). Keratins are intermediate filaments typically found in many epithelial cells including HERS. Antibodies against the recombinant M179 amelogenin (Simmer *et al.* 1994) that we have used probably pose the best choice but have not been used in any of the other studies. One study has reported on a novel enamel protein, amelin, in cells embedded in cementum (Fong *et al.,* 1996). The significance of this finding remains to be determined since the function of amelin is unknown.

In situ hybridization detection of amelogenin transcription signals demonstrated that amelogenin transcripts were limited to coronal ameloblasts and were absent in Hertwig's epithelial root sheath or in other cells of the developing root surface confirming an earlier study by Luo *et al.* (1991). Previously, immunological similarities between cementum and enamel proteins had been postulated (Slavkin and Boyde, 1974; Schonfeld, 1975; Slavkin, 1976; Schonfeld and Slavkin 1977) and amelogenin would have been a candidate molecule to be present both in enamel and cementum. Since the original publication by Luo *et al.* (1991) some critics have expressed concerns about the exact position of the crown/root boundary, which might have been obscured in darkfield micrographs. In order to address this concern and to clearly identify the crown/root boundary on the same micrograph, we used brightfield illumination and hematoxylin as a counterstain. Using this technique, our data confirmed the statement that HERS cells do not transcribe full-length amelogenin (Luo *et al.,* 1991) at all stages investigated (Fig. 8). Cells along the root surface were completely devoid of amelogenin hybridization products (Fig. 8). Amelogenin-specific hybridization signals were exclusively detected in the crown ameloblasts. In addition, we also did not detect any amelogenin in the cervical-most ameloblasts indicating that amelogenin expression was limited exclusively to crown ameloblasts and did not extend toward the root surface (Fig. 8). We have earlier discussed the significance of the absence of amelogenin from the cervical crown margin in relationship to coronal cementoid deposition. The absence of amelogenin signals from the cervical crown margin also answers the question of a possible oversight of amelogenin expression toward the root surface due to a less than defined crown margin. An interesting observation related to root mineralization was the detection of low-level amelogenin hybridization signals in the odontoblast layer which might support recent discoveries on the presence of polypeptides containing an amino-terminal amelogenin fragment in dentin matrix extracts (Nebgen *et al.,* 1999).

The presence or absence of amelogenin epitopes along the developing root surface has been a topic of great interest (Thomas *et al.* 1986; Slavkin *et al.* 1988). While not completely eliminating the possibility of antibody trapping, immunoperoxidase techniques exclude the possibility of false-positive results due to autofluorescence. We therefore used an immunoperoxidase strategy and antibodies against a recombinant M179 to define the localization of amelogenins at the tooth cervix. Confirming earlier results (Thomas *et al.,* 1986), we detected amelogenin exclusively in the enamel layer and in secretory ameloblasts but not in the root area (Fig. 9). Even under conditions optimized for maximum amelogenin staining, amelogenin was not localized in cells along the root surface and was neither present in the initial enamel matrix nor in the ameloblasts of the cervical crown margin. The absence of amelogenin in the cervical-most enamel matrix corresponds with the lack of organized enamel-like hydroxy-apatite crystals in this region and has been discussed earlier (Fig. 4).

Using Western blotting, we did not detect any amelogenin cross-reactivity in porcine cementum extracts while porcine enamel extracts reacted positively (Fig. 10). Since we were using an antibody against mouse amelogenin on porcine tooth extracts, one might argue that the mouse antibody might not have recognized porcine amelogenins. This is highly unlikely however since the mouse

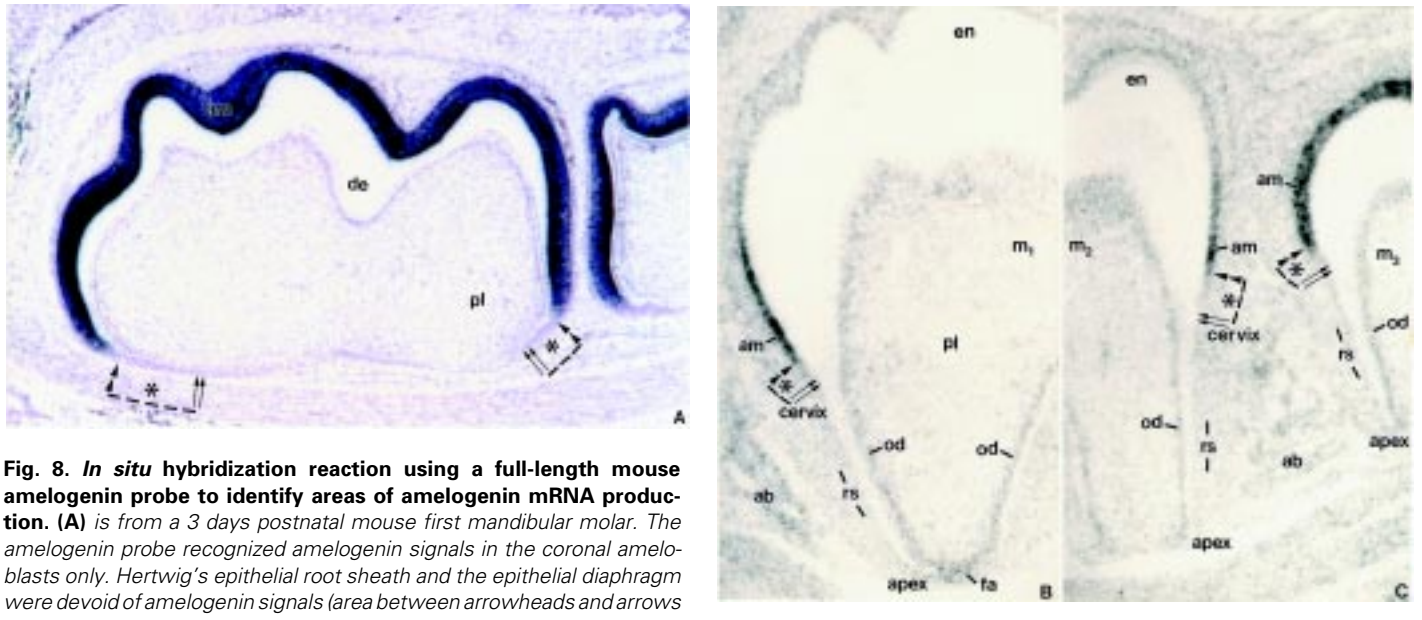

Fig. 8. *In situ* hybridization reaction using a full-length mouse amelogenin probe to identify areas of amelogenin mRNA production. (A) *is from a 3 days postnatal mouse first mandibular molar. The amelogenin probe recognized amelogenin signals in the coronal ameloblasts only. Hertwig's epithelial root sheath and the epithelial diaphragm were devoid of amelogenin signals (area between arrowheads and arrows marked by an asterix). The counterstain illustrated that the apical border of the amelogenin signals (arrowheads) was distinctly coronal of the tooth cervix. Dentin (de) and pulp (pl) were labeled for orientation purposes.* **(B,C)** *are from 10 days postnatal mouse molars. Amelogenin specific signals were detected in ameloblasts (am) of first (m_1), second (m_2), and third (m_3) mandibular molars. At this stage, the distance between the most apical amelogenin signal and the tooth cervix was 40 μm. The root surface (rs) was devoid of amelogenin specific signals. Enamel (en), odontoblasts (od), alveolar bone (ab) and tooth apex (apex) were labeled for orientation purposes. There was an above-background hybridization of amelogenin signals at the foramen apicale (fa). Magnification, 200x (A), 400x (B,C).*

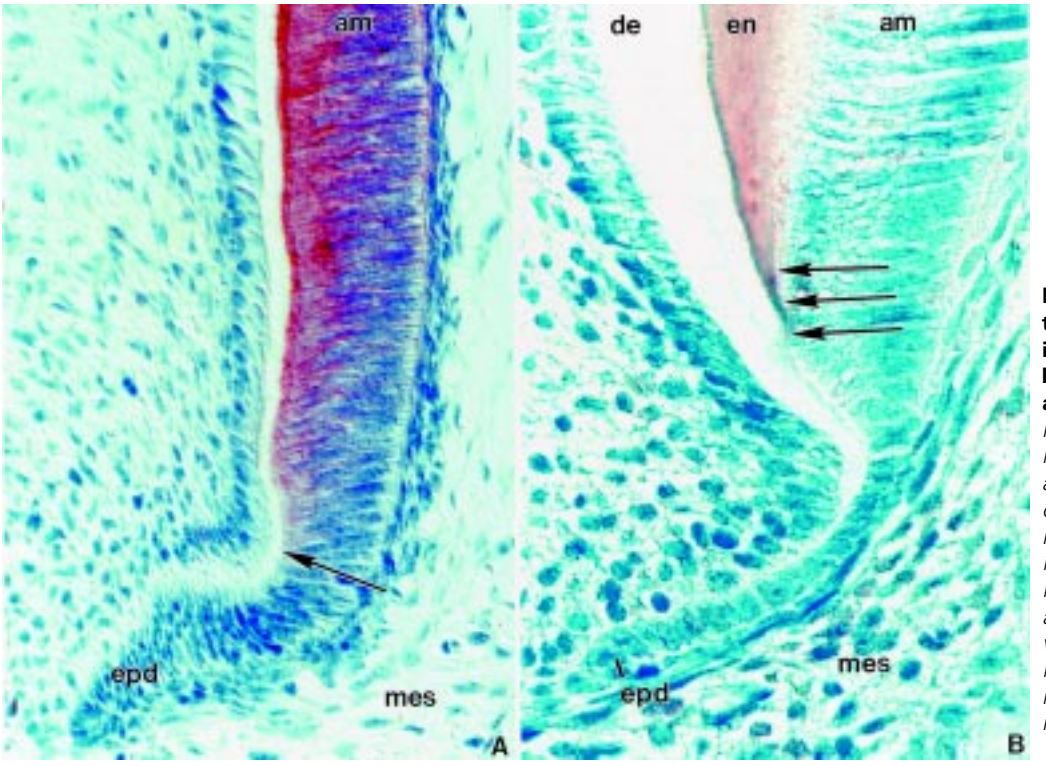

Fig. 9. Immunohistochemical distinction of amelogenin positive epitopes in the developing root using an antibody against a recombinant M179 amelogenin. *This antibody only recognized amelogenin epitopes in the enamel layer (en) and at the secretory pole of the ameloblast cells (am). No staining was observed in the epithelial diaphragm and in HERS (epd) nor in the developing root nor on the dentin (de) surface. The arrows indicate the cervical/apical limit of amelogenin protein distribution which was still within the coronal ameloblast layer.* **(A)** *is of a 3 days postnatal mouse molar while* **(B)** *is of a 12 days postnatal mouse molar. Magnification, 1,500x.*

antibody did detect porcine crown amelogenins in enamel extracts treated under identical conditions. The likelihood of a polyclonal mouse amelogenin antibody to react against pig amelogenins is enhanced by the high sequence similarity between mouse and porcine amelogenin (Simmer *et al.,* 1994, Hu *et al.,* 1996). One might further argue that amelogenins, if present, were localized at the dentin-cementum junction and not throughout the cementum layer. In order to account for this criticism, we chose fairly extensive root surface preparations that extended well into the root dentin surface. Even under these conditions, there was no evidence for amelogenins on the pig root surface, indicating that amelogenins do not play a role in cementogenesis.

The discussion about the presence or absence of enamel proteins on the root surface also raises the overall question about the functional importance of enamel proteins as constitutive factors during cementogenesis. This question is not about whether trace amounts of minor enamel proteins can be detected in small areas or isolated cells of the root surface, nor is it about whether fragments of enamel proteins are capable of stimulating mineralized tissue formation on the root surface. The question is rather whether enamel proteins play a functionally significant role during cementogenesis. In order to answer this question it is important to consider the predominance of collagen and bone-like proteins as principle matrix components of the bone-like cementum matrix (Somerman *et al.,* 1993; d'Errico *et al.,* 1997). The similarities between cementum and bone mesenchymal matrix structure become particularly obvious in comparison to the massive effects of the amelogenins as typical epithelial matrix proteins on the formation of enamel crystals (Diekwisch *et al.,* 1993). It further needs to be considered that cellular cementum is continuously remodeled and re-formed throughout the lifetime of mammalian teeth and at later stages of development completely surrounded by mesenchymal cells. From this perspective it becomes clear that cementum is a product of mesenchymal secretory cells and any putative role of epithelial cells is marginal. There have been reports on the successful clinical use of enamel matrix protein extracts (Emdogain) to stimulate acellular cementum formation (Hirooka, 1998). These studies are relatively recent and it is not clear which component of the enamel matrix protein extract is associated with the formation of new acellular cementum. While we cannot exclude the possibility that enamel proteins might have the capability to stimulate aspects of periodontal regeneration this alone is no evidence that enamel proteins stimulate cementogenesis during normal root development. Considering the evidence provided in this study on the mesenchymal origins of cementum, the putative

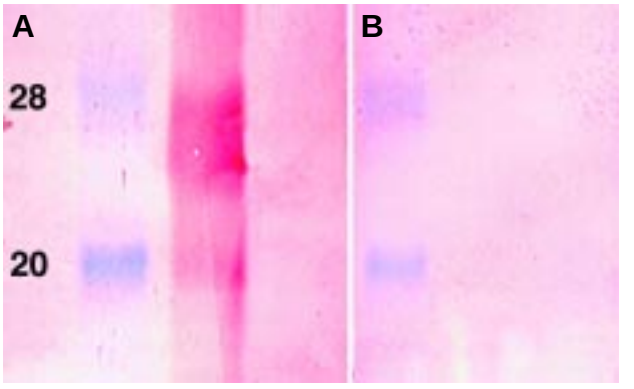

Fig. 10. Western blotting with mouse recombinant M179 amelogenin antibodies. (A) *In porcine enamel protein extracts, strong bands in the region of 20, 25, and 28 kDa were detected following Western blotting with mouse recombinant M179 amelogenin antibodies.* **(B)** *No signals were detected in cementum extracts using the same amounts of proteins and identical technique.*

induction of new acellular cementum formation using enamel protein extracts, if there is any, may not be based on a role of amelogenins during normal development.

Possible mechanism of cementoblast induction

Based on our findings amelogenin does not appear to be the major inductive agent to stimulate cells of the dental follicle to become cementoblasts and secrete cementum. At present, the mechanism of cementum formation remains unclear, but recent advances in our understanding of tooth developmental biology have presented a number of possibilities, including (i) the induction of cementoblasts by the underlying dentin matrix and (ii) the induction of cementoblasts by extracellular matrix molecules. Components of the underlying dentin matrix might be a possible source of signals contributing to the onset of cementogenesis. Wachtel *et al.* (1989) have provided evidence for an inductive role of hydroxyapatite in cell culture. There has also been evidence for amelogenin splice products in the pulp that might have a mineral-inducing role (Nebgen *et al.,* 1999, Veis *et al.,* 2000). Recent data on a possible migration of dentin matrix proteins from the pulp toward the ameloblast layer (Goldberg *et al.,* 2000) present the possibility of pulp-derived signals that might impact cell differentiation in ameloblasts as well as HERS cells or cementoblasts. Lastly, numerous extracellular matrix components might play a similar role during cementoblast differentiation as they play at the onset of ameloblast differentiation (Thesleff and Hurmerinta, 1981). Candidate molecules include basement membrane components such as laminin and fibronectin, integrin cell surface receptors, and peptide growth factors that interact with extracellular matrix components. Further understanding of the signaling cascades involved in the onset of cementogenesis will elucidate the mechanisms contributing to the formation of this intriguing tissue.

Summary

In conclusion, we have reviewed an extensive literature on early cementogenesis and performed a detailed morphological and molecular analysis to illustrate and verify key issues in the current debate about epithelial and mesenchymal contributions to root cementum. We have demonstrated that prior to cementogenesis, Hertwig's epithelial root sheath disintegrates and dental follicle cells penetrate the epithelial layer to invade the root surface. Our studies confirmed that HERS became disrupted or disintegrated prior to cementum deposition. We visualized how mesenchymal cells from the dental follicle penetrated the HERS bilayer and deposited initial cementum, while immediately adjacent epithelial cells were separated from the root surface by a basal lamina and did not secrete any cementum. Human specimen from the Gottlieb collection indicated that HERS was removed from the root surface prior to cementum deposition. Our *in situ* hybridization and immolocalization data revealed that both amelogenin mRNAs and enamel proteins were restricted to the crown enamel and were absent from the root surface and from the cervical-most ameloblasts adjacent to the root margin. On Western blots, cementum protein extracts did not cross-react with amelogenin antibodies. Our studies in conjunction with our literature review together confirmed the classical theory of cementum as a dental follicle derived connective tissue that forms subsequent to HERS disintegration.

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