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Molecular factors regulating development and regeneration of cementum

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Prior to the awareness of the importance of non-collagenous proteins in the regulation of mineralized tissues it was generally thought that bone and cementum were identical in composition (1). This was based in part on histology and relative percent of inorganic to organic compositions of these two tissues. However, cementum does have several distinct features: first, cementum is avascular; second, cementum has no innervation; third, cementum does not undergo significant remodeling, (i.e. active formation and resorption of tissue) as does bone, but instead, there is a slow deposition of cementum; and fourth, cementum has different functions from bone. As an individual tissue, cementum has been subclassified into specific types (e.g. acellular vs. cellular; afibrillar vs. fibrillar) based on various histological criteria; differences in cementum morphology and function have been the focus of a number of excellent scientific and review articles (2–6). The main function of cementum in health is to attach periodontal ligament fibers to the root surface. After periodontal disease, cementum is thought to contribute to the process of repair (7) and in fact some studies suggest that without the presence of cementum the periodontium cannot be restored to health (8, 9). Thus, a requirement for clinical regeneration of the periodontium, i.e. formation of new bone, new cementum and connective tissue attachment, may include treatments directed at stimulating or augmenting cementum formation.

Before reliable clinical strategies aimed towards periodontal regeneration can be developed, it is obvious that we must first understand the cellular and molecular mechanisms which regulate destruction and formation of component periodontal tissues. As illustrated in Fig. 1, many of the cells and molecular mediators operative during periodontal development, destruction, repair and regeneration are similar although the precise manner in which these function during each individual process may vary. Unfortunately, our current understanding of the processes which control and direct cementum formation is limited. Considerably more information is available regarding factors which regulate formation of the other hard tissues associated with the periodontium, i.e. dentin and alveolar bone. As these two calcified tissues develop in close spatial and temporal relationship to cementum, an understanding of the mediators associated with the genesis of dentin and bone may contribute valuable information on basic cellular and molecular mechanisms operative during cementogenesis.

Dentinogenesis can be predicted prior to actual matrix formation by the clinical orientation and polarization of preodontoblasts along the basement membrane which separates dental papilla cells from Hertwig's epithelial root sheath (HERS). Recent studies indicate that compositional and structural modification in this basement membrane is instrumental in the cytodifferentiation of preodontoblasts into functional odontoblasts (10–12). Initially rich in type IV collagen and fibronectin, the basement membrane appears to be important in the attraction and stabilization of preodontoblasts (10–11). The subsequent deposition of predentin matrix is associated with the expression of

various noncollagenous proteins, including dentin phosphoprotein-DPP (or phosphophoryn) (13) and a sialic-rich glycoprotein, dentin sialoprotein-DSP (14). Although the biological function of these proteins is yet undetermined, DPP and DSP are considered reliable molecular markers for the odontoblast cell lineage (14–15).

Similar to odontoblasts, osteoblasts are believed to be derived from undifferential mesenchymal progenitor cells. The process of osteoblast differentiation is associated with the expression of a variety of bone-related extracellular proteins, including bone-Gla protein (osteoclacin), SPARC (osteonectin) and bone sialoprotein (BSP); these proteins are believed to mediate interactions between cells and the extracellular matrix, including cell movement, attachment, and biomineralization (16–17). The process of endochondral bone formation following implantation of bone matrix in mature, non-mineralized tissues also appears to include chemotactic attraction and attachment of cells to matrix elements prior to proliferation and chondrogenic differentiation (18–21). While the inductive ability of implanted demineralized bone was originally attributed to one protein, bone morphogenetic protein (BMP) (22), it is now apparent that a number of BMP-type molecules exist, and many are members of the transforming growth factor/inhibin family of genes (23). The ability of these proteins to induce bone *in vivo* and *in vitro* strongly suggests that they play a significant role in the recruitment of osteoprogenitor cells to sites of bone formation and repair (20–21, 24).

In contrast to the abundance of information available concerning dentin and bone formation, relatively little information is available regarding molecular factors which regulate development and regeneration of cementum. Cementogenesis not only occurs during neonatal/adolescent development (here termed 'primary cementum' formation), but also during phases of normal physiological repair and maintenance ('secondary cementum' formation), and during therapeutically-induced regeneration ('regenerative cementum' formation). The processes which regulate primary, secondary or regenerative cementogenesis may be related in a manner similar to that observed when comparing bone development, regeneration, and repair. Studies directed at determining timed and spatial expression of factors/proteins during bone formation (25) versus fracture repair (26) have enhanced our understanding of the function of specific proteins during tissue mineralization in general. These studies have also demonstrated that many of the basic physiologic processes occurring during tissue development are also operative during postdevelopmental or reparative situations, e.g. maintenance/repair/regeneration. The possibility that developmental and repair share a number of cellular and/or molecular aspects is an especially exciting concept in the field of cementum research. The developing tooth lends itself as an ideal model for experimentation since tooth development is well described in a variety of species, is morphologically and chronologically linked, and is much more predictable and repeatable than any available regenerative model.

Over the past five years, our laboratory and others have attempted to identify and map the expression of specific matrix proteins during early tooth development, with emphasis upon those proteins which are expressed during formative cementogenesis, i.e. from late pre-cementogenesis through complete root formation (27–29). In addition, attempts have been made to characterize proteins entrapped or archieved within the mineralized matrix of mature cementum isolated from extracted, mature teeth (30–36). By comparing information from both sources, it is now possible to describe a profile of molecular factors which are temporally and/or spatially related to cementum formation. Although the functon of these proteins has not been established, it is highly probable that certain of these molecular factors have inductive or regenerative properties which are operative during various stages of cementogenesis (37). Table 1 describes the proteins which have thus far been described as either a) expressed during cementogenesis or b) present in mature cementum, and their probable role in the inductive events which support cementum development or regeneration.

It is becoming increasing evident that cell-matrix interactions play a significant regulatory role in tissue and organ morphogenesis (37). During tooth root formation, recent studies (38) indicate that cell/matrix interactions between primordial periodontal cells and matrix proteins expressed by these cells or adjacent cell populations may control the intricately timed and sequenced cellular events which accompany cementogenesis, i.e. *cell proliferation (mitogenesis), migration, attachment, (adhesion) of undifferentiated stem cells to the root dentin surface, differentiation of cementoblasts, and biosynthesis of cemental matrix* (39–40). Once believed to be independently occurring events, these processes may actually overlap and be reactivated during the progressive stages of root development. For example, during early tooth formation, primordial cells within the dental papilla are thought to undergo rapid proliferation before migrating and contributing to the investing layer of the dental follicle (41); later, as these follicular cells migrate and anchor to the forming root dentin surface, a secondary proliferative response may also occur

Table 1. Regenerative factors associated with cementum

Regenerative activity	Developing cementum	Mature cementum
1. Adhesion	• OPN-identified in area of HERS immediately prior to cementum deposition; localized to periodontal ligament at time of cementum deposiution. *In vitro*, DEM cells attach persistently to OPN[29,42] • FN-identified in area of odontoblasts during root formation[38] • LM–prior to cementogenesis expressed in area of HERS[54] • BSP[27]	• BSP[34] • Tenascin (surface)[70] • 55 kDa protein[31] • Fibronectin[34]
2. Chemoattraction	• FN-localized to area of root[38]	• Protein extract of cementum[32]
3. Differentiation	• HERS – may secrete factors/proteins e.g. epithelial-mesenchymal interactions important to cementum formation[56,92] • Osteonectin/sparc – present but function unknown – binds to calcium and hydroxyapatite, thus may have a role in mineralization[98] • TGF_{β} – Temporal and spatial pattern of expression during tooth development suggests a role in cell differentiation[99] • BSP[27]	• Osteonectin/sparc present, but function unknown[97,98] • 'Gla' proteins[101] • BSP[34] • Proteoglycans[118]
4. Mitogens	• TGF_{β} – identified prior to cementogenesis; mitogenic activity of TGF_{β} not established, nor established if present in cementum[99] • EGF – suggested role in tooth eruption[106]	• Mitogenic factors for gingival fibroblast[30,36]
5. Matrix biosynthesis	• Biosynthetic activity of developing cementum not yet established	• Protein extracts of cementum enhance collagen and total protein production by PDL cells[107]

Abbreviations: OPN-osteopontin/Spp1; FN-fibronectin; LM-laminin; HERS – Hertwig's epithelial root sheath; DEM-dental ectomesenchymal cells; TGFβ-transforming growth factor β; EGF-epidermal growth factor.

(42). As described in Fig. 1, the processes of proliferation, migration, attachment, differentiation, and biosynthesis are also thought to occur during situations of periodontal repair and regeneration, although the nature and origin of the stem cell population supporting regenerative cementum formation remains unknown (43–47).

There is strong experimental evidence that dental ectomesenchymal (DEM) cells residing within the dental papilla and dental follicle regions are the source of cells giving rise to cementoblasts during tooth development. This hypothesis is supported by a series of studies in which tooth germ development has been artificially manipulated in an attempt to determine the important of various component tissues in cementogenesis (39, 41, 48–52). It has been demonstrated that when tooth germs are transplanted to ectopic sites, cementum will form only when dental papillary or follicular cells are included in the transplanted tissues (41). Cell labeling and autoradiographic analysis of developing and transplanted tooth germs further suggest that cells of the dental papilla proliferate, migrate into and contribute to the dental follicle proper (39, 50–51); as HERS disrupts, follicular cells peripheral to HERS are thought to migrate and attach to the peripheral root dentin surface where differentiation into functional cementoblasts occurs (52). While little is known about the molecular factors which may regulate the directed migration of these undifferentiated mesenchymal cells from the internal papilla area to the external root surface, the most likely candidates are extracellular matrix proteins (37) found in the local area associated with cementum formation. Within the follicle itself, it appears that only those cells within the inner, investing zone closest to the root surface are capable of cementoblast differentiation (39). The complex series of histological events which accompany cementoblast differentiation and explanations as to their relative importance in root development are the subject of an excellent and extensive review by Schroeder (6).

As cell-matrix interactions may play a key regulatory role durng tooth root formation (38), it is appropriate to correlate the cellular events which accompany cementum formation (chemotaxis/migration, attachment, differentiation, cell proliferation and matrix synthesis) with those molecular factors which are currently known or potentially expressed during the same time period.

Chemotaxis/migration

Chemotaxis, the directed migration of cells in response to a chemmical gradient, is thought to play

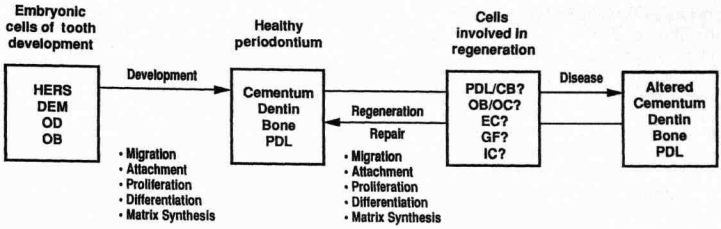

Fig. 1. Cells and proteins/factors associated with periodontal development and regeneration. This figure demonstrates the cellular activities required to promote both development and regeneration of periodontal tissues. The text itself and Table 1 describe the proteins/factors that have been implicated as having a role in development and/or regeneration of periodontal tissues. Abbreviations: HERS – Hertwig's epithelial root sheath; DEM – dental ectomesenchymal cells; OD – odontoblasts; OB – osteoblasts; PDL/CB – periodontal ligament cells/cementoblasts; OB/OC – osteoblasts/osteoclasts; EC – epithelial cells; GF – gingival fibroblasts; IC – inflammatory cells.

a major role in embryonic development and wound healing. While it is apparent that cell migration is an integral part of early tooth root development, it is currently unclear which specific biological mediators influence this phenomenon. Root development begins when cells of the cervical loop (a region where cells of the inner and outer enamel epithelium are contiguous) proliferate and form HERS. Cellular division within HERS results in apical migration of HERS cells through underlying dental ectomesenchymal (DEM) tissues; this migration serves to subdivide DEM into dental papilla and dental follicle subcomponents. With continued root development and formation of root dentin, it is believed that the intact epithelial sheath fenestrates with subsequent migration of individual epithelial cells away from the root surface and into the region of the future PDL space (44, 53). The mediators/factors responsible for initiating the timed disruption of HERS and the migration of epithelial cells away from the root surface remain undefined although various researchers propose that epithelio-mesenchymal interactions between HERS and surrounding mesenchyme regulate the timed expression of molecular products which trigger HERS fenestration (38). The disruption of the HERS network facilitates two important endpoints: a) the separation of HERS from its basement membrane, which remains virtually intact on the root dentin surface (54–55) and, b) the subsequent migration of undifferentiated DEM cells from the dental follicle proper toward the basement membrane/root dentin surface (39, 54, 56). It has been suggested that the directed migration of precementoblasts may be initiated by a chemoattractant found within the root-associated basement membrane (52, 55–56) or promoted by an adhesion gradient within the local environment (52). Owens (56–57), Schroeder (6) and Slavkin *et al.* (58) have suggested that HERS may deposit an epithelial product(s) in its basement membrane before its structural demise. Thomas & Kollar (38), using immunohistochemical techniques, have reported that two chemotactic glycoproteins, fibronectin and laminin, are expressed on the root aspect of HERS during early tooth formation and are components of the post-HERS root basement membrane. Fibronectin has been shown to be chemoattractive for a variety of mesenchymal cell types (59) while laminin promotes epithelial cell chemotaxis, adhesion and growth (60); therefore, based on physiologic potentials alone, both proteins are candidates in the directed migration of DEM and HERS cell during early cementogenesis. Fibronectin is also preserved within the mineralized matrix of mature cementum (34) and continues to be expressed in the post-development PDL where it is believed to function in maintenance-type cell-matrix interactions (61–62). Nishimura *et al.* have isolated additional proteins from mature cementum extracts which posses chemotactic properties for human gingival cells. In conclusion, there is considerable evidence to suggest that root-associated agents are involved in the directed migration of precementoblasts although the specific molecular factors involved in this chemotactic event have not yet been identified.

Attachment

Once migrating cells achieve their destination, they must then anchor to the selected substratum through the process of cellular adhesion, or attachment. It has been known for some time that cementum contains substances which promote the attachment of periodontal fibroblasts, and some of these may be responsible for the initial attachment of DEM cells during primary cementum formation.

The importance of cell adhesion in cementogenesis is vividly illustrated where environmental conditions exist which inhibit attachment, e.g., the inability to achieve new periodontal attachment formation on diseased root surfaces is primarily attributed to the presence of bacterial toxins and other inhibitors which block cell attachment activity (63–66). Many adhesion proteins mediate cell attachment through arg-gly-asp (RGD) peptide sequences (31, 42) which interact with integrin-type receptors on cell membrane surfaces. Integrins are a family of related receptors which are thought to be involved in a variety of cell/extracellular matrix interactions, including cell adhesion (67). The exact nature of the integrins associated with cementum formation and regeneration are yet undetermined. However, recent studies have shown that integrins belonging to the β_1 and β_3 families are present in periodontal tissues (42); the β_1 integrin is important for fibronectin-mediated cell attachment while the $\alpha_5\beta_1$ integrin appears to be selective for fibronectin (FN) (68). Fibronectin, an adhesion molecule found in high concentrations in most tissues, including those of the periodontium, also interacts with several other molecules, including proteoglycans, heparin, and collagen (69). FN has been identified throughout the periodontal ligament of permanent teeth, including the area of acellular cementum, using immunohistochemical techniques (62, 70). The localization of FN in the area of precementum formation during root development indirectly implies a role in attachment and spreading of DEM cells on the nonmineralized, collagenous matrix of first-formed root dentin; this hypothesis is supported by the demonstration that FN promotes attachment of both DEM and PDL fibroblasts *in vitro* (42). Analysis of cementum extracts by ion-exchange chromatography (31, 33–34) has confirmed that FN is a major noncollagenous component of the mineralized matrix of mature cementum. The question then arises, "What cells are responsible for deposition of FN and what is the precise role for this molecule in cementum formation?" As stated earlier, Thomas & Kollar (38) have shown that FN is expressed within the root-associated basement membrane, formed in part by HERS cells; however, it is currently unclear whether epithelial (i.e. HERS) cells or mesenchymal (i.e. DEM) cells, or both are primarily responsible for FN production in the developing tooth. Indeed, root-associated FN may play a variety of roles during tooth root morphogenesis, including migration, attachment, and proliferation of cells on the forming root surface (38); FN may serve similar functions in the mature PDL under conditions of normal cell/matrix turnover and cemental repair (61).

It is well established that in a given tissue different adhesion molecules can regulate different aspects of cell function (71) and that expression of these regulatory factors often occurs in a programmed fashion during progressive stages of development (72). This phenomenon appears to occur during cementum development where a number of known cell attachment proteins, and possibly novel, yet unidentified adhesion proteins, have been identified either *in situ* or in the mineralized component of the cementum matrix. Somerman *et al.* (34) demonstrated that a mineral-specific adhesion protein, BSP, a glycoprotein associated with bone and containing an RGD sequence (73–74), is present in cementum. BSP has been shown to promote both periodontal cell (75) and bone cell (76) attachment *in vitro*. Studies by Oldberg *et al.* (77), using ROS 17/2.8 osteoblast-like cells, have demonstrated that one of the cell receptors for BSP is the integrin $\alpha_v\beta_3$, a vitronectin receptor; this receptor is known to be present on periodontal cells (42). More recent studies (27) report that BSP is also expressed in the cementum matrix at the critical stage of early cementogenesis. Importantly, since BSP is considered to be specific to mineralized tissues (78), this protein may prove to be a useful and reliable marker for cementoblast differentiation (27). To date BSP appears to be selective for mineralized tissues except for trophoblasts located in placental tissues (79).

Attachment proteins classically associated with cell/matrix interaction during dentinogenesis (80–83), i.e. collagen, laminin and tenascin, may also participate in cementum formation although their function is largely unknown. Type I collagen is the major collagen species in cementum accounting for approximately 95% of total collagens (84–86) while Type V and possibly Type VI (87) represent other collagen types. Laminin, a glycoprotein associated with epithelial cell adhesion is expressed during HERS-directed root development and is localized on the root surface prior to cementogenesis (54–55). Tenascin, a molecule expressed during pre-odontogenesis and then lost during odontoblast differentiation (28, 88) is also found in the cementoblast – precementum layer and in the fiber attachment zones of mature cementum (70); its co-expression with FN in the cementogenic root area strongly suggests a regulatory role in cementoblast differentiation (70). Similar evidence suggests that osteopontin (OPN), a phosphoprotein expressed along the HERS-dental papilla interface during early root formation (29), may also participate in this process. The identification of OPN in tissues prior to mineralization supports a role for this molecule in initiation of mineralization activity (89–90). OPN promotes adhesion of

a diverse group of cells including DEM and periodontal cells (29) but the specific receptors mediating cell attachment are only partially understood. Indirect evidence indicates that $\alpha_v\beta_3$ is one of the receptors that mediates OPN cell attachment. This receptor integrin has been identified on mature periodontal cells (42, 90); however, it is not yet known if similar receptors exist on DEM cells.

Evidence also indicates that cementum may contain novel, yet-unidentified proteins which may be of significant value in understanding the genesis or regeneration of this tissue. Protein separation techniques have shown that mature cementum harbors numerous "phantom" proteins (34), including a 55 kDa protein shown to possess fibroblast attachment activity (31, 33); whether these reported proteins represent truly unique species or are degradation products of known proteins has not been fully determined. Analysis and identification of mineral associated cementum proteins will continue to be a major focus of periodontal research due to the potential importance of these proteins in cementum formation.

Differentiation

The process of acquisition of specialized properties and phenotypic features characteristic of mature cells is termed differentiation. There is considerable and longstanding debate concerning the factors or regulatory stimuli which control differentiation of cementoblasts. For almost thirty years, investigators have suggested that HERS cells, upon interacting with surrounding cells and matrix, secrete factors or proteins important to cementoblast differentiation (91–93). The idea that epithelial cells may be required to initiate mineralized cementum formation is founded in observations from classical embryology where certain types of cartilage and bone differentiation require epithelial stimuli; for example, chondrogeneis is initiated in limb bud by limb bud epithelium, in otic mesenchyme by epithelium from the otic vesicle, and in cranial (neural crest-derived) mesenchyme by pharyngeal, cranial and facial epithelium (94). The most convincing evidence that epithelial cells are required in cementum formation is derived from studies of tooth and craniofacial development (50, 54–55, 92, 95–97). The known ability of epithelial cells to undergo epithelial-mesenchymal transformation in other tissues, e.g. palatal tissue during midline fusion (96), has prompted some investigators to propose that HERS cells may be directly involved in cementum formation (38). Slavkin *et al.* (92) have demonstrated tht HERS cells synthesize and secrete enamel-related proteins along the forming root surface during *in situ* development and during *in vitro* organ culture; meanwhile, MacNeil and Thomas (54–55), through artificial manipulation of the developing tooth germ, have shown that cementum and PDL will not form in the absence of HERS and HERS-derived basement membrane. These latter findings, combined with the fact that disruption and release of products from HERS occurs at the time of cementum formation, support the hypothesis that HERS-derived proteins are required for cementoblast differentiation. While this hypothesis may explain the role of epithelium in the complex phenomenon of primary cementum formation, it fails to elucidate the mechanism of regenerative cementum formation in the apparent absence of epithelial cells; although remnants of HERS cells remain in the mature PDL as epithelial cell rests of Malassez, it is currently unknown if these cells participate in secondary or regenerative cementum formation.

Tooth root formation is also associated with the local expression of a number of other proteins/factors including osteonectin (SPARC) (98–99), TGF_β (99–100) and bone and matrix-'gla' protein (101–102). Reichert *et al.* (98), using immunohistochemical techniques, reported that osteonectin is first seen after disruption of HERS and with early primary cementum formation; cementum-producing cells were described as osteonectin-positive while the cemental matrix itself remained osteonectin-negative. In addition, BSP, expressed early in formation of bone both *in vivo* (78–79) and *in vitro* (17) may have an important role in promotion of cementoblast differentiation (27).

Mitogens/matrix synthesis

The awareness that proteins/factors that control cell proliferation and biosynthetic activity are important in the regulation of cell behavior during development and regeneration has lead to efforts focused on identifying and establishing the role of these molecules in specific tissues, including those of the periodontium. Importantly, growth factors and adhesion molecules interact with each other to regulate cell function (103–104). For example $TGF_{\beta 1}$ can regulate the expression of several integrins on the cell surface, *in vitro* (105). Thus, the temporal and spatial expression of growth factors during tooth development may be critical not only to cell proliferation, but to controlling the attachment of appropriate cells at a given site. Both EGF (106) and TGF-β_1 (99) are expressed during tooth development; however, the role they have in cementogenesis has not been established. In mature cementum, protein extracts have been shown to stimulate proliferation (30) and protein production

(107) in periodontal cells, *in vitro*. Most recently, Yonemura *et al.* (36) have identified a growth factor that may be selective to cementum. In addition, growth factors present in bone, including the TGF_{β} family, basic fibroblast growth factor (βFGF), insulin growth factor I and II (IGF I and II), and platelet derived growth factor (PDGF) may be present in cementum. The importance of these factors for regeneration of cementum is just beginning to be explored (108–109), but it is clear that determining the growth factors present in healthy cemental tissues will provide clues as to the necessary factors required to restore cementum lost to disease processes.

These discussions have focused on the factors/proteins important to the processes of formation and regeneration of cementum. However, equally important to the understanding of cementum is the identification of the cells involved in development and regeneration of cementum. While there is a general agreement that tissues of the periodontium harbor cells with the capacity to regenerate the periodontium (46–47, 110), the specific cells responsible for stimulating new cementum formation in the post developmental stage have yet to be clarified. This has resulted in research focused at isolating and characterizing cells from mature periodontal tissues using *in vitro* systems. Convincing evidence exists indicating that progenitors cells, having regenerative capacity, are in close association with vascular spaces (47, 110). Analysis of progenitor cell kinetic patterns following experimental regenerative procedures suggests that cells either from the periodontal ligament (43) or from surrounding alveolar bone (111) possess the ability to migrate and attach to denuded root surfaces and to differentiate into cementoblasts. Biochemical data from *in vitro* studies indicate that the periodontal ligament contains a heterogeneous cell population (47, 112) where some cells exhibit osteoblastic properties including high alkaline phosphatase (43, 44), a PTH-mediated cAMP response (113–114), an ability to form mineralized-like nodules (116) and increased synthesis of bone 'gla' protein in response to calcitriol (115). In contrast, other studies suggest that mature cells, from the ligament itself, may not have osteogenic potential. In fact, there is some indication that, *in vitro*, PDL cells, or their secretory products, may actually have an inhibitory role in the regulation of mineralization (117).

The research directions discussed here provide promise of a better understanding of the dynamic mineralized tissue called cementum. As studies progress, the traditional concept of cementum as a static or terminal tissue will be undoubtedly replaced by one which more accurately portrays it as biologically active and responsive. However, at this time, the factors or stimuli which regulate cementum activity (i.e., formation) during development, maintenance, repair and regeneration of the periodontium remain ill-defined and a common link between these separate but potentially related processes has not yet been established. Elucidation of these questions appears critical in the design of treatment modalities directed towards predictable formation of new cementum (e.g. periodontal regeneration). This information will also enhance our overall understanding of the process of tissue mineralization.

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