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Keratins as components of the enamel organic matrix

Olivier Duverger[1], **Elia Beniash**[2,*], and **Maria I. Morasso**[1,*]

[1]Laboratory of Skin Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD

[2]Department of Oral Biology, Center for Craniofacial Regeneration, University of Pittsburgh School of Dental Medicine; Department of Bioengineering, University of Pittsburgh Swanson School of Engineering; McGowan Institute for Regenerative Medicine, Pittsburgh, PA

Abstract

Dental enamel is a hardest tissue in the human body, and although it starts as a tissue rich in proteins, by the time of eruption of the tooth in the oral cavity only a small fraction of the protein remains. While this organic matrix of enamel represents less than 1% by weight it plays essential roles in improving both toughness and resilience to chemical attacks. Despite the fact that the first studies of the enamel matrix began in the 19th century its exact composition and mechanisms of its function remain poorly understood. It was proposed that keratin or a keratin-like primitive epithelial component exists in mature enamel, however due to the extreme insolubility of its organic matrix the presence of keratins there was never clearly established. We have recently identified expression of a number of hair keratins in ameloblasts, the enamel secreting cells, and demonstrated their incorporation into mature enamel. Mutation in epithelial hair keratin *KRT75* leads to a skin condition called pseudofollicularis barbae. Carriers of this mutation have an altered enamel structure and mechanical properties. Importantly, these individuals have a much higher prevalence of caries. To the best of our knowledge, this is the first study showing a direct link between a mutation in a protein-coding region of a gene and increased caries rates. In this paper we present an overview of the evidence of keratin-like material in enamel that has accumulated over the last 150 years. Furthermore, we propose potential mechanisms of action of KTR75 in enamel and highlight the clinical implications of the link between mutations in KRT75 and caries. Finally, we discuss the potential use of keratins for enamel repair.

Enamel: a brief overview

Dental enamel comprises the outer layer of a tooth crown and is the hardest tissue of the human body. It is composed of ~96% carbonated apatite, ~3% of water and less than 1% of organic matrix by weight. Although the organic matrix is a minor component of mature enamel, it plays a very important role in the mechanical toughening of this tissue [1-3]. The basic building block of enamel is the enamel rod, which consists of elongated crystals,

*Corresponding authors: ; Email: ebeniash@pitt.edu, ; Email: morasso@nih.gov.

arranged in parallel arrays with their crystallographic c-axes perfectly co-aligned (Figure 1A). Enamel rods are approximately 2-3 μm in diameter and are wrapped in a thin layer of organic matrix called enamel rod sheaths. Even though the organic matrix is present throughout the enamel thickness, its concentration is greater in the inner enamel layer where, in addition to the rod sheaths, larger organic structures called enamel tufts are present at the interface with dentin [4].

Ameloblasts are epithelial cells responsible for enamel deposition. They start to secrete a mineralized extracellular matrix on top of the dentin soon after the onset of dentin mineralization, and this stage of enamel deposition is called secretory stage. The composition of secretory enamel is very different from that of mature enamel; it consists of roughly equal parts of mineral, organics and water by weight. Importantly the structural organization of crystals in secretory and mature enamel is similar; the only difference is that the nascent crystallites are much thinner. The organic matrix of secretory enamel is composed primarily of a protein amelogenin, which accounts for 90% of the total protein [5, 6]. Other matrix components include the structural proteins enamelin and ameloblastin, and a proteinase MMP20 [6]. When the full thickness of enamel is deposited, secretory ameloblasts transform into maturation stage ameloblasts. During the maturation stage, the enamel matrix proteins are degraded by proteinases such as KLK4 and replaced by fluid in which enamel crystals grow laterally, until the density of mature enamel is reached [6, 7]. A very small organic fraction made of small peptides, amino acids and an insoluble proteinaceous material in the tufts and enamel rod sheaths remains in mature enamel.

A historical perspective

Early studies of mature enamel composition in the second part of the 19th century were focused on the question of enamel vitality, with some researchers proposing that enamel is a vital tissue due to its sensitivity to instruments, and others suggesting that enamel is a mineralized tissue lacking vital functions [8]. Attempts by Thompson to identify a vital material in enamel led to the discovery of an insoluble organic residue after dissolution of mineral by acid, and he suggested that this substance was keratin [9].

In 1930, Rosebury conducted an extensive biochemical study of enamel matrix and concluded that the material was keratin-like, based on its proteinaceous nature, its remarkable resilience to chemical attack, presence of sulfur in its composition and its reaction with a number of stains [8]. Importantly, in the mid-twentieth century some evidence of the crucial role of the organic matrix of enamel started to emerge [10, 11].

Systematic studies of enamel composition begun in the 1960s, when Eastoe identified a new class of proteins (named amelogenins) in forming enamel with a unique amino acid composition [12]. These proteins regulate enamel mineral nucleation, morphology and organization [13], and are degraded during enamel maturation with only a small fraction of insoluble protein remains in the mature enamel [14]. Importantly, the amino acid composition of enamel matrix of forming and mature enamel are drastically different [15, 16]. The discovery of the major organic component in forming enamel shifted the focus of the scientific community towards understanding the role of the organic matrix in the process

of amelogenesis, while interest in the organic matrix remaining in the mature enamel slowly faded.

Changes in the organic matrix composition from secretory to mature enamel were extensively studied in the 1970s by Robinson and colleagues who reported several important findings. Specifically, they analyzed the amino acid composition of the enamel matrix at different stages of amelogenesis and showed that there was a small fraction of enamel matrix deposited during secretory stage that remained in place post-maturation, while the major fraction of the secretory enamel matrix was degraded during the maturation stage [4, 17, 18]. This protein fraction remaining in mature enamel, named tuft, is rich in Ser and Gly and resembles skin keratins [4]. The similarities of tuft protein with keratins led to a hypothesis that the tuft protein complex might be “a primitive epithelial component of a more specialized enamel matrix” [4]. Its amino acid composition is very different from the amino acid composition of the enamel matrix at the secretory stage, which is predominantly comprised of amelogenin, rich in Pro, His, and Gln residues (Table 1). Furthermore, immunochemical studies showed that antibodies raised against tuft protein fraction react with a material in secretory vesicles of ameloblasts and with multivesicular bodies in cells of skin epithelium [19]. Altogether, these earlier studies strongly suggested that a protein with characteristics similar to keratin is present in mature enamel. However, the exact composition of the tuft protein has remained undetermined, primarily since it is heavily cross-linked and insoluble in a broad range of solvents [20].

A recent paradigm-shifting study shows for the first time that a set of epithelial hair keratins is present in teeth and are essential components of the organic material present in mineralized tooth enamel [21]. Keratins are fibrous structural proteins that are highly cross-linked and give a unique strength to epithelial tissues. Epithelial hair keratins are expressed in the supporting tissue surrounding the hair shaft (inner root sheath and companion layer). These layers of the hair follicle are vital for structural support and proper anchorage of the hair shaft into the skin [22]. Expression and functional analysis of mature human enamel demonstrated the presence of the epithelial hair keratin KRT75 in the organic matrix, primarily at the periphery of the enamel rods where enamel rod sheaths are located (Figure 1). Furthermore, genetic association was found between a common missense polymorphism in KRT75 ($KRT75^{A161T}$) previously identified as causal in pseudofolliculitis barbae, and the incidence of dental caries in a cohort of children and adults assessed using standard dental caries indices (The Center for Oral Health Research in Appalachia (COHRA) study) [23]. Linear regression analysis showed that the missense polymorphism in the *KRT75* gene significantly increased susceptibility to dental caries in adults but not in children.

In depth functional analysis of teeth showed that the pseudofolliculitis barbae-associated $KRT75^{A161T}$ mutation correlated with defects in the structural and mechanical properties of enamel exemplified by altered morphology and arrangement of enamel rods and significantly reduced inner enamel hardness, and a higher incidence of ‘channel-like’ carious lesions, which differ drastically from the broad funnel shaped lesions observed in clinical radiographs [21]. This discovery represents a transformative milestone in the understanding the genetic basis of caries pathology.

A second rarer missense polymorphism in KRT75 ($KRT75^{E337K}$), linked to loose anagen hair syndrome, is associated with increased caries in children only [21]. It is significant that the association of the E337K polymorphism was found in primary dentition. It might be hypothesized that subsets of epithelial hair keratins are distinct in the functional keratin networks required for the mechanical stability of primary versus permanent tooth enamel. These results strengthen the importance of targeting the keratin gene family in genetic studies of tooth decay, and point towards the possibility that mutations in other epithelial hair keratins will be associated with enamel defects.

We have conducted a correlation analysis between the amino acid composition of the rat enamel matrix at different stages of amelogenesis, as reported by Robinson et al. [18], and the amino acid compositions of rat amelogenin and rat KRT75. This analysis shows that, while the amino acid composition of secretory enamel matrix strongly resembles amelogenin, the amino acid composition of KRT75 correlates with late maturation and mature enamel (Table 2).

Function of hair keratins in tooth enamel

Based on the defects in enamel structure observed in individuals carrying the A161T polymorphism in *KRT75*, it appears that the epithelial hair keratins identified in enamel rod sheaths are involved in the proper arrangement and cohesion of enamel rods during their formation [22]. Considering their unique biochemical properties (elasticity and toughness), it can be hypothesized that hair keratins stabilize the enamel rod sheaths, and contribute to the perfect balance between hardness and fracture toughness of enamel. It is worth noting that epithelial hair keratins in the companion layer and the inner root sheath of the hair follicle are essential for proper guidance of the growing hair and for its anchorage into the skin [22]. Given that keratins are cytoskeletal proteins, it remains to be determined whether they are delivered through exocytosis or deposited in enamel with ameloblast fragments that have been show to be retained in enamel [24, 25].

Our data suggest that epithelial hair keratins may also contribute to the resistance of enamel to caries [21], potentially by stabilizing the enamel rods. Indeed, the periphery of enamel rods has long been considered a pathway of entry for lactic acid as well as the exit route for dissolved minerals [10]. Moreover, the proteins located at the rod sheath were shown to be more chemically stable in enamel from patients with healthy teeth than in sound enamel from patients prone to develop caries [11]. A later analysis of the tuft protein distribution in mature enamel points toward the possibility that it can inhibit progression of caries [26]. Any defect in protein composition/structure at this location may lead to destabilization of enamel rod sheaths and facilitate the initiation and development of caries. Therefore it is possible that, by compromising the stability of enamel rod sheaths, mutations in KRT75 reduce the resilience of enamel to progression of carious lesions.

Clinical implications: pseudofolliculitis barbae and tooth decay risk

Dental caries is by far the most common chronic infectious disease that affects individuals of all ages [27, 28]. Etiology of caries is complex and involves numerous factors including

socioeconomic and behavioral factors, such as diet, lifestyle oral hygiene, as well as genetic factors affecting enamel structure and saliva composition [29-32]. Knowing the genetic markers associated with a higher risk of caries may lead to more effective prevention and treatment options.

Our studies show that individuals with polymorphism in KRT75 associated with pseudofolliculitis barbae present ‘channel-like’ carious lesions potentially forming due to a more unstable organic material supporting the enamel rod. The diameter of these lesions (about 100 μm) suggests that they form along groups of enamel rods, and most likely correspond to the dissolution of previously described enamel features formed of bundles of enamel rods [33, 34]. The small diameter of these lesions makes them difficult to detect at early stages using conventional caries detection methods. It would therefore be essential when treating individuals with pseudofolliculitis barbae to use more aggressive prophylaxis approaches such as frequent application of fissure sealants throughout the lifetime [35]. In individuals with polymorphism in KRT75 the mechanical properties of the inner enamel are compromised [21] which needs to be taken into consideration. Therefore extra-care should be taken when treating these patients, specifically when preparing for a filling or a crown. As indicated above, children diagnosed with loose anagen syndrome that present the KRT75 E337K mutation have higher risk for developing caries in their primary dentition. Therefore, these patients would also benefit from early detection and more vigorous preventive care.

Although the A161T polymorphism in KRT75 has been associated with pseudofolliculitis barbae, not all carriers of the genetic variant exhibit the characteristic barber rash phenotype; it is more prevalent in individuals with curly hair (e.g., African Americans). However, we found that this polymorphism affected the enamel regardless of ethnicity [21]. A genetic test would therefore be essential for personalized prevention approaches to benefit individuals carrying this KRT75 polymorphism, whether they show visible signs of pseudofolliculitis barbae or not.

Potential impact in tissue engineering: hair keratins in enamel restoration

The discovery that hair keratins are present in mature enamel and play a crucial role in forming a cohesive and biomechanically stable enamel may have a significant impact on the design of novel enamel repair strategies. Unlike other mineralized tissues such as bone and dentin, which are deposited by cells that are present throughout life (osteoblasts and odontoblasts, respectively), the enamel of erupted teeth does not have any cellular component. It is therefore not possible to repair enamel defects such as those caused by caries by acting on the cells forming the enamel. Classical approaches to enamel restoration involve filling of enamel defects or capping tooth crowns using metals, ceramics or composite materials. Novel enamel repair strategies are emerging which focus on biomimetic mineralization of enamel [36-38]. These strategies typically include three components: (1) an etching agent (e.g., phosphoric acid) to prepare the enamel for proper anchorage of the artificial enamel, (2) proteins or protein analogs (e.g., amelogenin, peptides) to promote and control hydroxyapatite crystal formation and (3) a source of calcium and phosphorus usually delivered in the form of a hydrogel. Although these techniques have led to some degree of success in reproducing rod-like mineralized

structures, none of them reproduces all the physical and mechanical properties of mature enamel.

Due to the fact that mature enamel comprises ~96% of mineral, ~3% of water and less than 1% of proteins, no permanent scaffold (natural or synthetic) has been used in these biomimetic strategies of enamel repair, unlike strategies developed for the repair of collagenous mineralized tissues such as bone. And yet, the small portion of organic material in mature enamel plays a crucial role in the biomechanical properties of enamel [2, 3, 39]. Our recent findings further support this hypothesis and reveal that this organic material contains hair keratins which, through their unique biochemical properties, confer strength and flexibility to the enamel rod sheaths supporting enamel rods.

In recent years, the use of hair keratins isolated from human hair has shown promising results for the engineering of biomaterials for diverse biomedical applications [40, 41]. Keratins have the ability to spontaneously self-assemble and polymerize, which makes it possible to develop various types of biomaterials such as porous scaffold, films and hydrogels. Incorporating hair keratin hydrogels in the process of biomimetic mineralization of enamel may improve the biomechanical properties of the synthetic substance in a way that makes it more similar to natural enamel.

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Highlights

- The organic material in mature enamel matrix plays a crucial role in its biomechanical properties.
- A recent study shows that epithelial hair keratins are incorporated into mature enamel.
- Mutations in Keratin 75 are associated with increased susceptibility to dental caries.
- These findings have strong implications for the development of personalized oral care.
- Keratins may be used in novel enamel repair strategies.

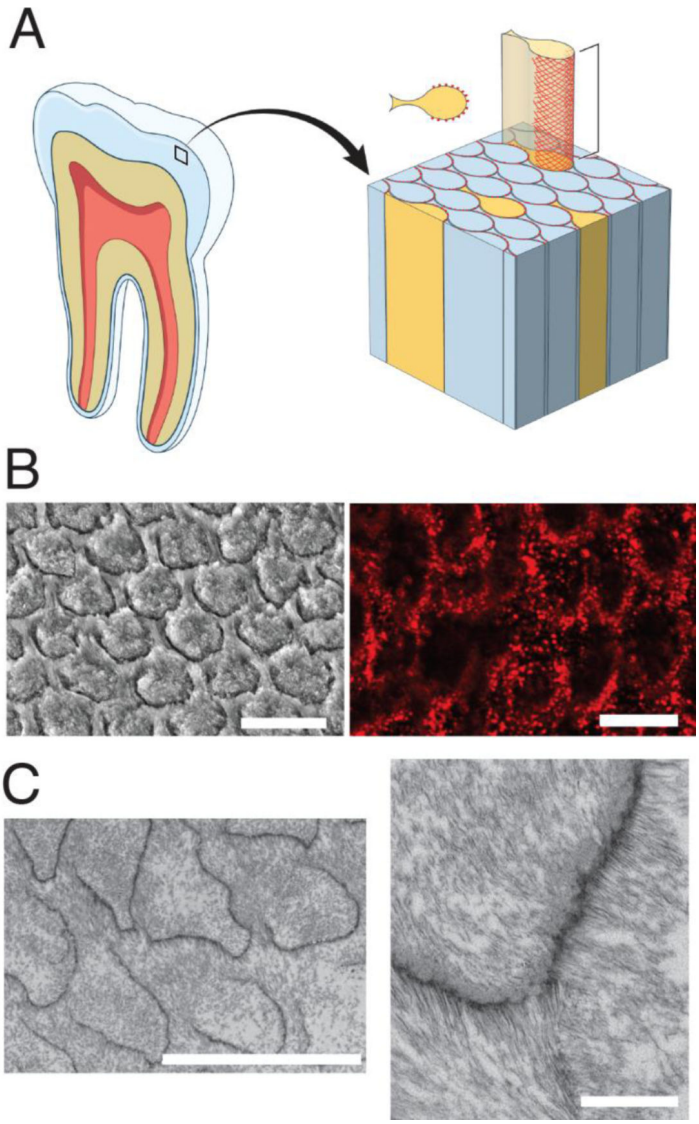

Figure 1. Enamel structure and the presence of hair keratins in enamel rod sheaths

A) Schematics showing the arrangement of enamel rods in mature enamel and their association with enamel rod sheaths made of organic material accumulated along a semicircle at the periphery of each rod. **B)** Scanning electron microscopy analysis of ground, polished and etched human molars showing the characteristic keyhole pattern of enamel rods (left panel; scale bar: 10 μm). Immunochemical detection of KRT75 performed on a similar surface showing staining primarily where enamel rod sheaths are located (right panel; scale bar: 10 μm). Primary antibody: anti-KRT75 (LifeSpan BioSciences Inc.). Secondary antibody: Alexa 555 conjugated goat anti-guinea-pig antibody (Life technologies). **C)** Transmission electron microscopy of enamel rod sheaths after demineralization of human enamel showing the semi-circular pattern of sheaths surrounding each individual rod. Scale bars: left panel 10 μm; right panel 1 μm.

Table 1

Amino acid composition of the enamel matrix at different stages of amelogenesis [16], rat amelogenin and keratin 75

		Me	Lme	Se	Amel	KRT75
Ala		8.7	7.8	5.5	1.5	6.5
Arg		5.7	3.4	2.3	1.0	7.2
Asx	Asn				0.6	4.2
	Asp	8.7	8.6	3.3	2.2	4.5
Glx	Gln				12.8	5.1
	Glu	12.3	12.7	15.3	3.7	7.2
Gly		21.5*	12.3	10.3	3.1	10.8
His		4	3.4	6.5	7.1	1.0
Ile		2.4	3.7	3.1	3.6	3.7
Leu		6	6.7	8.1	6.6	8.6
Lys		1.2	6	0	1.5	4.7
Met		2	1.5	0	4.6	1.7
Phe		2.3	2.2	2.9	1.0	3.5
Pro		7.3	6.7	20.4	25.0	2.8
Ser		9.2	11.9	10.5	3.1	11.7
Thr		3.6	5.6	3.3	3.6	5.8
Tyr		2.2	2.2	2.4	3.6	2.8
Val		4.4	5.2	4.7	4.6	6.8
Trp		0	0	0	1.5	0.5
Cys		0	0	0	0.0	1.0

Me- mature enamel, Lme- late maturation enamel, Se- secretory enamel, Amel- amelogenin, KRT75- Keratin 75

*The abnormally high Gly levels in mature enamel, are potentially due to contamination [4]

Table 2

Results of Pearson correlation analysis (r-values) of the amino acid compositions of enamel matrix from different stages of amelogenesis, amelogenin and keratin 75

	Lme	Se	Amel	KRT75
Me	**0.86**	**0.63**	0.24	**0.75**
Lme		**0.68**	0.31	**0.89**
Se			**0.85**	0.44
Amel				0.25

Me- mature enamel, Lme- late maturation enamel, Se- secretory enamel, Amel- amelogenin, KRT75- Keratin 75, statistically significant r-values are in bold.