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REVIEW

A post-classical theory of enamel biomineralization… and why we need one

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Enamel crystals are unique in shape, orientation and organization. They are hundreds of thousands times longer than they are wide, run parallel to each other, are oriented with respect to the ameloblast membrane at the mineralization front and are organized into rod or interrod enamel. The classical theory of amelogenesis postulates that extracellular matrix proteins shape crystallites by specifically inhibiting ion deposition on the crystal sides, orient them by binding multiple crystallites and establish higher levels of crystal organization. Elements of the classical theory are supported in principle by *in vitro* studies; however, the classical theory does not explain how enamel forms *in vivo*. In this review, we describe how amelogenesis is highly integrated with ameloblast cell activities and how the shape, orientation and organization of enamel mineral ribbons are established by a mineralization front apparatus along the secretory surface of the ameloblast cell membrane.
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INTRODUCTION

Dental enamel is comprised of highly oriented, thread-like crystallites of calcium hydroxyapatite (HAP) organized into rod and interrod structures.[1] The rods are about 5 μm in cross-sectional diameter[2] and comprised of bundles of parallel crystallites (about 26 nm×68 nm in cross-section) packed at a density of ~550 crystallites·μm^{-2}, or roughly 40 thousand crystallites per rod.[3] Enamel crystallites appear to extend all the way from the dentino-enamel junction (DEJ) to the surface of the tooth.[4]

Understanding the mechanism of dental enamel formation requires reconciliation of ideas from scientists who grow crystals *in vitro* and scientists who study the biological process of amelogenesis *in vivo*. These two perspectives have never assimilated satisfactorily and seem to be on different courses. Better characterization of amelogenesis at the biochemical and genetic levels and in knockout mice with developmental enamel malformations has greatly advanced our understanding of events *in vivo*. Advances on the biological side are yielding new theories that are replacing the 'classical' theory[5–6] of dental enamel formation. This review states the case for a model of enamel formation postulating that the initial mineral in enamel is amorphous calcium phosphate (ACP) and that enamel mineral ribbons are the product of a specialized mineralization front: an apparatus along the secretory surface of the ameloblast plasma membrane that generates, shapes and elongates enamel mineral ribbons.

BASEMENT MEMBRANE

Enamel crystallites initiate at the DEJ immediately following fenestration and removal of the basement membrane beneath fully differentiated preameloblasts.[7] The basement membrane is replaced by a membrane-associated apparatus comprised of enamel proteins that coats the secretory surface of the ameloblast plasma membrane and is necessary for the initiation and elongation of enamel mineral ribbons. The genes encoding enamel proteins are themselves descended from a basement membrane gene: SPARC-like protein 1 (*Sparcl1*).[8–10] The initiation of enamel formation requires successful replacement of the basement membrane with the mineralization front apparatus. This does not occur properly when proteins associated with the basement membrane, such as collagen 17,[11–13] α6/β4 integrin[14–16] or laminin-332[17–20] or proteins associated with the mineralization front, such as enamelin[21] or ameloblastin,[22–23] are defective or missing.[24–26] Amelogenin (the most abundant enamel matrix protein) appears to be sparse at the mineralization front[23,27–28] and a thin layer of enamel (~15 μm *vs.* ~110 μm) is deposited in *AmelX* knockout mice.[29]

When the initial enamel starts to form, the distal surface of ameloblasts is folded, with cell processes extending to contact the irregular surface of the underlying predentin.[30] The initial enamel has the same characteristic shape of mineral ribbons that is observed throughout the secretory stage of amelogenesis. At the ameloblast membrane (mineralization front), the mineral ribbons are thin slits (1.5 nm× 15 nm) in cross-section.[3] On the dentin surface, these ribbons are closely associated with collagen, which can be recognized by its characteristic cross-banding.[30] From the onset, the initial enamel ribbons are tightly integrated (attached) to dentin, principally through connections to its organic matrix[31–33] and extend from there to the mineralization front (Figure 1) where the ribbons are actively elongated by the addition of ions or ACP to the tips of the mineral ribbons. At first,

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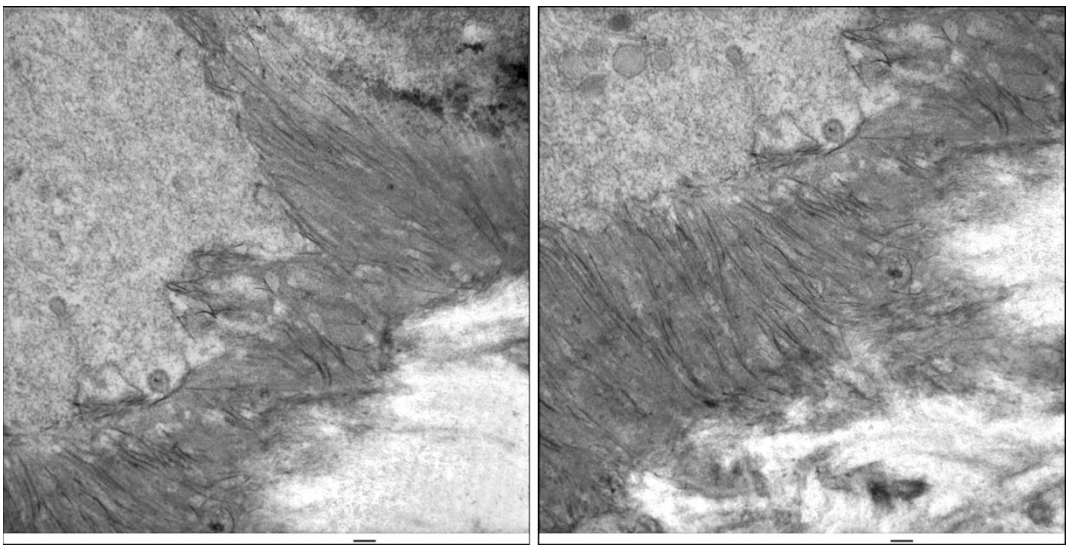

Figure 1 Formation of initial enamel. Day 7 mouse mandibles were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer and post-fixed with osmium tetroxide. Sections were stained with uranyl acetate, then lead citrate, and viewed by TEM. Ameloblasts are on the upper left. Banded collagen fibers are on the lower right. The enamel ribbons initiate on the dentin surface in close association with collagen and the mineralization front on the ameloblast membrane. Scale bars=100 nm. TEM, transmission electron microscopy.

irregular depressions on the dentin surface are filled, creating 'islands of enamel'. The ameloblast cell processes retreat as the ribbons elongate. The cell membrane becomes smooth, forming an uninterrupted mineralization front, and the underlying enamel layer becomes continuous.[30,34] As ribbon elongation proceeds, each ameloblast develops a Tomes' process. The mineralization front becomes discontinuous again, interrupted by the non-secretory portions of the Tomes' processes that do not support mineral ribbon elongation. The architecture of the ameloblast distal membrane and its division into secretory and non-secretory surfaces establishes the rod/interrod structural hierarchy characteristic of enamel.[30,35–36] In rat incisors, ameloblasts move away from the dentin at a rate of 13.5 μm per day (0.56 $\mu m \cdot h^{-1}$ or about 9.3 $nm \cdot min^{-1}$).[37] As the ribbons generally form at an angle to the line perpendicular to the DEJ, this represents a minimum rate for the lengthening of enamel ribbons at the mineralization front.

PROBLEMS WITH THE CLASSICAL THEORY

The classical theory of enamel formation held that proteins bind specifically and selectively to the sides of crystalline ribbons, inhibiting their growth in width and thickness and permitting growth only in the *C*-axis direction.[38] The purpose of proteolytic cleavages was to remove the protein inhibitors from the sides of the crystallites to allow them to mature (to grow in width and thickness). HAP's sixfold symmetry[39] might have precluded the molecular recognition of selected crystal faces that was required to produce the observed slit-like cross-sections of enamel ribbons. This problem and others were solved by proposing the existence of an octacalcium phosphate precursor phase.[40–42] Substantial evidence supports the classical theory in principle, but the classical theory is not consistent with observations of how enamel forms *in vivo*.

Enamel proteins bind HAP *in vitro* and inhibit their growth, and this growth inhibition can be reduced by the addition of enamel proteases such as matrix metalloproteinase 20 (MMP20)[43] or kallikrein 4 (KLK4).[44] *In vivo*, however, there is a high rate of mineralization on the sides of the enamel crystals in the transition/early maturation stage when enamel proteins are still abundant and should be inhibiting such growth.[45] There is also a substantial amount of crystal maturation in *Klk4* null mice, even though the enamel proteins are not effectively removed from the enamel layer during the maturation stage of amelogenesis.[46]

Electron diffraction and electron probe microanalysis of enamel ribbons near the mineralization front reveal a very poorly crystalline HAP with a low and variable Ca/P molar ratio and no evidence of octacalcium phosphate.[47] Newly formed enamel mineral ribbons are ACP that transforms into HAP.[32,48] The key finding is that the size, shape and spatial organization of the enamel ribbons are established prior to their crystallization. The characteristic enamel ribbons with slit-like cross-sections are not dictated by 'stereochemical' interactions with selected crystal faces, as they are not yet crystalline. The mineral in the ribbons has no shape of its own. The ribbon shape must be due to an external influence, such as the shape of the space, or mold, within which it forms. Like the classical theory based upon an octacalcium phosphate precursor, the concept of an ACP precursor is also supported in principle by *in vitro* studies. The first synthetic HAP crystals suitable for X-ray diffraction were made by hydrolyzing solid $CaHPO_4$ (monetite) into HAP.[49] The solid–solid conversion of ACP into HAP occurs *in vitro*.[50] Amelogenin stabilizes ACP and delays its conversion to HAP[51] *in vitro*, although the ACP phase in enamel *in vivo* seems to be relatively short-lived.[32] An ACP precursor phase is also thought to play a role in dentin and bone mineralization.[52]

INITIAL ENAMEL RIBBONS

Enamel proteins, such as amelogenin, are secreted prior to the onset of enamel mineralization as the basement membrane is degraded and ameloblasts come into contact with the collagen-rich predentin matrix.[53] Ameloblastin, enamelin, MMP20 and dentin sialophosphoprotein are also expressed by early ameloblasts[54] and could be part of the organic component that helps fasten the incipient enamel ribbons to collagen. MMP20 activity seems to be critical for this attachment as a thin line of hypermineralized enamel at the DEJ in wild-type mice is missing in *Mmp20* null mice[55] and enamel

delaminates at the DEJ (Figure 2). All forming enamel, including the initial enamel in contact with dentin, is directly associated with the ameloblast membrane and the ribbons are elongated by cycles of mineral deposition at the mineralization front throughout the secretory stage of amelogenesis.

THE MINERALIZATION FRONT APPARATUS

The classical theory ignores the mineralization front apparatus; however, *in vivo* studies demonstrate that the mineralization front apparatus is literally the essence of enamel formation. When enamelin or ameloblastin are missing or defective, the mineralization front apparatus fails and the enamel layer is absent.[25–26] No mineralization front apparatus equals no enamel. The mineralization front shapes the enamel ribbons before they are crystalline[48] and orients them. In humans, the growing enamel ribbons are oriented perpendicular to the mineralization front.[35] When ameloblasts develop Tomes' processes, the contour of the mineralization front changes so that different faces of the mineralization front are oriented in different directions. This is the basis for the hierarchical organization of enamel ribbons into rod and interrod structures (Figure 3).[34,56] In rodents, the Tomes' processes in one row of ameloblasts are inclined in the same direction, while those of adjacent rows are inclined in the opposite direction,[57] resulting in a decussating (X-shaped) pattern of enamel rods, each filled with enamel ribbons oriented along the long axis of the rod, but at an angle to the rod in the adjacent row. Near the end of the secretory stage of amelogenesis, ameloblasts retract their Tomes' processes and the final enamel, like the initial enamel deposited prior to formation of the Tomes' processes, lacks rod/interrod divisions and the ribbons run perpendicular to the enamel surface. The mineralization front determines the number, shape and orientation of the mineral ribbons and the topography of the mineralization front establishes rod/interrod organization.

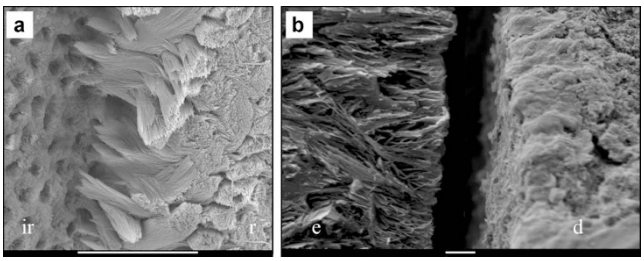

Figure 2 Preferred fracture levels in *Klk4* and *Mmp20* null mice. (**a**) *Klk4* null mouse enamel tends to fracture where the initial enamel forms the first interrod enamel (ir) and the base of the rods (r) just above the DEJ.[46] Bar=10 μm. (**b**) *Mmp20* null mouse enamel (e) separates from dentin (d) right at the DEJ. Bar=1 μm. DEJ, dentino-enamel junction; KLK4, kallikrein 4; MMP20, matrix metalloproteinase 20.

There is virtually no evidence that describes in detail how enamel ribbons are extended at the mineralization front apparatus; however, the requirements of the process limit the possibilities and allow working hypotheses to be proposed. Perhaps calcium and phosphate channels in the plasma membrane above slits in the mineralization front apparatus concentrate these ions in a restricted extracellular space where ACP precipitates. Mineral precipitation may reduce the free calcium and phosphate ion concentrations within the space to allow more ions to be channeled into it to support continued ribbon elongation. The mechanism of calcium ion entry into the enamel matrix is unknown, but several intriguing observations have been reported. Ameloblasts do not concentrate calcium in anticipation of mineralization.[58] Calcium flux into enamel is regulated by ameloblasts and is slower than it would be if it were not regulated.[59] Calcium release-activated calcium modulator 1 acts as the pore-forming subunit for calcium

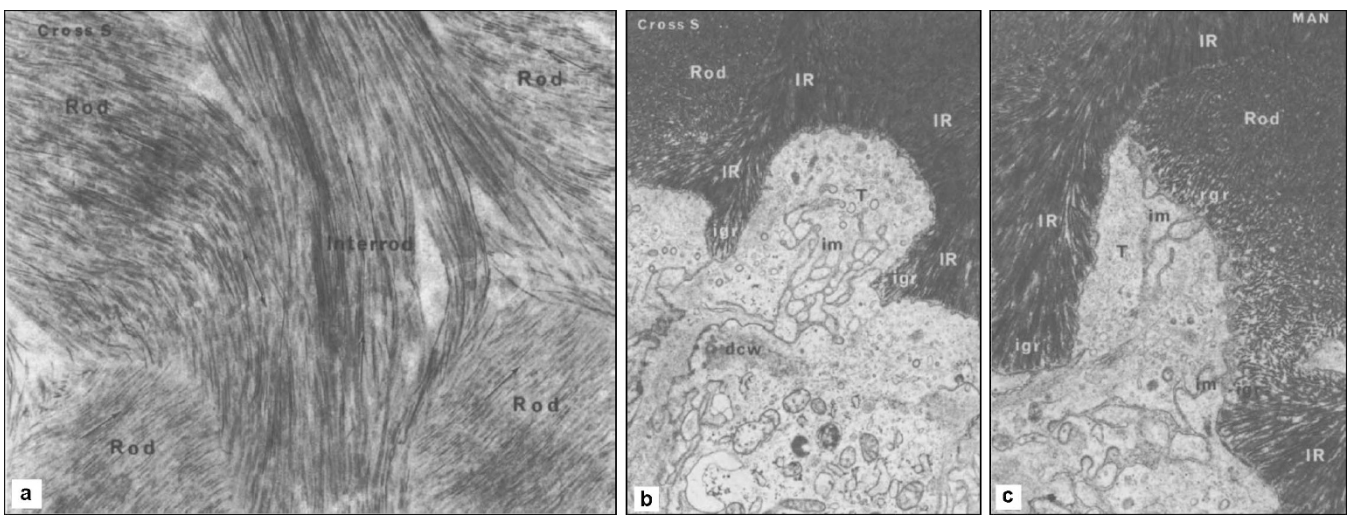

Figure 3 Crystal shape, orientation, and organization are determined at the mineralization front. Three TEMs of an undecalcified sections of secretory stage inner enamel stained with uranyl acetate and lead citrate reproduced with permission from[34] (**a**) Rat incisor section showing that within the rods, crystallites run parallel to the rod axis (×70 000). Between adjacent rods the interrod crystallites run at almost right angles. (**b**) Developing human tooth showing the relationship of the ameloblasts to the interrod and rod growth regions (×20 000). The Tomes' process (T) is surrounded by interrod enamel (IR) and is lined by a relatively smooth membrane. Interrod growth regions (igr) are seen at the prong tips on either side. The section has cut the rod growth region tangentially and it thus appears in the center of the process (im). dcw: distal cell web. (**c**) The rod growth region (rgr) and the forming rod are seen on one surface of Tomes' process (T). The opposite surface faces interrod enamel (IR). Interrod growth regions (igr) are seen at the prong tips on either side. Infolded membranes (im) are seen at interrod and rod growth regions. TEM, transmission electron microscopy.

release-activated calcium channels in the plasma membrane.[60] Mutations in *ORAI1* result in dental enamel malformations.[61]

Although the defining feature of the secretory stage of amelogenesis is the expansion of the enamel layer by the lengthening of mineral ribbons by the mineralization front apparatus, the mineral ribbons also grow in width and thickness,[3] so that calcium deposition as a whole is relatively uniform throughout the matrix.[62]

SECRETORY STAGE ENAMEL PROTEINS

The major proteins in the secretory stage enamel extracellular matrix are amelogenin,[63] ameloblastin[64] and enamelin[21] (which is not tuftelin). These proteins are cleaved by MMP20[65] in the extracellular matrix. *Amelx*, *Ambn*, *Enam* and *Mmp20* are genes specialized for dental enamel formation. Only dental enamel defects are observed in the corresponding four null mice and these genes degenerate in vertebrates that have stopped making teeth or enamel during evolution.[66–68] In the pig, where tooth size is sufficient to isolate enamel protein cleavage products in quantity, the cleavage sites of enamel proteins have been characterized and correspond to the exact sites that are cleaved by MMP20 *in vitro*.[69–72] Secretory stage enamel proteins in *Mmp20* null mice are largely intact (uncleaved).[73] Typical enamel ribbons are observed near the DEJ in *Mmp20* null mice.[74]

Based upon the classical theory, one might expect that *Mmp20* null mice would produce a normal enamel layer with crystallites that do not mature. One might also expect that the *Amelx*, *Ambn* or *Enam* null mice would make thicker hexagonal or plate-like crystals. None of these expectations are met. The *Enam* and *Ambn* null mice fail to make enamel and show significant ameloblast cell pathology. Tomes' processes do not form properly in *Mmp20* null mouse ameloblasts, which produce a disorganized, thin enamel.

TRANSITION

As long as the mineralization front is sustained by ameloblasts, the enamel ribbons grow longer and the enamel layer thickens.[75] After laying down a final layer of aprismatic enamel, secretory stage ameloblasts transition into modulating, maturation stage ameloblasts, with ~25% of ameloblasts undergoing apoptosis.[45,76] Ameloblast transition involves major changes in cell size and architecture,[77] the onset of KLK4 secretion,[78] and replacement of the mineralization front apparatus with a novel basement membrane containing amelotin[79] and odontogenic, ameloblast-associated[80] and other proteins. When the mineralization front apparatus is gone, the lengthening of enamel ribbons is over. All subsequent mineralization involves the widening and thickening of ribbon-like crystallites previously formed during the secretory stage.

ENAMEL MATURATION

During the maturation stage of amelogenesis, the enamel crystallites deposited during the secretory stage grow exclusively in width and thickness and the enamel proteins are removed.[45] In humans, the maturation stage for the permanent teeth lasts about 4 or 5 years. Maturation stage ameloblasts modulate between ruffle-ended and smooth-ended morphologies.[81] In rat mandibular incisors, maturation ameloblasts modulate three times a day with most of their time being spent in the ruffle-ended form.[82] Calcium entry into the matrix appears to be mainly through ruffle-ended ameloblasts.[83] The enamel beneath ruffle-ended ameloblasts is mildly acidic (pH ~6) as a consequence of mineral deposition and is neutralized by bicarbonate to physiologic pH (~7.2) under smooth-ended ameloblasts.[84] As mineralization proceeds, enamel proteins are progressively degraded by KLK4 and the digestion products are reabsorbed into ameloblasts so that the crystallites can grow together and interlock.[46] In developing teeth, KLK4 is specifically expressed by transition and maturation stage ameloblasts.[85] There is trace expression of KLK4 in other tissues besides teeth,[86] but the only phenotype in mice and humans that lack KLK4 is found in the enamel.[87] In the absence of KLK4 expression, there is substantial retention of enamel proteins within the enamel matrix layer even as the teeth erupt. The enamel layer is increasingly hypomineralized from the enamel surface to the DEJ.[55,88] Recently, there has been much interest in defining the mechanisms of ion transport (Ca^{2+}, PO_4^{3-}, H^+, HCO_3^{2-})[75,89–91] to improve our understanding of enamel maturation.

CONCLUSION

Dental enamel forms by the deposition of characteristic, non-crystalline, mineral ribbons by a mineralization front apparatus closely associated with the secretory surfaces of the ameloblast plasma membrane. The shape and orientation of enamel mineral ribbons is established at the mineralization front and is not due to stereospecific inhibition of mineral deposition on selected crystal faces by acidic enamel proteins. The hierarchical organization of enamel ribbons into rod and interrod enamel is established by the topographical re-configuration of the mineralization front that occurs with formation of the Tomes' process. The mineralization front apparatus is the key to enamel formation, and significant advances in our understanding of amelogenesis will be realized by gaining a better understanding of molecular events occurring at the enamel mineralization front.

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