



Review Article

Molecular Networking in Amelogenesis

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ABSTRACT

Amelogenesis is a tightly regulated and intricate process of enamel formation. It involves epithelial-mesenchymal interactions at the embryological level by means of enamel proteins expression. During this process, ameloblasts undergo morphological changes which correspond to the pre-synthetic, synthetic and post-synthetic phases. Regulatory changes within these cells have been studied at light, ultramicroscopic and molecular levels. This paper focuses on the intricate pathways in Amelogenesis at various stages and also cites, clinical correlations wherever deemed appropriate.

Key words: Amelogenesis, Epithelial- mesenchymal interactions, dysregulations.

INTRODUCTION

Enamel formation is prestaged by definitive changes in enamel organ components. Towards the end stages of advanced bell stage, the outer enamel epithelial cells become squamoid and their continuity is disrupted by intercellular gaps. These gaps provide spaces for invasion of connective tissue elements from the dental follicle or sac. As the blood vessels of the dental follicle advance through the discontinuities, the stellate reticulum cells start to collapse. Extension of vascular

channels into stellate reticulum brings the substances essential for metabolism and enamel matrix synthesis closer to the cells responsible for amelogenesis i.e., stratum intermedium and ameloblasts. During the appositional stage, ameloblasts require their maximum height, their organelles increase in numbers and become polarized. At this stage, the nucleus occupies the proximal third of the cell i.e., the part adjacent to stratum intermedium. The distal secretory end is filled with rough endoplasmic reticulum and secretory vesicles. The Golgi complex and smooth endoplasmic reticulum

occupy most of the middle third of the cell. Terminal bars are formed at both the proximal and distal ends of the ameloblasts. Tomes' processes extend downwards from the distal terminal bar. Enamel formation begins immediately after initiation of dentinogenesis. The first layer of dentin is juxtaposed by a "thin" layer of aprismatic enamel. After depositing the "scaffold" enamel layer, the maturing ameloblasts retreat, completely differentiate and begin to deposit enamel organic matrix in daily increments averaging 4µm thickness. Therefore, enamel organic matrix deposition takes place in three phases- [1,2]

1. Formation of Tomes' process.
2. Formation of enamel prism template.
3. Matrix completion (maturation) and mineralization of enamel prism.

This review focuses on the functional stages of amelogenesis with focus on matrix components, crystal nucleation and enamel growth.

Fine structure of ameloblast

Katchburian [1972] studied the cellular and fine structural changes taking place during ameloblast differentiation involve growth and elongation of cytoplasm; reversal of polarity and organelle development.^[3] The undifferentiated ameloblasts are mainly concerned with protein synthesis for internal metabolism. These cells are rich in free ribosomes and have only few rough endoplasmic reticulum. The first evidence of differentiation is elongation of cytoplasm and parallel orientation of microtubules in distal end of the cell. Further, cellular differentiation is marked by an increase in rough endoplasmic reticulum (rER) in distal cytoplasm and migration of golgi complex distally. Intracisternal accumulation of enamel matrix-like material in rough endoplasmic reticulum at this stage suggests that the golgi complex at this stage is unable to process for secretory material synthesized in the rER.

However, the presence of enamel matrix-like material in intercellular spaces indicates an attempt to secrete matrix by a mechanism which by-passes the golgi complex.

With further differentiation, the golgi complex reaches the distal cytoplasm where it develops and becomes very extensive. As soon as the enamel matrix begins to be deposited at the distal surface, ameloblasts withdraw leaving the cytoplasmic extension (Tomes' process) in the early calcifying enamel. The presence of microtubules and filaments in Tomes' process suggest that they play a role in the directional and localized growth of this constricted cellular extension. Cytometric and karyometric data provide good information about the cellular activities and functions of inner enamel epithelium, stellate reticulum and stratum intermedium layers. The activity of inner enamel epithelial cells is related to primary and young enamel formation. On the other hand, the stellate reticulum and stratum intermedium cells are associated with immature enamel formation, transformation of immature to mature enamel and enamel mineralization. Histochemical analysis shows that alkaline phosphatase is present in inner enamel epithelial cells and is localized in ameloblast nuclei whereas it is present in nuclei and cytoplasm of cells of stratum intermedium and stellate reticulum. [4]

Formation of Tomes' process

Immediately following the initial deposition of an enamel layer, pronounced changes occur in the distal region of the ameloblasts. As the cells begin to withdraw, many cytoplasmic protrusions appear on their distal surfaces. These protrusions contain numerous microtubules in different orientations. As enamel deposition proceeds, ameloblasts continue to withdraw, leaving behind a cone-shaped cytoplasmic process (Tomes' process) immersed in newly deposited enamel. The distal cytoplasm beyond the junctional complexes, and the

base and intermediate portions of the Tomes' process, possess many small secretory granules; coated and uncoated vesicles, vacuoles and elongated structures. There is a gradual decrease in number of elements from the base to tip of Tomes' process. Terminal bars and Tomes' processes of the ameloblasts are more developed in zones corresponding to primary enamel. However, greater development of cells of stellate reticulum and stratum intermedium is localized in zones of immature and transitional enamel and enamel mineralization. [4]

Stages of enamel formation on functional basis are classified as: [5]

1. Secretion of an organic matrix.
2. Crystal nucleation.
3. Crystal elongation.
4. Removal of organic matrix.
5. Crystal maturation.

Stages of ameloblast life-cycle and their genetic regulation

Neural crest cells migrating to the future dental arches induce thickening of the stomodeal epithelium. Tooth development requires interactions between the epithelium and the underlying mesenchymal cells. The five stages of ameloblast life-cycle are:

1. Presecretory stage: Differentiating inner enamel epithelial cells are not yet depositing mineralizing enamel matrix.
2. Secretory stage: Ameloblasts secrete enamel matrix proteins and proteinases.
3. Transitional stage: The stratum intermedium, stellate reticulum and outer enamel epithelium alter their cytoarchitecture to form papillary cells. The secretory ameloblasts become short in height.
4. Maturation stage: Ameloblasts undergo cyclical alterations between ruffle-ended and smooth-ended morphologies, with lateral membrane modulations and alterations of intercellular junctions. [6,7] Water and

organic matrix are removed and calcium and phosphate ions are deposited. There is a banded distribution of two different ameloblast morphologies in enamel organ. The extracellular processes comprising enamel maturation are highly cyclical and correlate precisely with, and on the same time as, the rapid modulation of maturation ameloblasts.

Repetitive alterations between ruffle-ended and smooth-ended morphologies are the principal functional activity of maturation stage ameloblasts. The predominant morphology for ameloblasts during the maturation stage is 'ruffle-ended'. The maintenance of this border requires microtubule assembly. In some species, where tooth formation is a rapid process as many as 45 of these reformative cycles take place in fifteen days. The ruffled-border possesses a characteristic morphology. These histological features include differences in the degree of vacuolation and density of contents seen within the infoldings of the apical plasma membrane which gradually becomes more dilated and foamy as enamel matures. These tubular channels are filled with electron-dense material at the beginning of the maturation stage. These structures disappear half-way through the maturation stage, when most proteins have been removed from the developing enamel. During the transition, the cells apparently first re-establish apical junctional complexes, then they reform the tubular channels and ultimately, form the deeply infolded apical plasma membrane surface.

The pH of forming enamel fluctuates during modulation but rarely drops below 6.1. There are only three likely mechanisms, ameloblasts can use to neutralize excess acid (H^+ ions) during periods of crystal growth. These are:

- a. via frequent flushing of the fluid-filled spaces in enamel, including the ions.

b. via a matrix protein, like amelogenin to absorb the hydrogen ions.

c. via a continuous release of bicarbonate ions into the enamel layer in proportion to the amount of excess hydrogen ions being generated by crystal growth.

Based on cytochemical and tracer studies, ameloblasts have been shown to possess resorptive function at secretory and maturation stages. [8] Ameloblasts take up exogenous proteins from the Tomes' processes and from the lateral cell surfaces.

5. Reduced ameloblast stage: Cells become flattened and are no longer active.

APC Gene expression:

Adenomatous Polyposis Coli (APC) gene expression is transient in various stages. No APC activity can be detected in early (differentiating) presecretory ameloblasts. APC expression is first detectable in the apical cytoplasm of presecretory ameloblasts when the odontoblasts secrete the first dentinoid layer. APC expressivity can also be seen in stratum intermedium. APC can be detected in the secretory ameloblasts from the Tomes' process to the terminal web apparatus near the base of the cell. APC expression decreases in ameloblasts and stratum intermedium cells at the end of the secretory stage. [9]

E-cadherin and APC co-expression:

The presecretory stage ameloblast express E-cadherin and no APC. In the secretory stage, when cell-cell adhesion must be down-regulated to allow sliding movements of ameloblasts, APC expression is up-regulated and E-cadherin is downregulated. In the transition stage, where the ameloblast morphology changes again, E-cadherin is strongly re-expressed and APC expression disappears. In the maturation stage, when junctions formation alternates between the apical and basal terminal web complexes, E-cadherin is

downregulated and APC expression reappears. In the maturation stage, the dental papillary cells express E-cadherin and catenin molecules. α - and γ -catenin are present in ameloblasts throughout amelogenesis. [9]

Enamel matrix:

Evidence derived from biochemical, immunocytochemical and autoradiographic studies show that ameloblasts start secreting enamel matrix proteins as they start differentiating. [10,11] Their secretory activity peaks during the secretory stage and continues at a lower rate in maturation stage. Enamel formation is an extracellular process involving enamel protein removal coinciding with subsequent replacement by carbonated hydroxyapatite crystallites. Assembly of enamel matrix, disassembly by proteases, and the growth of mineral phase at the expense of the organic phase imply that enamel formation is an extremely intricate biological process. Transient expression of dentin sialophosphoprotein by pre-secretory ameloblasts point towards early events of amelogenesis; dentinoenamel junction and adjacent "aprismatic" enamel formation.

Amelogenins are tissue-specific proteins that are primarily expressed by inner enamel epithelial cells and represent the major enamel matrix constituent in developing enamel. In humans, there are two amelogenin genes, AMELX and AMELY, located on the sex chromosomes; however, these are non-allelic. Approximately, 90% of amelogenin mRNA expression is from X chromosome. Mutations in AMELX cause X-linked amelogenesis imperfecta. During enamel development, extensive alternative splicing of the amelogenin primary transcript leads to up to 15 individual mRNAs. The translational mRNAs are progressively hydrolyzed by enamel proteases as enamel crystals grow. This process leads to a highly organized enamel

layer with less than 2% organic matrix, by the time tooth erupts into the oral cavity. [12]

The most abundant non-amelogenin proteins are ameloblastin and enamelin, expressed from the AMBN and ENAM genes, respectively. The human AMBN and ENAM genes are located on chromosome 4q13.2. Mutations in ENAM gene cause a severe form of autosomal-dominant smooth hypoplastic amelogenesis imperfecta. [11]

Ameloblastin (also known as amelin or sheathlin), is an extracellular protein that is expressed at high levels in secretory and post-secretory ameloblasts and also by Hertwig's epithelial root sheath cells during cementogenesis while Amelogenin and Tuftelin appear to be restricted in their expression to ameloblasts engaged in enamel formation. Ameloblastin is found at the interface between Tomes' process of the ameloblast and the newly secreted enamel organic matrix. An anchoring function between ameloblastin and enamel matrix. [13]

Both amelogenin and tuftelin proteins exhibit self-assembly domains. However, there is no affinity between these two proteins. The "two-phase" system for amelogenin and tuftelin bio-activity appears to be uninfluenced by ameloblastin.

Enamelin:

The term "Enamelin" is restricted to the enamelin gene (ENAM) and its protein products. Enamelin is present in minor quantities in the immature enamel matrix. Enamelin cleavage products concentrate in the rod and interrod enamel and are scarce in the sheath space. This "reverse honeycomb pattern" suggested that the enamelin cleavage products are apparently bound to enamel crystals. The enamelin expression for its N-terminus is uniform in the enamel matrix. This expression shows variation as it decreases for surface to dentinoenamel junction (DEJ). It is strongly expressed at the dentinoenamel junction (DEJ). Variations in enamelin expression suggested

that the N-terminus is rapidly degraded or re-absorbed into the ameloblasts. The most restricted localization pattern is exhibited by the enamelin C-terminus, beneath the secretory face of the Tomes' process at the mineralization front. This highly restricted enamelin precipitates in the elongation of enamel crystals at the mineralization front. The 32kDa enamelin component accumulates in the rod and interrod enamel, bound to depositing mineral, while the N-terminus Polypeptides do not bind mineral and concentrate in the sheath space. Enamelin is processed by MMP-20 almost immediately following its secretion. [14]

The human enamelysin (MMP-20) gene is located on chromosome 11. The MMP-20 codes for a calcium-dependent proteinase enzyme. MMP-20 expression has been described in ameloblasts, pre-ameloblasts, odontoblasts, odontogenic tumors and carcinoma cell lines originating from tongue. Kallikrein-4 (KLK-4) protein is a calcium-independent serine proteases. KLK4 is secreted as an inactive zymogen of 230 amino acids that become active protein comprising of 224 amino acids, formed by removal of six amino acids by MMP-20. KLK4 is actively present in the mineralized enamel at the dentinoenamel junction during the secretory stage. KLK4 is responsible for the degradation of TRAP amelogenin cleavage products to smaller fragments. [12]

DLX3 gene and protein:

DLX3 gene is a member of homeobox gene family that is homologous to the distalless (D11) gene of drosophila. It is expressed during development of chondrocranium, dermatocranium, sensory organs, brain, limbs, and appendages, and in osteogenesis and hematopoiesis. The encoded DLX3 human protein is a 31738 Da protein composed of 287 AA with a 60 AA homeodomain. This protein acts as a transcriptional activator and is essential for craniofacial, tooth, brain, hair and neural

development. Mutation in human DLX3 gene homeodomain is associated with amelogenesis imperfecta (hypoplastic-hypomaturation type), with taurodontism.

Till this date, mutations in five genes, AMELX, ENAM, KLK4, MMP-20 and DLX-3 have been found to cause amelogenesis imperfecta. [15,16]

Tuftelin:

Tuftelin protein has been suggested to function during enamel crystal nucleation. During mammalian tooth formation, the initial deposition of mineral is highly regulated. In developing tooth cusp, there is a center where differentiation is most advanced. A dramatic increase in matrix secretion occurs at these centers. It is presumed that dentin and enamel crystallites nucleate on organic matrix. Enamel crystals seem to initiate independent of dentin mineral suggesting that an ameloblastic secretion is required for nucleation. Organic macromolecules regulate crystals shape. Enamel crystallites are unique in growing principally I length while remaining only a few nanometers thick. This preferential growth of enamel crystallites in C-axis direction occurs due to inhibition of crystal growth on sides by organic macromolecules. Hence, tuftelin is the principal candidate molecule in enamel matrix required for nucleation of enamel crystallites. [15,16]

Apoptosis of ameloblasts:

Ameloblasts die in two phases during the maturation stages. [17] The first component of cell death occurs during post-secretory transition, when ameloblasts rapidly re-organize themselves from a predominantly secretory function to a transport/barrier function. Approximately 25% of the total ameloblast population entering post-secretory transitions die in a matter of hours, leaving 75% of original population that formed enamel rods to initiate rhythmic modulations on the surface

of maturing enamel. Apoptotic changes such as shrunken and condensed nuclear fragments are evident. Surviving ameloblasts engulf cellular debris.

The second phase of cellular death occurs the long modulation phase, when the enamel hardens. An additional 25% of ameloblast population dies as ameloblasts modulate, so that only 50% of original population which formed enamel rods, eventually finishes the maturation stage. The final component of apoptotic death apparently occurs in reduced ameloblasts during eruption, when the reduced enamel epithelium fuses with oral epithelium to form junctional epithelium.

Calcium movement during amelogenesis:

Calcium flux into forming enamel is regulated by regulation of the intracellular junctions of ameloblasts and calcium transport system. [18,19,20] In the secretory and smooth-ended ameloblast regions, calcium moves to the enamel surface through the intercellular spaces of ameloblasts and in rough-ended ameloblast regions via the ruffle-ended ameloblasts.

Biochemical aspects of enamel mineralization

Acid phosphatase is one of the major constituents of early enamel crystals formed during enamel matrix nucleation. [21] The surfaces of developing enamel crystallites are rich in hydrogen phosphate ions (HPO_4^-). Approximately 85% of calcium present in enamel fluid in secretory stage of amelogenesis is in non-ionic form. This calcium is probably bound to peptides derived from the enzymatic degradation of amelogenesis. The Ca-ATPase transport system is present on the entire cell membrane i.e., apical, basal and lateral aspects of the secretory ameloblasts. Therefore, the potential capacity for calcium transport is high in secretory stage ameloblasts. [22,23]

Assumptions based on studies by Moreno [1987] and Crenshaw [1982] on porcine enamel formation show that to deposit 1.2 mm thick enamel at the rate of 4-6µm/day: [22,23]

- a. Diameter of single enamel prism is 3 µm.
- b. The average tissue density during the secretory stage is 2.0 gm/cm³.
- c. The average mineral content in organic matrix (in secretory stage) is 40% by weight.
- d. each prism is formed by one ameloblast.

Fluoride ion substitutes calcium hydroxyl ions in apatite form; reducing the crystal volume and increasing the structural stability. In the process of enamel mineralization during amelogenesis, free fluoride ions in the enamel fluid accelerate the hydrolysis of acidic precursors, and accelerate the growth of apatite crystals. The incorporation of fluoride ions into the enamel reduces the mineral solubility thereby, modulating the ionic composition of enamel fluid and enhancing the matrix protein-mineral interaction. However, excessive fluoride concentration leads to abnormal enamel formation by retardation of enamel maturation. [24]

The fluoride content of enamel mineral increases up to an average of 100 ppm, during the secretory phase and continuously increases up to 1000 ppm, during the maturation stage of amelogenesis. Coating of hydroxyapatite crystals with enamel matrix proteins result in retardation of fluoride ion incorporation into the crystals. Calcium flux into forming enamel is regulated by the intercellular junctions of ameloblasts and the calcium transport system. [25]

CONCLUSION

Enamel is the highest mineralized tissue in the body. Its formation is a genetically regulated process which takes place by mechanisms inherent in the odontogenic epithelial and mesenchymal

tissues. The matrix deposited is a complex pot-pourri of various growth factors and cytokines that are formed by the ameloblasts. Mineralization of enamel matrix is an equally dynamic process that eventually results in a protective yet environmentally vulnerable hard tissue.

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