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Evolution and Development of Hertwig's Epithelial Root Sheath

Xianghong Luan, Yoshihiro Ito, and **Thomas G.H. Diekwisch**
Brodie Laboratory for Craniofacial Genetics and Department of Oral Biology, The University of Illinois College of Dentistry, Chicogo, IL

Abstract

Periodontal regeneration and tissue engineering has re-awakened interest in the role of Hertwig's Epithelial Root Sheath (HERS), an epithelial tissue layer first discovered in amphibians more than a century ago. Using developmental, evolutionary, and cell biological approaches we have therefore performed a careful analysis of the role of HERS in root formation and compared our data with clinical findings. Our developmental studies revealed HERS as a transient structure assembled in the early period of root formation and elongation and subsequently fenestrated and reduced to epithelial rests of Malassez (ERM). Our comparative evolutionary studies indicated that HERS fenestration was closely associated with the presence of a periodontal ligament and a gomphosis-type attachment apparatus in crocodilians and mammals. Based on these studies, we are proposing that HERS plays an important role in the regulation and maintenance of periodontal ligament space and function. Additional support for this hypothesis was rendered by our meta-analysis of recent clinical reports related to HERS function.

Keywords

Periodontium; Development; Cementum; Apoptosis; Darwinian Medicine

A. Hertwig's Epithelial Root Sheath - from Comparative Anatomy to Clinical Relevance

Hertwig's Epithelial Root Sheath (HERS) was first discovered as a bi-layered cell sheath surrounding the roots of amphibian teeth (e.g. in *Triturus* and *Salamandra)(flertwig,* 1874). HERS descends from the oral epithelium and the enamel organ to form a collar around the cervical part of the amphibian tooth root (Fig. 1). The image of a continuous epithelial cell layer covering the newly formed root surface like a glove or a second skin and seemingly logically contributing to the secretion of mineralized tissue on the dentinous root surface has found entry into many textbooks and also turned into a welcome scientific explanation for one of dentistry's first biotech enterprises (Hammarström et al., 1996; Hammarström, 1997). Yet, many current concepts of the function of HERS during root formation ignore the profound differences between amphibian and mammalian tooth roots. While Hertwig's original report faithfully recounted root formation in amphibian teeth, mammalian HERS is more or less a transitory entity, loosing its integrity already at the very moment of tooth root elongation.

Author for correspondence: Thomas G.H. Diekwisch, D.M.D., Ph.D. (sc.), Ph.D. (phil.), Professor and Head, Department of Oral Biology, Director, Brodie Laboratory for Craniofacial Genetics, Allan G. Brodie Endowed Chair for Orthodontic Research, Professor of Anatomy/Cell Biology, Bioengineering, Orthodontics, Periodontics, UIC College of Dentistry, 801 South Paulina Street, MC 841, Chicago, Illinois 60612, Phone: (312) 413 9683, FAX: (312) 996 6044, e-mail: tomdkw@uic.edu, Web: http://dentistry.uic.edu/CraniofacialGenetics, Oral Biol: http://dentistry.uic.edu/Depts/oralb/welcome.htm.

Current interest in regenerative dentistry and periodontal disease has sparked the debate over the roles of tissues, cells and factors in the development and regeneration of the periodontium. As a part of this trend, much attention has been devoted to the role of HERS as it relates to cementogenesis (Zeichner-David et al., 2003; Yamamoto et al., 2004). In the current review we are proposing that there is more to HERS than its proposed role in cementogenesis. We have therefore conducted a thorough developmental analysis to document the systematic and extensive fenestration of HERS since its inception, its collapse into rests of Malassez after root development, and its later-life fate during development and thereafter. In addition, and in order to synthesize the striking differences between amphibian and mammalian HERS and to infer implications toward HERS function in humans, we have performed an in-depth study of HERS in a variety of vertebrate lineages, ranging from chondrichthyans to men. Our analysis revealed a gradual transition from a coronal enamel organ cap in chondrichthyans and teleosts, over the continuous cervical epithelial collar facilitating apical ankylosis in amphibians, to the transient epithelial network of crocodilians and mammals, allowing for mesenchymal cells of the dental follicle to trespass the epithelial barrier and secrete cementum on the dentinous surface of the root. We have correlated these investigations on the developmental and evolutionary biology of HERS with several clinical reports, which are supporting a concept of HERS as a regulator of periodontal ligament biology as it relates to width, blood vessel homeostasis, and cementogenesis, as well as protection against resorption and ankylosis.

B. Development of Hertwig's Root Sheath in Mice

The development of Hertwig's root sheath begins with the formation of a bilayered extension of the inner and outer dental epithelium from the cervical loop of the enamel organ. The epithelial double layer continues to grow in apical direction outlining the shape of the future root of the tooth. During the initiation phase of root development, the transcription factor NHI-C/CTF appears to play a pivotal role in root odontoblasts (Steele-Perkins et al. 2003) that possibly interact with HERS. The morphological disintegration of HERS begins with the dissociation of the outer basement membrane of HERS. At this stage, HERS cells lose their cuboidal form and become flattened, and the outer epithelial layer breaks up before the inner one (Andujar et al., 1984, 1985; Owens, 1978). A final step prior to general dispersal of HERS is the disintegration of the basal lamina of the inner layer (Owens, 1978). During further root development, HERS breaks up into epithelial nests and cords, allowing for other cells to come in contact with the outer dentin surface (Owens, 1978; Diekwisch, 2001, 2002).

There has been considerable attention toward the onset of HERS development and root formation (reviewed in Diekwisch, 2001). Yet, questions related to the role of HERS in relationship to cementogenesis and root morphogenesis remain. We have therefore performed a systematic study of the temporo-spatial distribution and development of HERS throughout mouse molar root formation using keratin as a marker for epithelial cells along the root surface. Immunohistochemical cell-tagging via keratin labeling is based on previous studies in which keratin expression has been used as an epithelial cell marker during normal tooth development (Lesot et al., 1982; Smith et al., 1990, Lombardi et al. 1992). Moreover, several investigators have demonstrated keratin expression by HERS (Alatli et al., 1996; Kaneko et al., 1999; Onishi et al., 1999). Specifically, the basal keratinocyte marker Keratin 14 (Wetzels et al., 1989) has been documented in rests of Malassez using immunohistochemistry and monoclonal antibodies (Gao et al., 1988). In our study, we have performed immunoreactions using an anti-pan-keratin antibody as a marker for epithelial cells along the root surface (Figs. 2 and 3). In addition, we have generated Keratin 14 transgenic mice in which the KI4 gene served as a powerful marker for Hertwig's root

sheath and epithelial rests of Malassez (Figs. 4 and 10). In these studies, the K14 transgene was detected using the lacZ reporter gene and stained with p-galactosidase.

Onset of HERS development in mice as a bilayer extension of the outer and inner enamel epithelium

We have analyzed the beginning of HERS development in mice using both immunohistochemical and KI4 transgenic keratin-based marker systems (Figs. 2 and 4). At the onset of HERS elongation from the cervical loop, cells from the ameloblast layer and from the outer enamel epithelium extended in apical direction (Fig. 2). Subsequently, HERS formed a continuous bi-layer of flat, cuboidal cells, at the interface between dental follicle and dental pulp (Fig. 4C).

During mouse molar root development HERS epithelial cells became increasingly dissociated

In order to follow the fate and distribution of HERS cells along the developing roots surface, we have marked epithelial cells along the developing root surface with anti-keratin antibodies to follow the fate of HERS cells during root formation. The set of figures from mouse molar tooth organs between lday and 20 days postnatal illustrates the increasing distance between individual HERS cells as a consequence of root elongation (Fig. 2). From 5 days to 20 days postnatal there was a five-fold increase of the distance between individual HERS cells. While the distance between individual HERS cells increased progressively, mesenchymal cells populated the spaces between the epithelial cells. The epithelial diaphragm as the most apical portion of HERS remained intact throughout all stages investigated.

In 10 days postnatal mouse molars HERS formed a network of cells in proximity to but not in contact with the developing root surface

In order to understand the three-dimensional relationship between HERS cells and the developing root surface we performed three-dimensional reconstructions of anti-keratin labeled serial sections of a 10 days postnatal developing mouse first mandibular molar (Fig. 3). The parasagittal sections in Fig. 3B illustrate a line of epithelial cells in close proximity to the root surface. A 30° horizontal rotation of the parasagittal montage (Fig. 3 A) revealed that the row of epithelial cells apparent in the parasagittal view was the lateral view of a fenestrated network of epithelial cells in close proximity to the root surface.

Keratin 14 as a marker for Hertwig's root sheath in mice

Our studies established the Keratin 14 transgene as a highly specific marker gene for cells of Hertwig's epithelial root sheath (HERS) and epithelial rests of Malassez (ERM). Our findings indicate that the KI4 transgene was expressed by many epithelial tissues such as the oral epithelium or the enamel organ (Fig. 4). However, HERS and ERM were the only structures identified as carriers of the KI 4 transgene along the root surface and in its periphery (Figs. 4 and 10). Connective tissues such as root dentin, alveolar bone, pulp, odontoblasts, and periodontal ligament were not marked for the KI4 transgene (Figs. 4 and 10). Moreover, all epithelial tissues in the root area contained the K14 transgene (Figs. 4 and 10). We thus suggest that the KI4 transgene is a viable marker for HERS and ERM epithelial cells along the root surface.

Specifially, our KI4 transgene expression studies visualized the defined KI4 expression pattern of the HERS bilayer and the epithelial diaphragm (Fig. 4) in one week postnatal mice (Fig. 4). At this stage, the HERS bilayer was continuous (Fig. 4). Subsequent stages at two and four week postnatal revealed the fenestration of HERS into a network of HERS

cells (Fig. 4). In six months postnatal mice, the KI4 transgene identified the aggregated cell rests of Malassez along the root surface (Figs. 4 and 10).

Mammalian HERS as a transient tissue layer in development

In summary, our data indicate that in mice, there is only a short period during which HERS emerges from the cervical loop and forms a continuous sheath tightly surrounding the newly developed root surface. In humans, there is considerable distance between HERS and the root surface already at the onset of root formation (Diekwisch, 2001). Both, in mice and in humans, and in all other mammals to our knowledge as well, HERS breaks up into epithelial cords at the very beginning of cementogenesis (Diekwisch, 2001) and allows for mesenchymal cells of the dental follicle to access the root surface (Diekwisch, 2002). Throughout the period of root elongation, HERS continues to dissociate while windows of mesenchymal cells in between cords of the ever thinning HERS network increase in size until HERS' final collapse into epithelial rests of Malassez. We are proposing that based on our marker studies, mammalian HERS is a fleeting, transient structure and profoundly different from the highly organized dense layers of mineral secreting cells such as ameloblasts and odontoblasts. We further argue that a morphological mineral layer pendant to the reticulated structure of the HERS network as a mineralized deposit on the root surface has yet to be described. We have therefore applied an evolutionary developmental approach to further reveal the function of HERS in mammals.

C. Evolution of HERS from Fish to Human

Hertwig's root sheath is one of the few tissues that were first discovered in amphibians and not in mammals. The reason behind Hertwig's early discovery of HERS in amphibians was likely related to the longevity of HERS in the amphibian dentition - mid-saggital sections of amphibian jaws reveal ample opportunities to view root sheaths at all stages of development. In the current study, we have investigated trends in HERS patterning from fish to human as a means to deduce clues about its function and involvement in root formation. The approach we have taken is not unlike an evolutionary medicine (Darwinian medicine) approach as it has been suggested by others (Kaifu et al., 2003; Trevathan et al., 1999) to detect general trends in evolutionary biology to the benefit of developmental biology or medicine. A summary of the species and lineages investigated for the present study is presented in Fig. 12.

In non-mammalian vertebrates, HERS continuity was not disrupted during development allowing for cementum deposition on the root surface apical of HERS

An extensive analysis of HERS position and fate in non-mammalian vertebrates was performed to provide further information about the origins of the tissues involved in root formation. The results from our studies of non-mammalian vertebrates were compared to the position and fate of HERS in mammalian periodontia of mice and men. Here we are summarizing six common features of root development in basal vertebrates as they relate to this study: (i) In all basal species investigated, keratin-labeled dental epithelium extended only over the coronal aspect of the entire tooth length, including the coronal portion of the tooth root (Figs. 5-7). The apical portion of the root surface was always free from epithelial cells, even though HERS covered the coronal part of the root surface in some species such as the axolotl (Figs. 5-7). The extend to which HERS descended toward the root apex varied in different species. In gecko and iguana reptiles as well as in guppy fish and in the chondrichthyans shark and ray, the keratin-labeled dental epithelium formed an epithelial cap mostly covering the tooth crown (Figs. 5 and 7). In contrast, amphibian teeth were distinctly separated into three portions: a coronal enamel organ cap, a cervical extension of HERS, and an apical portion of the root surface that was not covered by HERS (Fig. 6). The

dimensions of the enamel organ, HERS, and the apical root end in relationship to the entire tooth length varied significantly between axolotl and frog. Axolotl teeth were characterized by a root sheath that covered most of the developing root surface and left only the lower fifth to one third of the root accessible to mesenchymal tissues (Fig. 6). Many Urodelian dentitions also feature islands of epithelial cells in the non-mineralized interproximal connective tissue between teeth (Fig. 6). In opposite, frog teeth featured a diminutive enamel organ and an overextended apical root end (Fig. 6). (ii) Basal vertebrate HERS formed a continuous layer throughout development; i.e. HERS in fishes, amphibians, and non-crocodilian reptiles was not fenestrated and did not form an epithelial diaphragm (Fig. 5-7). As a consequence of HERS continuity, there were no epithelial rests of Malassez in basal vertebrates (Figs. 5-7). (iii) In fishes, amphibians, and non-crocodilian reptiles, the apical root surface was covered by mesenchymal cells, cementum or bone (Figs. 5-7). (iv) In most of the non-mammalian vertebrates investigated, a bridge-like cementoid tissue connection was detected between individual teeth amongst each other as well as between individual teeth and mandibular bone (Figs. 6-7). The cementoid tissue between individual teeth was visible in the bone-carrying species gecko, iguana, frog, axolotl, and guppy (Figs. 5-7) while it was missing in the continuously erupting teeth of the chrondrichthyans ray and shark (Fig. 5). (v) The enamel organs of individual teeth were connected with each other as well as with the oral epithelium through a band of epithelium, the general lamina (e.g. Figs. 6,7).

Rare human specimen of the Gottlieb collection revealed characteristic reticulated shapes of human HERS in close association with nervous ganglia

The numerous preparations of HERS in developing human teeth belong to the most precious items of the Gottlieb collection at Baylor University in Dallas/Texas. In a previous study, we have described the significant distance of HERS from the developing root surface in humans (Diekwisch, 2001). Here, we are using Gottlieb's large size thick sections of human HERS to reveal the fine network of human epithelial cells spanning many areas of the root surface (Fig. 8). Our micrographs illustrate filigree epithelial cords measuring one or two cell layers in thickness and surrounding mesenchymal windows of 100pm in width (Fig. 8). Ganglia cells and nervous plexus were detected in close proximity of the "knots" of the cellular net spun by cords of HERS (Fig. 8). Our documentation of ganglia cells in close association with HERS is corroborated by earlier studies on periodontal neural endings intimately related to epithelial rest of Malassez in humans (Lambrichts et al., 1993). We suggest that the highly organized network structure of HERS in adult human teeth in tandem with the rich innervation indicate that HERS remains as an active tissue layer in the human periodontium.

Evolutionary stages in HERS evolution: cap, sheath, and net

Together, these studies reveal a gradual progression of HERS morphology and function from fish to human. Our studies allow for the distinction of three stages of HERS in vertebrates: (i) A narrow epithelial cap laterally and apically confined by the cervical loop in teleosts and chondrichthyans (Fig. 5). In chondrichthyans, tooth root attachment occurs via fibrous ligaments basal of the cervical loop. Teleosts feature deposition of cementoid tissues apical of the enamel organ, which anchor the teeth in the jaw. (ii) Continuous and elongated epithelial sheaths covering the coronal portion of the root shaft throughout the life of the tooth in amphibians and non-crocodilian reptiles (Figs. 6 and 7). At this stage, deposition of cementoid tissues occurs apical of HERS which in turn facilitates ankylotic attachment to the jaw bone. Moreover, islands of epithelial rests in the interproximal space further define non-mineralized regions in the coronal zone of the interdental space, (iii) In crocodilians and mammals, HERS has emerged as a transient structure evolving from a brief shaft associated with the initiation of root formation to a filigree network at later stages of root development

and a subsequent collapse of the network into epithelial rests of Malassez. At this stage, there is an intimate association between the penetration of HERS' epithelial cell barrier by dental follicle derived connective tissue cells and the subsequent establishment of a periodontal ligament replacing HERS as the principle tissue occupying the root surface. The similarities between mammalian and crocodilian dentition have been thoroughly documented in a previous study on the crocodilian attachment apparatus in *Caiman crocodilus* in which we have reported on the presence of a periodontal ligament together with a fenestrated HERS in the caiman (McIntosh et al., 2002). Together, our analysis closely links HERS morphology, evolution, and development with the evolution of the dental attachment apparatus from a simple ligamentous or ankylotic anchorage characteristic for basal vertebrates to the complex gomphosis-type embedding of the tooth organ featuring periodontal ligament and alveolar bone socket in mammals.

D. Fate of HERS

Cell proliferation assays have indicated significant proliferation of the cervical loop and HERS cells during initial root formation (Harada et al., 2002; Kawano et al., 2004; Ohshima et al., 2005). Moreover, it has been demonstrated that the number of HERS cells decreases throughout human development (Tertel-Kalweit and Donath, 1985; Yamamoto et al., 2004). It is therefore conceivable that only a portion of the cells that set out to form HERS remained viable and stayed within the root sheath. Four possible mechanisms for the reduction of the number of HERS cells are generally discussed, either that HERS cells undergo apoptosis (Kaneko et al., 1999; Cerri et al., 2000; Cerri and Katchburian, 2005), that they are incorporated into the advancing cementum front (Lester, 1969), that they undergo epithelial-mesenchymal transformation (Thomas, 1995), and/or that they migrate away from the root surface (Andujar et al., 1985).

Limitation of apoptosis signals to only a few cells in HERS and in the dental follicle

In our studies, we have used the TUNEL technique to detect indicators of apoptosis along the developing root surface (Fig. 9). Our studies revealed only sporadic labeling of isolated nuclei of HERS, of the developing dental follicle and of the periodontal ligament at a rate of 1-5 nuclei per section (Fig. 9). In previous studies, we have also documented the presence of crescent-shaped spaces indiciative of apoptosis in HERS cells using transmission electron microscopy (Diekwisch, 2001;Fig. 3E). These findings are in congruence with earlier reports who reported that only a few (Kaneko et al., 1999) or some (Cerri et al., 2000) HERS cells underwent apoptosis or cell death during root formation. Together, these studies indicate that while some HERS cells may undergo apoptosis at a rate representative for developing tissues, many HERS cells remain vital and become part of the adult periodontal ligament as rests of Malassez.

Incorporation of HERS cells into the developing cementum matrix

Our studies using KI4 as a marker for HERS cells and rests of Malassez revealed incorporation of epithelial cells along the root surface as well as at the apical tip of the root (Fig. 10). Our micrographs indicate that prior to incorporation into the cementum layer, epithelial cells became encapsulated and were engulfed by the mineralizing cementum matrix. In our studies, KI4 emerged as an exclusive marker for HERS epithelial cells and/or rests of Malassez along the root surface. There were no un-stained epithelial cells detected in the periodontal ligament, and KI4 did not label mesenchymal cells. Nevertheless, not all K14-labeled HERS cells or rests of Malassez were incorporated by the advancing cementum front - the majority of rests of Malassez remained in close proximity to the root surface (Fig. 4). Our labeling studies provide strong support for Lester's original 1969 discovery that epithelial cells of the root surface may be incorporated by cementum (Lester, 1969).

Where did all the epithelial cells go?

Our studies of mouse molar root development document the continuous growth of HERS during initial root formation (Figs. 2 and 4). The growth of HERS occurs by directed proliferation of the epithelial cells of the root sheath (Cho and Garant, 2000). At later stages of root development however, the distance between cords of HERS cells and the mesh diameter in the epithelial network covering the root surface increased (Figs. 3 and 4), lending support to the argument that during terminal stages of root growth and development, HERS proliferation rates do not match the proliferation rates of connective tissue cells contributing to root growth. It thus appears as if there were fewer HERS cells on the maturing root surface than on the early-onset root surface. Apoptosis occurred in all tissues in the proximity of the developing root (Fig. 9), supporting earlier studies by Kaneko et al. (1999) and Cerri et al. (2000). Yet, in light of the established role of apoptosis during normal development (Adams, 2002), our findings suggested that apoptosis rates of HERS did not exceed those of other tissues in the periphery of the developing root. Incorporation of HERS by the thickening cementum layer does occur (Fig. 10), but only in the mature root. Thus, epithelial-mesenchymal transformation as originally suggested by Thomas (1995) remains a viable alternative to explain the fate of HERS. Another possibility has been suggested by Andujar et al. (1985) and Wentz et al. (1950) who documented the migratory capacity of HERS cells and proposed that some of them migrate away from the root surface to form the rests of Malassez. Based on our studies of preparations of human HERS (Fig. 8; and Diekwisch 2001) which is removed several cell layers from the advancing cementum front, we are supportive of Andujar's and Wentz's concept, even though Yamamoto et al. (2004) dispute the possibility of HERS migration. Yet, it is not clear whether HERS cells simply move away from the root surface or whether other mechanisms contribute to their reduction and displacement over time.

E. Functional Implications

HERS and cementogenesis

It was Isaac Schour, who in his classic textbook wrote: "As soon as the dentin of the root begins to form, while the developing tooth is still within its bony crypt, connective tissue cells of the dental sac break through Hertwig's epithelial sheath, and arrange themselves along the dentinal surface" (Schour/Noyes, 1938). Using fluorescent dyes and transmission electron microscopy, we have provided experimental evidence visualizing the massive migration of dental follicle cells (Diekwisch, 2002) and their perforation of HERS (Diekwisch, 2001) in support of Schour's original concept. In sites of initial cementogenesis we have also documented that dental follicle cells accessed the root surface subsequent to penetration of the HERS barrier while HERS cells remained confined through a basal lamina, indicating that dental follicle cells and not HERS cells secrete initial cementum (Diekwisch, 2001). In the present study, we are adding evidence for the continuous fenestration of HERS and its collapse as rests of Malassez providing access for dental follicle/periodontal ligament cells to attach to the root surface. Together, our studies indicate that during root formation, HERS acts as a barrier that establishes root shape and may mediate cementum formation, but does not secrete cementum itself. Our results together with the clinical data presented above confirm Heretier's hypothesis that the absence rather than the presence of HERS epithelial cells is an essential requirement for the onset of cementogenesis (Heretier, 1982), yet, we are not excluding the possibility of an inductive role of HERS toward the initiation of acellular cementogenesis.

The possibility that HERS may in fact secrete cementum, or that HERS-derived products might be related to enamel-related molecules, and that these proteins might initiate acellular cementum formation has been favorably discussed by a number of authors (Owens, 1978;

Slavkin et al., 1989). A clinical product has been developed in association with the perceived role of enamel matrix proteins in cementogenesis, and a couple of studies have suggested that HERS cells are involved in the development of both acellular and cellular cementum (Alatli et al., 1996; Hammarström et al., 1996). However, others have questioned the presence of enamel proteins in cementum (Thomas et al., 1986), or the transcription of amelogenin (Luo et al., 1986; Fong et al., 1996; Diekwisch, 2001; Zeichner-David et al., 2003), or the presence of amelogenin proteins in cementum (Diekwisch, 2001). Consequentially, attention has been directed toward other enamel-related products of the enamel organ, especially ameloblastin and enamelin (Zeichner-David, 2001; Zeichner-David et al., 2003), which may explain possible clinical effects in addition to the broad spectrum of factors and molecules contained in such extracts,. Two molecules, ameloblastin, and dlx2, featured highly specific distribution patterns in HERS (Lezot et al., 2000; Zeichner-David et al., 2003) and may indeed play significant roles in HERS differentiation and function.

Another unique aspect of the present analysis is our meta-analysis of the position of HERS in the interface between jawbone, cementum, and pedestal (Fig. 11). Our summary-sketch (Fig. 11) illustrates that throughout vertebrate periodontal apparatus evolution, a plethora of closely related mineralized tissues contribute to the support and anchorage of teeth. Besides the jawbones (e.g. *Os dentale,* mandible, maxilla), these include the tooth-bound structures cementum, pedestal, and alveolar bone. While closely related on a morphological and molecular/biochemical level, they are nevertheless biomechanically separated and can be distinguished using various histochemical dyes. The present analysis suggests that cementum and pedestal are discernable in the non-mammalian periodontium, where cementum provides an interdental attachment between teeth while the pedestal establishes apical anchorage toward the jawbone. In crocodilians and mammals, the distinction between cementum and pedestal is less defined because of the loss of the ankylosed region between cementum and jawbone. While the mammalian alveolar bone appears to correspond to the non-mammalian pedestal, both in terms of position and cellular organization, it is not clear how the mammalian acellular and cellular cementum relate to their non-mammalian precursors. It is conceivable that the establishment of a non-mineralized zone via HERS-related gene products may have occurred in the center of a putative pedestal-precursor, leaving alveolar bone and cellular cementum as its derivatives on both borders of the periodontal ligament space.

Whether or not HERS is ultimately involved in cementogenesis, the quest for an alternative explanation for HERS function remains viable at this point. And while it is not impossible that HERS may have multiple functions, one might argue that a unique and defined structure such as HERS has only evolved because of a unique functional quality that gave organisms significant survival advantages to establish and support an entity as complex and discrete as mammalian HERS.

HERS and the periodontal ligament

The comparative anatomy studies presented here as well as a previous study on caiman teeth (McIntosh et al., 2002) have indicated that the presence of a fenestrated HERS is tightly linked with the establishment of a non-mineralized periodontal ligament in mammalian and crocodilian teeth that provides protected and elastic anchorage for highly evolved thecodont teeth via gomphosis. In non-crocodilian reptiles and amphibians, ankylotic acrodont and pleurodont attachment is missing from the cervical region covered by HERS, and cementoid tissues of attachment are confined to the apical portion of the root lacking HERS and allowing for a stable mineralized ankylosis-attachment in regions devoid of epithelial barriers. In teleosts or chondrichthyans, HERS is completely absent, allowing for complete ankylotic or ligamentous attachment apical of the enamel organ.

The classic theory on HERS function relates HERS with the establishment of root shape during root formation (Owens, 1978). This hypothesis has found support in an extensive study on autotransplantation of premolars in which variations in root growth were linked with damage to HERS (Andreasen et al., 1990). In addition, there have been a number of isolated clinical studies on the later-life remnants of HERS, the epithelial rests of Malassez (ERM), which together provide meaningful clues toward our understanding of the role of the epithelium in the mammalian periodontium. One paper reported on significant proliferation of Malassez epithelial rests during tooth movement indicative of their active role in the mature dentition (Talic et al., 2002). A second study from the field of orthodontics detected a loss of continuity of the ERM network and an incursion of blood vessels in tandem with orthodontic root resorption suggesting a loss of periodontal ligament homeostatic control possibly mediated by ERM (Kat et al., 2003). A third clinical study, this time on replantation of mandibular incisors in dogs, linked root resorption with an absence of ERM (Wallace and Vergona, 1990). This study indirectly recalls Gottlieb's original theory of the "Schutzzement" (Gottlieb, 1942). A final but pivotal study on the denervation of the inferior alveolar nerve revealed decreases in ERM population in tandem with a reduction of periodontal ligament space (Fujiyama et al., 2004). The results from this study also suggest that ERM may prevent root resorption and induce acellular cementum formation (Fujiyama et al., 2004).

Together, these clinically-oriented studies suggest that Malassez' epithelial rests are not only an accidental left-over of early embryonic development but rather play significant roles in (i) the regulation and maintenance of the periodontal ligament space, (ii) the prevention of root resorption and ankylosis, (iii) the maintenance of periodontal ligament homeostasis, (iv) induction (i.e. not secretion) of acellular cementum formation. In addition, several key molecules have been identified (e.g. HSP27, MMP-13, and BMP-2), which may support ERM in their roles related to cementum repair (Hasegawa et al., 2003) and cell migration (Leonardi et al., 2001, 2005).

We propose that these clinical reports in tandem with our developmental and evolutionary studies re-introduce HERS as the ultimate governor of the periodontal ligament, the regulator of its width and homeostasis and the shield against resorption and ankylosis. From an evolutionary biology perspective, HERS appears to have evolved first to provide elastic anchorage for and mediate eruption of amphibian teeth and then may have evolved to facilitate the formation of a non-mineralized periodontal ligament in crocodiles and mammals and maintain its functional integrity. During development, HERS fenestration allows mesenchymal cells from the dental follicle to penetrate the epithelial barrier and deposit cementum. A part of this function may be related to the induction of acellular cementogenesis, and future studies will provide definitive answers to address this important issue.

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Fig. 1. Sagital section of a tooth organ in the lower jaw of *Salamandra maculata* from the original drawing by Oscar Hertwig (1874)

The epithelial root sheath ("Epithelhülse", H) has been enhanced using blue color. Note Hertwig's intricate differentiation of tissues of the attachment apparatus, including *Processus dentalis* (F), *Os dentale* (Od), Cement (C), undecalcified zone between *Os dentale* and tooth crown (h), and tooth socket (So). Other tissues are labeled enamel (S), dentin (d), basement membrane (B), and enamel membrane (MS).

Fig. 2. Immunostaining of epithelial cells along the developing root surface using a polyclonal anti-keratin wide-spectrum screening antibody

Epithelial cells positively labeled with anti-keratin antibody were stained in brown color. Representative images of first mandibular molars of the following stages were selected: Fig. 2A: 1 day postnatal, Fig. 2B: 5 days postnatal, Fig. 2C: 10 days postnatal, and Fig. 2D: 20 days postnatal. For orientation purposes, the following cell layers were labeled: stratum intermedium (si), ameloblasts (am), enamel (en), dentin (de), pre-dentin (pd), odontoblasts (od) and HERS (hers). In Fig. 2A the ameloblast cell layer (am) and HERS were not separated. HERS developed as a cervical loop extension of the outer enamel epithelium (oee) and ameloblast (am) cell layers. From Fig. 2B (5 days postnatal) to Fig. 2C (10 days postnatal), the distance between the cervical margin of the ameloblast cell layer (am) and the most cervical HERS (hers) cell had increased more than five-fold. Between Fig. 2C (10 days postnatal) and Fig. 2D (20 days postnatal), the distance between single epithelial cells (ep) had significantly increased. The distance mark (d) in Figs. 2C and 2D demonstrates the two-fold increased distance between two epithelial cells (ep) proximal to the apical margin of the ameloblast cell layer (dj=50pm, d2= 100pm). Note the fibers (fib) inserting at the cervical portion of the developing root surface in Fig. 2D (20 days postnatal).

Fig. 3. Three-dimensional reconstruction of anti-keratin labeled serial sections of a 10 days postnatal developing mouse first mandibular molar

The reconstructed image consists of images of nine adjacent 5pm thick paraffin sections that were stained using anti-keratin antibodies and immunoperoxidase detection methods. Green (dentin, de) and dark blue (enamel, en) colors represent the mineralized tissues. The light blue color marks the epithelial cells layers ameloblasts (am), HERS (hers), and the epithelial cells between HERS and ameloblasts. Fig. 3A is a 30° horizontal rotation of the montage shown in Fig. 3B. Note the fenestrated network of epithelial cells covering the root surface in Fig. 3 A and the space (arrows) between root surface and epithelial cells demonstrated in Fig. 3B.

Fig. 4. Hertwig's root sheath as visualized in Keratin-14 transgenic mice

The KI4 transgene was detected using the lacZ reporter gene and stained with P-galactosidase. Fig. 4A is a sagital section through the mouse molar region (mi, m2, m3) of the lower jaw of a one week postnatal mouse. Oral epithelium (oe), enamel organ (eno), and Hertwig's epithelial root sheath (hers) were intensely labeled via the K14 transgene. Note the clearly outlined position of the epithelial diaphragm (dia) in the second molar (m2). In the further developed first molar (mi), HERS continuity was already interrupted, while HERS was continuous in the second molar (m2). Fig. 4B illustrates the network or HERS in two weeks postnatal mice (hers) covering the developing root surfaces of the first molar (mi). The position of the second molar (m2) and of the enamel layer (en) are marked for the purpose of orientation. This and the preparation in Fig. 4C are whole mount sections, in which the superficial aspect of the tooth and jaw had been removed to allow visual access toward the root surface. Fig. 4C. Whole mount preparation of a four weeks postnatal mouse jaw carrying the K14 transgene. At this stage, root formation was almost complete and a network of Hertwig's root sheath (hers) was outlining the circumference of the tooth roots. The three molars (mi, m2, m3) and the enamel layer of the second molar (en) are marked for

orientation purposes. Fig. 4D is a higher magnification of the apical tip of the enamel organ of a one week postnatal mouse carrying the KI 4 transgene. Hertwig's epithelial root sheath (hers) and ameloblasts (amel) were labeled at this point, while dentin (de), odontoblasts (od), dental follicle (df), and alveolar bone (ab) were not. At this stage of initial root formation, Hertwig's root sheath was continuous. Fig. 4E. Sagital section through the anterior root of a second lower jaw molar at six weeks postnatal. At this stage, HERS had been reduced to Epithelial rests of Malassez (M), which reacted positive for the KI4 transgene. Alveolar bone (ab), dentin (de), root cementum (cem), pulp (pip), and periodontal ligament (lig) were negative. Note the greatly interspersed distribution of the epithelial rests of Malassez (M) allowing for periodontal ligament cells to attach to the root surface.

Fig. 5. Histochemical analysis of epithelial and mesenchymal tissues in chondrichthyan and actiopterygian teeth

Figs. 5A,B, and C were from a guppy fish *(Poecilia reticularis).* 5D-G were from a Shovelnosed Guitarfish *(Rhinobatos productus),* Fig. 5H was from a Pacific Homshark *{Heterodontus franciscii).* The enamel organs (eo) of developing guppy teeth (Figs. 5A-C) were attached to the oral epithelium (oe) via a general lamina. The root dentin (de) between individual teeth and *Os dentale* (Od) were connected by a cementoid bridge (cem). The Shovelnosed Guitarfish *{Rhinobatos productus)(f ig.* 5D-G) and the Pacific Homshark *{Heterodontus franciscii)(f*ig. 5H) demonstrated a clear separation between enamel organ epithelial cells (cl, cervical loop) and mesenchymal cells of the periodontal ligament (pl) in chondrichthyan teeth. The root surface apical of the cervical loop (cl) was devoid of any epithelial cells. Periodontal ligament and cervical loop were separated from each other by a basement membrane (arrowheads). Tooth enameloid (en), oral epithelium (oe) and root dentin (rd) were labeled for orientation purposes.

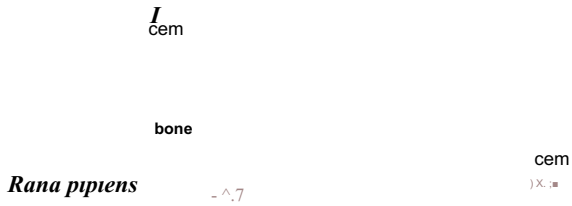

Fig. 6. Immunohistochemical staining of epithelial tissues in amphibian teeth using anti-keratin wide-spectrum screening antibodies

Figs. 6A and B were from a Leopard Frog *(Rana pipiens),* Figs. 6C-E were from a Mexican Axolotl *(Ambystoma mexicanum).* In the frog jaw (Figs. 6A and B) Hertwig's epithelial root sheath (hers) measured less than a quarter in length compared to the entire length of the tooth root. The remaining root surface was covered with cementoid of attachment between adjacent tooth roots and mandibular bone. Borders between root dentin (de), cementoid of attachment (cem), and *Os dentale* (bone) were difficult to distinguish. The anti-keratin antibody labeled Hertwig's epithelial root sheaths (hers) as well as oral epithelium (oe). The axolotl jaw (Figs. 6C-E) was similar to the frog jaw in that a cementoid tissue (cem) connected teeth amongst each other as well as individual teeth with the Os dentale (Od). In comparison to the frog, the cementoid tissue (cem) of the axolotl was less prominent. Hertwig's epithelial root sheath (hers) covered between one half and two thirds of the root dentin (rd) surface. Note the presence of islands of epithelial cells (isl) in the interdental region and distant from HERS. The apical portion of the root dentin surface was occupied by mesenchymal cells. The anti-keratin antibody recognized oral epithelium (oe), epithelial islands (isl), and Hertwig's epithelial root sheath (hers).

Fig. 7. Anti-keratin immunohistochemical staining of epithelial tissues in mammalian and reptilian teeth

Figs. 7A and F are micrographs of a developing tooth organ of a Texas Banded Gecko *(Coleonyx brevis)* immunostained with anti-keratin antibodies. Hertwig's root sheath (HERS), ameloblasts (amel), and general lamina (general lamina) were labeled by the anti-keratin antibody. A typical characteristic of reptilian jaws was the position of bone of attachment (cem) connecting adjacent teeth among each other. The body of the jaw bone (Od, *Os dentale)* was in distinct distance to the cementum-derived tooth-carrying bone. Note that the epithelial cell layer was limited to the tooth crown allowing for cementoid tissue and bone of attachment (cem) forming between adjacent teeth. Figs. 7B, C, and D are anti-keratin immunoreactions in the epithelial tissues of erupting iguana teeth. Fig. 7C is an overview; Figs. 7B and 7D are higher magnifications. The anti-keratin antibody labeled oral epithelium (oe) and Hertwig's epithelial root sheath (hers). The cementum layer (cem) covered the entire root area and facilitated an ankylotic attachment between tooth root and

alveolar bone (alv). Adjacent teeth were attached to a basal *Os dentale* (Od) instead of being directly connected with each other via a cementoid bridge as seen in the gecko and frog. In Fig. 7F, the anti-keratin antibody was applied to sagital paraffin sections through a caiman tooth organ *(Caiman crocodilus)*. Here, the antibody discretely labeled the coronal ameloblasts (am) and remnants of Hertwig's root sheath (hers) interspersed along the root surface. Note the wide spaces between individual HERS cell rests allowing for access of the caiman periodontal ligament (pdl) to attach to the root surface. Fig. 7G is a micrograph from a mouse first mandibular molar prior to eruption. Oral epithelium (oe), ameloblast cell layer (amel), and papillary layer (pap) were distinctly stained with anti-keratin antibodies. While the entire tooth crown (crown) was surrounded by keratin-positive epithelial cells, the root surface (root) and the alveolar bone (alv) were not labeled. Only the most apical portion of HERS (ap, arrow) was recognized by the anti-keratin antibody.

Fig. 8. Hertwig's Epithelial Root Sheath in human teeth (specimens from the Gottlieb Collection, Dallas, Texas)

Fig. 8A. Network of Hertwig's root sheath in humans in *en face* orientation toward the root surface. Note the wide open regions containing periodontal ligament fibroblasts (lig) between individual cords of HERS (hers) cells. Fig. 8b. Sagital section through the network of HERS cells in perpendicular orientation to Fig. 8 A. The network of HERS cells (hers) formed a defined layer adjacent to the root surface and embedded in periodontal ligament fibroblasts (lig). Fig. 8c. Close relationship between HERS and nerve cells. Note the presence of nervous plexus (pl) and ganglion cells (gg) within knots of the HERS network. Fig. 8d. Another demonstration of the close association between HERS cells and nervous

plexus within the fibroblast-rich (fib) periodontal ligament space. These preparations are from the historic Gottlieb collection at Baylor University. Bar = 100pm.

Fig. 9. Demonstration of apoptotic cells in the periodontal ligament using the TUNEL technique

Figs. 9 A and 9C were DAPI labeled fluorographs of the same areas as in the TUNEL stainings in Figs. 9B and 9D. The position of alveolar bone (ab), dental follicle (df), ameloblast nuclei (am), and odontoblast nuclei (od) was indicated for orientation purposes. Ameloblast mitochondria (am) provided orientation marks in the TUNEL stained Fig. 9B. Using the TUNEL technique, numerous apoptotic cell nuclei in the dental follicle were visualized (arrows, Figs. 9B and D). Figs. 9E and 9F are double exposures of the same area using both propidium iodide staining and ApopTag Red apoptosis labeling. Apoptotic nuclei were marked by arrows. Note the two adjacent apoptotic nuclei in the proximity of the root surface (Fig. 9F). Alveolar bone (ab), dental follicle (df), root dentin (rd), and enamel (en) were labeled for orientation purposes.

Fig. 10. Fate of Hertwig's root sheath in six weeks old mice visualized in Keratin-14 transgenic mice

In this study, the KI4 transgene was detected using the lacZ reporter gene and stained with p-galactosidase. Micrographs illustrate the incorporation of epithelial rests of Malassez (M) (Figs. 10A, B) as well as individual cells of Hertwig's epithelial root sheath (hers) into the mineralizing cementum layer (cem)(Fig. 10C). Tissues labeled for orientation are periodontal ligament (pdl), alveolar bone (ab), and root dentin (den). Note the strong staining intensity for the KI4 transgene in Malassez' rests of 6 weeks old mice. Figs. 10A and 10B illustrate the incorporation of KI 4 Malassez' rests into the cementum matrix of the root shaft at multiple sites. Fig. 10C documents the encapsulation and incorporation of individual HERS cells in the mineralizing cementum mass of the root apex.

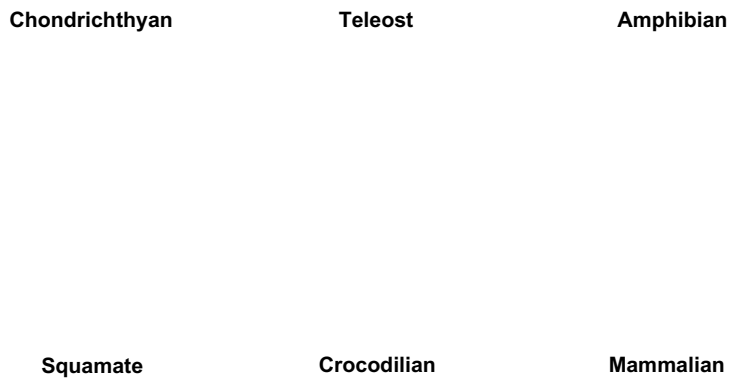

Fig. 11. Schematic relationship between Hertwig's root sheath, periodontal ligament, and mineralized tissues of tooth anchorage and attachment throughout vertebrate evolution and development

This sketch illustrates the position of mineralized tissues of attachment basal of HERS in tooth-bearing gnathostomes as well as the relationship between HERS and periodontal ligament anchorage in thecodont vertebrates. Fig. 11A summarizes the ligamenteous anchorage of teeth in chondrichthyans. The ligament (pdl) is positioned basal of the enameloid organ (eo). In Fig. 1 IB (many teleosts), the *Os dentale* (Od) of the jaw is surrounded by two other, closely related mineralized tissues, the cementum (cem) which interconnects individual tooth units, and the pedestal (ped) on which the tooth crown is anchored. Mineralized tissues of anchorage are all positioned basal of the enameloid organ (eo). The amphibian tooth attachment apparatus (Fig. 11C) features an elongated Hertwig's root sheath (hers) which covers part of the tooth root. The apical tip of HERS coincides with the coronal border of the cementoid of attachment (cem). Similar as in teleosts, individual amphibian tooth units are supported by pedestals (ped). All three mineralized tissues of attachment, cementum, pedestal, and Os dentale, are fused together via ankylosis. In amphibians, the extension of mineralized tissues in coronal direction is defined by HERS and other epithelial rests. The acrodont and pleurodont dentitions of many reptiles (Fig. 1 ID) are characterized by ankylotic attachment of tooth roots to the *Os dentale* (Od) via cementum (cem) and the pedestal (ped). While the entire root surface apical of HERS is covered by cementum (cem), adjacent teeth and gingival tissues are also interconnected by ligamenteous tissues (pdl) in the cervical region of the tooth. In contrast, Crocodilian dentitions (Fig. 1 IE) are truly thecodont. Their teeth are embedded in sockets provided by alveolar bone (ab). Alveolar bone (ab) and root cementum (cem) are separated by Hertwig's root sheath and by a periodontal ligament (pdl). In contrast to mammals, the crocodilian periodontal ligament is partially mineralized (Macintosh et al. 2002). Fig. 1 IF illustrates a cross section through a mammalian tooth. The mammalian periodontal ligament (pdl) is well organized into parallel fiber bundles and lacks the mineral deposits found in crocodilians. During mammalian root development, HERS stretches along the entire root surface (hers) and extends into an epithelial diaphragm (dia) at the root apex. A theca formed by ankylotic fusion of alveolar (alv) and mandibular/maxillary bone (mand) provides anchorage for the tooth root. Figure legends: enamel/enameloid (en, yellow), dentin (de, green), enamel organ

(eo, red), cementum (cem, blue dots), pedestal (ped, mauve), Os dentale/mandible (Od, blue graticule), periodontal ligament (pdl, blue interrupted lines), Hertwig's Epithelial Root Sheath (hers, red dots), Epithelial Diaphragm (dia, red oval). The orientation of illustration patterns in the periodontal ligament space does not follow physiological fiber orientation.

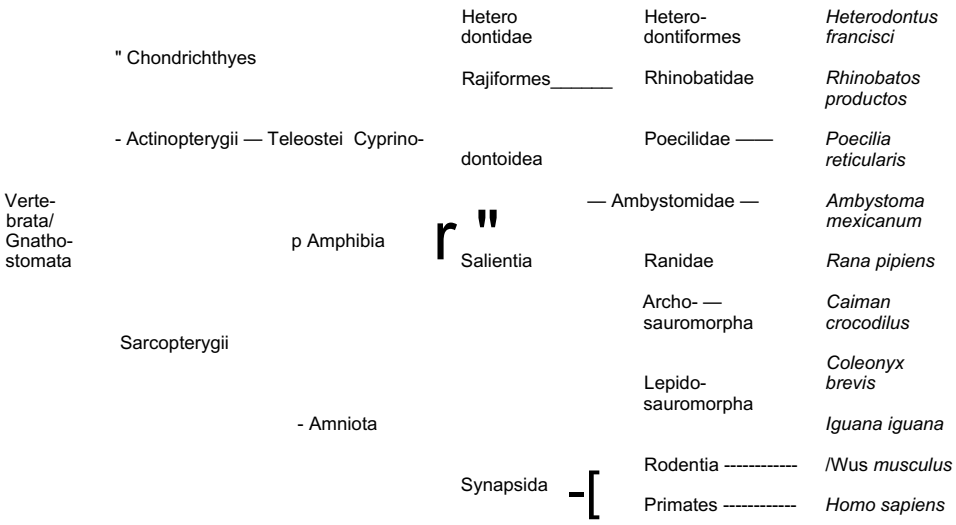

Fig. 12. Schematic phylogeny of species involved in the current study

Note that the thecodont archosaurs are more distant from the thecodont mammals than the pleurodont/acrodont squamates (Lepidosauromorpha) suggesting convergent evolution of mammals and crocodilians in respect to tooth morphogenesis.