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Biom mineralization of a self-assembled-, soft-matrix precursor: Enamel

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Abstract

Enamel is the bioceramic covering of teeth, a composite tissue composed of hierarchical organized hydroxyapatite crystallites fabricated by cells under physiologic pH and temperature. Enamel material properties resist wear and fracture to serve a lifetime of chewing. Understanding the cellular and molecular mechanisms for enamel formation may allow a biology-inspired approach to material fabrication based on self-assembling proteins that control form and function. Genetic understanding of human diseases expose insight from Nature's errors by exposing critical fabrication events that can be validated experimentally and duplicated in mice using genetic engineering to phenocopy the human disease so that it can be explored in detail. This approach led to assessment of amelogenin protein self-assembly which, when altered, disrupts fabrication of the soft enamel protein matrix. A misassembled protein matrix precursor results in loss of cell to matrix contacts essential to fabrication and mineralization.

Development of the enamel organ epithelia and enamel formation

The developing tooth is a well-recognized model for epithelial-mesenchyme dependent organ formation, where it has been used to identify reciprocal-signals exchanged between the ectoderm-derived epithelia (ameloblasts) and the neuroectoderm-derived mesenchyme (odontoblasts). These signals determine cell fate, organ morphogenesis and terminal cell differentiation [1-3] with formation of enamel, dentin and cementum, the cell-specific bioceramic composite dental tissues. Here, I focus on the less appreciated exchange of signals that continue through enamel formation that are required to ensure proper formation and function.

These instructive signals culminates in the activation of transcription factors that initiate enamel gene expression with the epithelial derived ameloblast cell exiting the cell cycle to initiate formation of the enamel extracellular matrix precursor [4-6]. The enamel matrix is composed principally of amelogenin protein, with modest contributions from other enamel matrix proteins, namely ameloblastin, enamelin, tuftelin, amelotin [7, 8] and short-lived sulfated proteins [9].

At the start of enamel matrix formation, an increase in the transcription factor CAAT/enhancer binding protein- α (C/EBP- α) drives the ameloblasts out of the cell cycle, an action that is opposed by Msx2 [10]. C/EBP- α also serves to up-regulate the expression of enamel matrix proteins, such as amelogenin, ameloblastin and other structural proteins [5, 11, 12]. Ameloblasts are remarkably cells serving all of the requirements for producing a bioceramic composite. Along with enamel protein synthesis, the ameloblasts are simultaneously responsible for synthesizing the proteins responsible for degrading the enamel matrix proteins, for synthesizing the transmembrane proteins and for the intracellular machinery required to re-absorb and further process degraded matrix proteins. Ameloblast cells also biosynthesize and control the ion solute transporter proteins responsible for calcium and phosphate transport needed to produce the mineral precursor and to modulate the extracellular pH to remove hydrogen ions produced with hydroxyapatite (HAP) mineral deposition. [13-19]. These complicated and uncorrectable production steps require the ameloblasts to remain precisely orchestrated with respect to a specific segment of the forming matrix that it created. The fundamental unit of enamel formation is known as the enamel rod, with each rod created by a single ameloblast cell (Figs. 1, 2), the so-called interrod matrix precursor and final mineral phase is produced cooperatively in the area shared by adjacent ameloblasts (Fig. 2B, 2C). The hierarchical complexity of mouse incisor enamel is elegant, resembling a fabric (see Fig. 1A) composed by thousands of individual HAP crystallites bundled together by an ameloblast cell (Fig. 1B, 1C). Reciprocal cell-to-matrix interactions are accomplished by linking one ameloblast cell to one enamel rod [20]. The cell must stay in contact with the cylinder of matrix proteins it synthesized during a migration of thousands of micrometers (Fig. 1A) during enamel formation [21].

The first wave of enamel matrix protein occurs at the dentine enamel junction (DEJ), in an anatomic area of “specialized” enamel that serves to link two dissimilar bioceramic tissues, the enamel with the dentine [22, 23]. We focus our attention on the interactions between the ameloblast cells with the enamel protein extracellular matrix. We describe the molecular pathways used by the ameloblasts to retain their physical location over the sea of enamel matrix proteins. We show that changes to the ability of the cell to interact with the matrix alters enamel formation and such interruptions are regularly seen in human diseases of the dentition [24, 25] and in genetically altered mice [26-28]. The presence of a highly conserved domain in amelogenin protein provides a functional motif for cell-to-matrix interactions through receptors located on the specialized secretory end piece of the ameloblast cell, the Tomes’ process. [29]. The possible origin of lesions arising from altered cell-to-matrix interaction has generally been overlooked since the enamel mineral phase provides to many observers the impression of an inert, non-living ceramic, rather than the vibrant tissue that is reliant on cell-to-matrix interactions for proper formation. Formation of a functional and correctly organized enamel matrix must precede the formation of the mineral phase. Enamel does not remodel, therefore errors in matrix formation are permanently recorded in the mineral phase and result in alterations to the material properties of the teeth, making them less tough and prone to early failure. Such defects would provide a strong negative selection pressure during ancestor evolution, even though they are compensated for in humans by medical intervention

Ameloblast cells mold the enamel extracellular matrix made of protein

Ameloblasts are the cells responsible for enamel formation (see Fig. 2). Enamel begins as a 100% protein extracellular matrix precursor, with amelogenin being the most abundant protein. Enamel proteins self-assemble to create a matrix that serves to guide its replacement by the mineral phase [30-33] (see Fig. 2A). In just days, the rodent tooth erupts into the oral cavity, only now the enamel contains less than 0.5% protein [34]. There are no second chances to correct enamel matrix production defects since enamel does not remodel and with eruption, the cells responsible for its fabrication cease to exist.

Nanci and co-workers found that mineral deposition occurs some distance away from the site of secretion at the Tomes' process of ameloblasts [35]. In this mineral-free zone, enamel matrix proteins undergo assembly into a matrix competent to guide the mineral habit as long, thin crystallites aligned along their carboxyl-terminus axis (long axis) parallel to the path that the ameloblast moves, secreting a cylinder of matrix to form the enamel rod (prism). The matrix at this stage is not mineralized, but rather is in the form of a hydrated gel. The ameloblasts hover over this protein gel with the Tomes' process embedded in the gel (see Figure 4). Each ameloblast retains a relationship between one ameloblast and one cylinder of enamel matrix defining the rod (Figs 1, 2 & 4).

Ameloblast-to-matrix registration is thought to result from the sum of cell-to-matrix interactions mechanisms (Fig. 2, 4, 5). Among these are the integrins, the well-known receptors located on the ameloblast secretory termini (Tomes' processes) that recognize amino acid motifs within enamel matrix proteins. Next is the action of lectin-like regions within the amelogenin protein that bind N-acetyl glucosamine residues on membrane protein receptors (glycocalyx), which are embedded on the secretory surface of ameloblasts (Tomes' processes). This sugar binding function of amelogenin resides in thirteen amino acid residues within the self-assembly domain of amelogenin [36, 37], (see Figs. 3B, 5). In the presence of a defective enamel matrix, disruption to these two pathways can reach a critical level, allowing the ameloblast cells to lose contact with the matrix and re-enter the cell cycle (Fig. 5). In vivo, ameloblast cells from developing teeth suffer from loss of interaction with the forming enamel extracellular matrix when their genome is engineered to express amelogenin that lack the self-assembly domain defined by amino acid residues 1-42 (A). The cells detach from the matrix, and re-entry into the cell cycle. In vitro, we confirmed that ameloblast-like LS8 cells interact poorly with amelogenin 1-42 protein and instead prefer wild type amelogenin [27].

Amelogenin Protein and Self-Assembly of Nanospheres

Ameloblasts secrete the enamel matrix through a specialized membrane termed the "Tomes' process". One ameloblast cell is responsible for the formation of one-enamel rod (also known as an enamel prism). One can envision the ameloblast secreting a cylinder of enamel matrix, approximately the same diameter as the ameloblast, with Tomes' process embedded in the enamel matrix (see Fig. 2, 5). Secretion starts at the dentine (dentine to enamel junction) and extends outward towards the chewing surface of the enamel (Fig. 1). The cell continues to secrete more enamel matrix protein to grow the future enamel thickness, similar

to squeezing a tube of toothpaste, while remaining in contact with each cylinder of enamel matrix proteins (Fig. 2). Cell-to-matrix registration is preserved over the entire path of secretion, a distance of several hundred to thousands of micrometers in length (Fig. 1). The basic design of enamel revolves around the organization of these matrix cylinders one to another, with the ameloblasts weaving the cylinders of enamel matrix proteins so that they coalesce on their inferior surface (Fig. 2). This produces continuity to the matrix known as the interrod. The unique physical properties of the final mineralized enamel are dependent upon the weaving of these cylinders of protein matrix and their coalescence to form the continuity of the interrod enamel [38, 39]. Within each enamel rod, thousands of substituted hydroxyapatite crystallites form, first as thin crystallites surrounded by amelogenin nanospheres that promote growth along the crystal ends [40, 41] (Fig. 2). Enamel matrix proteinases degrade the amelogenin rich matrix while the crystallites grow in thickness, eventually lying next to one another in the space previously occupied by the enamel matrix proteins [13, 42, 43] (Fig. 1).

The dominant protein of the enamel matrix is amelogenin. Amelogenin undergoes self-assembly to form nanospheres and, along with other enamel proteins, self-organizes as an extracellular matrix that is competent to orchestrate its own replacement by the mineral phase [44]. The ameloblast first creates a 100% organic enamel extracellular matrix in which enamel formation is initiated. However, over a short period of time, the matrix is converted to greater than 99.5% inorganic hydroxyapatite crystals [30, 31, 34, 45]. This metamorphosis is not achieved in a single step, but rather by a series of incremental steps in which amelogenin undergoes precisely regulated proteolytic degradation during removal. Recent evidence has shown that enamel formation is punctuated by a circadian cycle, in which ameloblasts cycle between 12 hours of matrix production, followed by 12 hours of matrix degradation and mineral growth [46]. With matrix degradation and removal space is created allowing the crystallites to expand in width and length, forming a polycrystalline enamel rod, that contributes to the unique material properties of enamel that allow it to last a lifetime [38]. The interface formed by the weaving of enamel rods and the small defects, which retain enamel matrix protein such as amelogenin among stacked crystallites, serve to favorably influence enamel toughness [22]. We recently created a knock in mouse in which enamel matrix was made by only the wild type mouse 180 amino acid long amelogenin. Removing all of the other amelogenin protein isoforms that contribute to matrix formation resulted in more than a 10-fold reduction in protein complexity, but only a 10% reduction in toughness [47].

Analysis from an evolutionary perspective has identified highly conserved domains within amelogenin protein [48-50]. The origin of the amelogenin gene has been traced to 630 million years ago to the Precambrian period [51, 52] with the identification of the conserved amino acid sequence at the amino-terminus hinting at a new function. We used the yeast two hybrid assay to identify domains within amelogenin that promote self-assembly (see Fig. 3A). This assay is based on the ability of the Gal4 transcription factor to be split into two function pieces, the binding domain (Gal4-BD) and the activation domain (Gal4-AD). Each of the Gal4 segments can receive a “query” protein fused “in frame” with the Gal4 segments. If the query proteins interact with one another, the Gal4 is brought back together where it initiated expression of a reporter, Lac-Z. Yeast cells carrying the interacting pair of

proteins become colored when assayed with a chromogenic substrate, signaling the positive interaction between the amelogenin proteins. The affinity of the interaction can be estimated by the extent of chromogenic substrate conversion in a simple calorimetric assay. Two domains within mouse 180 amino acid amelogenin (M180) responsible for protein-to-protein interaction were defined by residues 1-42 (A-domain) and by 157-173 (B-domain) to be required for self-assembly of the matrix through formation of nanospheres [29, 53, 54] (see Fig. 3B).

Patients with the hereditary enamel formation defect, *amelogenesis imperfecta*, exhibit a mutation at Pro³³ that lies within the highly conserved A-domain (amino acids 1-42) required for amelogenin assembly into nanospheres [53, 54] (Fig. 3B). Residues 33-45 within the A-domain also serve to define a lectin-like region that binds N-acetyl glucosamine with high affinity [36] and is thus able to provide a molecular basis for matrix-to-cell interaction.

We removed domain A from amelogenin (A) by genetically engineering a “knock in mouse”. Here, the mouse genome is specifically altered by homologous recombination to target a gene of interest in the mouse genome. This approach serves to replace the wild-type amelogenin with an engineered amelogenin lacking the self-assembly domain A (A). Importantly, all of the mouse DNA that provides regulatory control over amelogenin gene expression is preserved, so that the effect of the engineered protein on enamel formation can be ascertained in its full complexity [27] without changes to the protein abundance that can occur with transgenic mice. Moreover, a transgenic approach retains functions the wild type gene making interpretation of a phenotype difficult [55]. In contrast, we used knock in genetic strategy in which the wild type gene is removed and replaced with the engineered amelogenin protein so that interpretation of the changes to enamel hierarchy can be more readily identified. The incisor teeth from these animals reveal a profound defect to enamel formation (Fig 4). This effect of the knock in is most readily seen in the forming enamel from an incisor of a heterozygous female (Fig 3D). Because the amelogenin gene is located on the mouse and human X-chromosome [56], random inactivation of one the two X-chromosome occurs [57]. In this way, a defective matrix is made by cells expressing the chromosome bearing the knock in amelogenin that lacks the self-assembly domain-A (Fig 3C, arrows) while normal matrix is made adjacent to it by a cohort of cells expressing wild type amelogenin with its complete functional repertoire (Fig 3C). To better observe the changes in cell-to-matrix interaction, we removed ameloblast cells from the forming matrix and observed the indentations made by Tomes’ processes in forming enamel. Enamel made with wild-type amelogenin shows a repeating pattern for the indentation of Tomes’ process revealed by the rod and interrod boundaries in the soft-protein matrix (Fig 4B). In contrast, the shape and contour of the Tomes’ processes was altered from this repeating shape in the enamel made by cells expressing an amelogenin protein lacking the A-domain (A) (Fig. 4A).

Light microscopy was used to interrogate the forming incisor tissue and revealed that the incisors made with wild type amelogenin possess well-formed and highly defined Tomes’ processes that were covered by integrin-alpha 6. Compared to the wild type, the incisor from the A amelogenin mice showed significant distortion to the shape of the Tomes’ process

along with reduced abundance of integrin- α 6, suggesting the defective matrix no longer supported cell-to matrix interaction to the same extent as did the wild type matrix. In slightly older animals we observed detachment of the ameloblasts cells from the matrix (Fig. 4E) with cell proliferation and the formation of multiple layers of ameloblasts. Ameloblast cells are normally post-mitotic and cease their proliferation prior to enamel matrix formation [58], however, in mice expressing the A-domain deleted (A) amelogenin, the cells re-enter the cell cycle as shown by incorporation of the DNA nucleotide precursor, 5'-bromodeoxyuridine (BrdU) (Fig. 4E, 4F). The cell-to-matrix interactions that dominate enamel formation during this essential soft-phase of early enamel formation are altered and result in profound defects to the mineral phase [27, 59]. Defects in the protein matrix do result in defects to the mineral.

Matrix Assembly and Cell-to-Matrix interaction: a Model

Enamel formation proceeds through a soft-phase, in which ameloblast cells synthesize and organize the soft protein matrix precursor as shown in figure 5A. With time, the proteins are almost completely removed and replaced by substituted hydroxyapatite (Fig. 5B). This unique form of fabrication produces a hierarchically integrated composite material. Cell-to-matrix interactions serve to manipulate the soft-precursor phase, molding it before mineral replacement. The molecular basis of the cell-to-matrix interaction stem from receptors on the Tomes' processes and the lectin-like domain of amelogenin that self-assemble to form nanospheres (Fig. 5). Biochemical knowledge for the amelogenin protein was coupled with insights gained from human mutations of the amelogenin gene, which, when coupled with advances in mouse genetics permitted us to test predictions of protein function in whole animals. Understanding the cellular and molecular basis for enamel formation offers insights into nanotechnology approaches to enamel regeneration.

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Figure 1.

SEM image of fully mineralized mouse incisor enamel illustrating the hierarchical complexity achieved by cells weaving the soft enamel matrix precursor phase.

Panel A, backscattered electron image of full thickness enamel revealing the alternating decussation of enamel rods (R). At both the dentine enamel junction (arrow scale bar) and at the chewing surface, the enamel rods do not decussate (Courtesy of Drs. Lacruz and Bromage, New York University);

Panel B, SEM image of a ground and etched en face surface of mouse incisor enamel which reveals the third orientation of HAP crystallites corresponding to the interrod enamel (IR) whose crystallites are oriented approximately normal to the long axis of the HAP crystallites from the enamel rods (R). The interrod surrounds the rod (see Fig.2); and

Panel C, the fundamental unit of enamel is the “rod”, which is fabricated by a single cell and is composed of thousands of HAP crystallites organized by and within the now absent protein precursor soft phase.

Figure 2.

The hierarchical organization of enamel is established during the soft precursor phase composed by enamel matrix proteins.

Panel A, Mesoscale (cell) through nanoscale (protein) hierarchical enamel organization.

Panel A1, fluorescent light microscope image of ameloblasts secreting red fluorescent-labeled amelogenin protein into the forming enamel; **panel A2**, SEM image of the position of ameloblast cells following mechanical removal of cells to reveal the underlying cell fabricated enamel extracellular matrix visualized by SEM; **panel A3**, SEM image of rod and interrod enamel, each rod is composed by bundles of thousands of HAP nanocrystallites which are surrounded by the interrod enamel; **panel A4**, TEM image reveals self-assembled wild type amelogenin nanospheres decorating the HAP crystallites perimeter; **panel A5**, AFM tapping mode image of wild type amelogenin nanospheres self-assembled from recombinant produced mouse amelogenin.

Panel B, Cartoon image of time-progression for enamel formation at the interface for cell-to-enamel ECM of rod and interrod boundaries drawn to emphasize the progressive conversion of the soft protein phase to the mineral phase. **Panel B1**, illustrates the earliest secretory phase, with amelogenin nanosphere assembly occurring upon secretion into the extracellular space to form a soft- precursor phase that is molded by the cells as they are pushed away from the matrix under pressure from protein secretion. The ameloblast

specialized secretory end piece, the Tomes' process, is responsible for secretion and molding of the enamel extracellular matrix, as well as for transport of calcium and phosphate into the matrix for HAP mineral formation and for reabsorption of the matrix proteins as they are degraded to allow the mineral phase to expand, all while controlling the extracellular pH; **panel B2**, the second most abundant enamel matrix protein ameloblastin (double line), serves to delimit the lateral boundary of the enamel rod while octacalcium phosphate and amorphous calcium phosphate precursors are converted to HAP.

Ameloblastin has also been shown to self assemble [60]; **panel B3**, HAP crystallite long-axis parallels the secretory path of the ameloblast within the rod and are surrounded by the interrod HAP crystallites with the long-axis of interrod crystallites oriented approximately normal to the HAP crystallites within the rod which fabricates a mineral phase continuum. **Panel C**, Enamel formation at the cell-to-enamel matrix interface. **Panel C1**, illustrates the immunodetected distribution of ameloblastin protein (light microscopic image taken from [61]) along the enamel rod perimeter to define its lateral boundaries; **panel C2**, SEM image of a newly formed segment of enamel after the ameloblast cells have been removed. The conical indentations are the depression into which the Tomes' processes that forms the enamel rod fit (R), while the interrod (IR) forms from the collaborative secretion of neighboring ameloblasts, forming a continuum for the matrix which is converted to HAP crystallite. The approximate location of the ameloblastin protein is shown as a double line on the periphery of the enamel rod.

Figure 3.

Yeast two-hybrid assay used to identify amelogenin assembly domains and the alteration to hierarchical enamel organization with elimination using genetic engineering of the amino terminal A-domain for amelogenin self-assembly. **Panel A**, GAL4 is separated into two independent domains: the Binding Domain (BD) and the Activation Domain (AD) neither of which alone results in expression of the reporter gene. Recombinant DNA allows the mouse 180 amino acid (M180) amelogenin to be joined in frame to either the Gal4-AD or the Gal4-BD, neither of which alone can activate the lac-Z reporter. However, when both constructs are transfected into the same yeast cell, interaction between the amelogenin self-assembly domains allows the Gal4 transcriptional activity to be reconstituted, with the yeast colony developing a blue colour in the presence of a chromogenic substrate revealing the protein-to-protein interaction; **panel B**, cartoon diagram of the two amelogenin self-assembly domains, an “A-domain” comprising amino acid residues 1-42 which includes the N-Acetyl-Glucosamine lectin binding domain and a “B-domain” comprising residues 167-172; **panel C**, genetic knock-in of an mouse amelogenin gene in which the “A-domain” was deleted from the expressed protein and the resulting mouse incisor enamel from a chimeric female. Amelogenin is on the X-chromosome; consequently females have two copies, a wild-type amelogenin and an “A-domain” deleted amelogenin. Random inactivation of one copy of the X-chromosome results in cohorts of ameloblast cells making enamel with either the wild type amelogenin or amelogenin lacking the “A-domain” for self-assembly (arrow), resulting in a chimeric tooth composed of normal enamel interspersed with abnormal enamel (arrow).

This permits each tooth to have both a control and an experimental genotype and phenotype. The elimination of the A-domain permits the creation of a phenocopy of the human enamel formation disease, *amelogenesis imperfecta*; **panel D**, RT-PCR validation for the expression of A-domain deleted amelogenin (A) in the animal shown in panel C.

Figure 4.

Cell-to-matrix interactions during enamel formation.

Panel A, SEM image of forming enamel following removal of the ameloblast cells, the loss of amelogenin self-assembly domain defined by amino acid residues 1-42 (A-domain) in this homozygous knock in mouse ($\Delta A/\Delta A$) results in alteration to the shape of Tomes' process, compared to **panel B**, where the canonical shape for Tomes' process is seen in wild-type (Wt/Wt) enamel matrix; **panel C**, immunodetection of $\alpha 6$ integrin in $\Delta A/\Delta A$ mouse incisors showing the integrin is localized and enriched on the blunted (see Fig. 4A) Tomes' processes (arrow) compared to the extended Tomes' processes (arrow) seen in the wild-type (Wt/Wt) animal shown in **panel D**. In the $\Delta A/\Delta A$ mouse incisor, multi-layered ameloblasts are typically found after postnatal day three (**panel E**) seen this hematoxylin-eosin stained sagittal tissue section, while BrdU incorporation into the DNA of ameloblasts indicates cell proliferation as the cells loose contact with the matrix made with ΔA amelogenin (**panel F**) as they re-entry the cell cycle from a post-mitotic state. The A-domain defines not only a self-assembly domain, but also an amelogenin domain required for cell-to-matrix contact and maintenance of the ameloblast phenotype.

Figure 5.

Cartoon model for ameloblast cell-to-matrix interactions in enamel. This model illustrates the proposed molecular basis for cell-to-matrix interactions in which a lectin-like domain in the wild-type amelogenin nanospheres interacts with the N-acetyl glucosamine contained on the ameloblast glycocalyx. The absence of the lectin-like activity in the A-domain deleted amelogenin in is hypothesized to alter the balance of interactions allowing the ameloblast to detach from the forming matrix and re-enter the cell cycle. Also participating are integrins that bind amelogenin protein domains (see Fig. 4C and 4D).