THE ROLE OF ENAMEL MATRIX PROTEIN AMELOTIN ON BIOMINERALIZATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Faculty of Dentistry
University of Toronto

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Abstract

Dental caries is one of the most common chronic diseases of people worldwide. Despite recent advances in oral health care and restorative dentistry, the problem of dental caries and erosion remains unresolved due to the lack of knowledge of the natural biomineralization process of tooth tissues. Dental enamel plays a crucial role in preventing the tooth from destruction. However, if damaged or lost, it cannot be regenerated. Understanding the molecular mechanisms behind formation of enamel would not only make restoration of the tissue possible, but would also provide new insights for optimum design of calcium phosphate based biomaterials for dental and orthopedic applications. Amelotin (AMTN) is a recently discovered protein that is primarily expressed during the maturation stage of enamel formation and is localized at the cell-mineral interface on the surface enamel layer. *In vivo* studies using transgenic mice suggest a direct regulatory function for AMTN in enamel biomineralization. The aim of this PhD project was to test this hypothesis using different *in vitro* model systems of mineralization. I first showed that recombinant human (rh) AMTN accelerates hydroxyapatite mineralization in a dose-dependent manner when dissolved in the mineralization SBF buffer. Inactivation of a conserved SSEEL motif resulted in significant reduction in the mineralizing ability of the full-length molecule. I also evaluated the importance of phosphorylation in mineralization by testing a synthetic peptide
containing a short sequence of AMTN including phosphorylated SSEEL in the crystallization assay and showed that it promoted mineralization albeit to a lesser degree than rh-AMTN. Detailed characterization of secretory enamel matrix in overexpressor mice showed rapid and uncontrolled mineralization. I also tested the mineralizing ability of AMTN in an established osteoblast cell line and demonstrated that AMTN transfected or introduced in the culture media in the recombinant form both accelerate the formation of mineralized nodules. AMTN molecules embedded in collagen gel matrix were also able to mineralize the collagen material in the SBF buffer within a few hours. The findings of this PhD project provide solid evidence that AMTN is a promoter of hydroxyapatite mineralization and likely a key player in the establishment of surface enamel layer.
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# Table of Contents

Acknowledgments........................................................................................................ iv

Table of Contents........................................................................................................ vi

List of Tables .................................................................................................................... x

List of Figures ................................................................................................................... xi

Chapter 1 ......................................................................................................................... 1

1. Introduction ................................................................................................................ 1

1.1. Biomineralization: an overview .............................................................................. 2

1.2. Enamel: a unique model to study biomineralization .............................................. 5

1.3. Amelogenesis ........................................................................................................... 9

1.3.1. Pre-secretory stage ............................................................................................ 10

1.3.2. Secretory stage .................................................................................................. 11

1.3.3. Maturation stage ............................................................................................... 14

1.4. Enamel Matrix Proteins and Biomineralization .................................................... 20

1.4.1. Amelogenin (AMEL) ....................................................................................... 21

1.4.2. Ameloblastin (AMBN) ..................................................................................... 26

1.4.3. Enamelin (ENAM) ......................................................................................... 27

1.4.4. Matrix Metalloproteinase-20 (MMP-20) ......................................................... 28

1.4.5. Kallikrein-4 (KLK4) ....................................................................................... 29

1.4.6. Amelotin (AMTN) .......................................................................................... 31

1.4.7. Odontogenic Ameloblast-associated (ODAM) ................................................ 35

1.4.8. Other Enamel Proteins ..................................................................................... 38

1.5. Rationale, Hypothesis and Objectives .................................................................. 40

Chapter 2 ....................................................................................................................... 41

2. The Enamel Protein Amelotin is a Promoter of Hydroxyapatite Mineralization ........ 41
Abstract ........................................................................................................................................... 42

2.1. Introduction ................................................................................................................................. 43

2.2. Materials and Methods .............................................................................................................. 45
   2.2.1. Recombinant human protein production .......................................................................... 45
   2.2.2. AMTN short peptides ....................................................................................................... 46
   2.2.3. Protein-hydroxyapatite binding ....................................................................................... 47
   2.2.4. In vitro mineralization assay ............................................................................................. 48
   2.2.5. Characterization of the Calcium Phosphate Precipitates .................................................. 49
   2.2.6. Colloidal gold immunolabeling .......................................................................................... 50
   2.2.7. Cell culture assay ............................................................................................................. 51
   2.2.8. TEM analysis of transgenic mice ....................................................................................... 51

2.3. Results ......................................................................................................................................... 52
   2.3.1. Adsorption Isotherms ........................................................................................................ 52
   2.3.2. Rh-AMTN promotes HA mineralization in vitro ............................................................... 54
   2.3.3. AMTN overexpression in mouse calvaria cells promotes mineralization ....................... 57
   2.3.4. Rapid enamel mineralization in transgenic mice ............................................................. 58
   2.3.5. The conserved SSEEL motif regulates the HA mineralizing ability of rh-AMTN ............. 59
   2.3.6. The effect of phosphorylation ............................................................................................ 61

2.4. Discussion .................................................................................................................................... 61

2.5. Conclusions .................................................................................................................................. 66

2.6. Disclosures ................................................................................................................................... 66

2.7. Acknowledgments ....................................................................................................................... 66

Chapter 3 ........................................................................................................................................... 68

3. The Enamel Protein Amelotin Promotes Mineralization in Collagen-based Systems In vitro .......... 68
Abstract ................................................................................................................................. 69

3.1. Introduction ..................................................................................................................... 70

3.2. Materials and Methods .................................................................................................. 71
  3.2.1. Recombinant AMTN protein production ..................................................................... 71
  3.2.2. Cell culture experiments ............................................................................................ 71
  3.2.3. RNA isolation and real-time PCR analysis ................................................................. 72
  3.2.4. Preparation of collagen gels ....................................................................................... 73

3.3. Results .............................................................................................................................. 73
  3.3.1. Rh-AMTN promotes mineralization in osteoblast cultures ....................................... 73
  3.3.2. Mineralization of AMTN-embedded collagen gels .................................................... 74

3.4. Discussion ........................................................................................................................ 75

3.5. Acknowledgments .......................................................................................................... 79

Chapter 4 .............................................................................................................................. 80

4. Other Studies Related to the Mineralization Capabilities of AMTN ................................. 80
  4.1. HA-Binding Affinity and effect on In vitro Mineralization of Various Proteins .......... 81
    4.1.1. Introduction .............................................................................................................. 81
    4.1.2. Materials and Methods .......................................................................................... 82
    4.1.3. Results ................................................................................................................... 83
  4.2. Cell Culture Observations ............................................................................................. 89
    4.2.1. Introduction ........................................................................................................... 89
    4.2.2. Materials and Methods .......................................................................................... 90
    4.2.3. Results ................................................................................................................... 92
  4.3. Mineralization of AMTN-coated collagen membranes in vitro ..................................... 95
    4.3.1. Introduction ........................................................................................................... 95
    4.3.2. Materials and Methods .......................................................................................... 96
List of Tables

Table 1 Mouse gene-specific primers used for RT-PCR reactions......................................................... 73

Table 2 HA adsorption isotherm results of various proteins obtained compared to values reported in the literature. ........................................................................................................................................ 88
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Mineralization of extracellular matrix.</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Scanning electron micrograph of rods and interrods in enamel composed of arrays of HA crystallites.</td>
<td>7</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Scanning electron micrograph of enamel microstructure in a transverse section of the mouse mandibular incisor.</td>
<td>8</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Top panel: histology of developing porcine incisor and schematic of ameloblast morphology during different stages of amelogenesis.</td>
<td>10</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Formation and mineralization of rods and interrods.</td>
<td>13</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>A schematic of maturation stage smooth-ended and ruffle-ended ameloblast morphologies and their functional features.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>A schematic representation of the maturation stage of amelogenesis.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>The SCPP gene family.</td>
<td>21</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>The primary sequence of secreted human AMEL protein.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 1.10</td>
<td>The proposed mechanism for the formation of aligned bundles of HA crystals guided by AMEL nanospheres.</td>
<td>25</td>
</tr>
<tr>
<td>Figure 1.11</td>
<td>Human AMTN sequence including the signal peptide.</td>
<td>32</td>
</tr>
<tr>
<td>Figure 1.12</td>
<td>Transgenic mice overexpressing AMTN or expressing no AMTN.</td>
<td>35</td>
</tr>
</tbody>
</table>
Figure 2.1 (A) Coomassie blue-stained 15% SDS/polyacrylamide gel of rh-AMTN expressed in 
E.Coli bacteria and affinity purified. (B) The amino acid sequence of rh AMTN protein, AMTN 
mutants, and the synthesized peptides. ................................................................. 47

Figure 2.2 Linear adsorption isotherms of amelotin, osteopontin, and myoglobin. ............. 54

Figure 2.3 Mineral precipitates from the mineralization buffer containing 100 µg/ml rh-AMTN 
after 48 hours of incubation at 37°C........................................................................ 55

Figure 2.4 Light scattering plots of mineralization buffers containing (A) 0-40µg/ml AMTN and 
(B) AMTN, Myo, and OPN in equimolar concentrations, up to 7 days.............................. 56

Figure 2.5 TEM images of HA minerals precipitated from the mineralization buffer containing 
200µg/ml rh-AMTN immunolabeled with primary AMTN antibody and secondary protein A-
gold complex.................................................................................................................. 57

Figure 2.6 Transfection of mouse calvaria cell line MC3T3-E1 with murine AMTN.............. 58

Figure 2.7 TEM images of early secretory enamel matrix in WT and tg 57 mice overexpressing 
AMTN.......................................................................................................................... 59

Figure 2.8 Light scattering results of mineralization buffers containing 100µg/ml rh-AMTN or 
equimolar concentrations of AMTN mutants after 7 days of incubation at 37°C.............. 60

Figure 2.9 Light scattering results of mineralization buffers containing 100µg/ml rh-AMTN or 
equimolar concentrations of AMTN short peptides after 7 days of incubation at 37°C........ 62

Figure 2.10 Supplemental HPLC and mass spectrometry results of non-phosphorylated and 
phosphorylated AMTN peptides.................................................................................... 67
Figure 3.1 Alizarin red stained MC3T3 cells on day 10 supplemented with 100µg/ml rh-AMTN................................................................. 74

Figure 3.2 RT-PCR results of bone markers in MC3T3-E1 mineralizing cultures with or without 100µg/ml rh-AMTN in their media. ................................................................. 77

Figure 3.3 Incorporation of rh-AMTN with collagen................................................................. 77

Figure 4.1 Human ODAM sequence without the signal peptide. ........................................... 82

Figure 4.2 Coomassie-blue-stained 15% SDS/polyacrylamide gel of rh-AMTN, rh-ODAM peptide, rh-AMEL, and rh-SCPPP-Q1................................................................. 84

Figure 4.3 Light scattering plots of mineralization buffers containing 100µg/ml AMTN, 145 µg/ml ODAM, or 100µg/ml AMTN+145 µg/ml ODAM up to 10 days.. ......................... 85

Figure 4.4 Mineral precipitates from the mineralization buffers containing rh-AMTN, rh-ODAM peptide, or ODAM+AMTN after 14 days of incubation at 37°C.. ......................... 86

Figure 4.5 Light scattering plots of mineralization buffers containing AMTN, AMEL, or SCPPP-Q1 in equimolar concentrations, up to 15 days......................................................................................... 87

Figure 4.6 ALC and LS8 cell lines grown to about 70% confluence and imaged by phase contrast microscopy........................................................................................................................................... 91

Figure 4.7 ALC cultures in a 6-well plate up to 14 days and stained with Alizarin red........... 92

Figure 4.8 ALCs after 21 days of culture in DMEM, 10% FBS, and antibiotics.................... 93

Figure 4.9 2.5% agarose gel of the RT-PCR results for different RNA samples.. .............. 94
Figure 4.10 MC3T3-E1 subclones cultured in αMEM media supplemented with inorganic or organic phosphate source and stained with alizarin red after 2 weeks. ........................................... 95

Figure 4.11 Cytoplast membranes coated with rh-AMTN or without any protein coating and after incubation in the mineralization buffer for 6 days. .................................................................................................. 97

Figure 5.1 Silver-stained 15% SDS-PAGE showing FLAG-tagged mouse and human AMTN proteins expressed in ALC. ...................................................................................................................... 108
Chapter 1

1. Introduction
1.1. Biomineralization: an overview

Biomineralization is a controlled process by which live organisms produce minerals. The biomineralized tissues have unique composite structures designed to serve specific purposes such as mechanical support and protecting underlying soft tissues. The important feature of these biominerals is their complex morphologies and shapes in comparison with their synthetically produced counterparts (Weiner 2003). Synthetic minerals form from a supersaturated solution and further grow to a single crystal or a poly-crystalline material with a regular structure that is reflective of atomic organization. A biogenic mineral on the other hand, is not just formed by an inorganic phase but by a combined action of the inorganic and an organic phase. The latter consists of a variety of biological molecules that cooperatively regulate the formation of the mineralized structure and define its final morphology and shape. The flexible organic phase provides a framework for nucleation of early minerals and directs their growth and development to highly complex shapes with remarkable structural properties that are not producible by non-biogenic mechanisms. Toward the end of the mineralization process the organic phase may be removed to a great extent to result in a mostly inorganic mineralized structure or may partially remain to generate composite organic-inorganic mineralized tissues. More than 60 different mineral types have been identified to exist in 5 different living kingdoms of protists, monera (bacteria), fungi, plants, and animals (Nudelman and Sommerdijk 2012; Weiner 2003). These main minerals are silica, magnetite, calcium carbonates, and calcium phosphates. The mineral of bone and teeth in vertebrates is hydroxyapatite (HA), a crystalline form of calcium phosphate with a chemical formula of $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$. The biogenic apatite is non-stoichiometric and contains variable amounts of ionic impurities such as Na, Mg, or K substituting for Ca, F or Cl.
substituting for OH, and CO$_3$ substituting for PO$_4$ or OH (Buerlein et al., 2007). In human enamel for example (Aoba 2004), the putative apatite stoichiometry is 

$\text{(Ca)}_{4.56}(\text{Mg})_{0.03}(\text{Na})_{0.11}(\text{HPO}_4)_{0.10}(\text{CO}_3)_{0.23}(\text{PO}_4)_{2.66}(\text{OH,F})_{0.65}$.

Different organisms use a variety of crystallization mechanisms to produce mineralized tissues. A recent review (Weiner and Addadi 2011) has summarized these mechanisms into three main pathways: 1) mineralization of extracellular matrix, 2) mineralization within a large vesicle, and 3) mineralization within intracellular vesicles. The proposed mechanism for the formation of mineralized tissues in vertebrates (mainly collagen-mediated such as in bone, dentin, and calcified cartilage) is the mineralization of extracellular matrix. Figure 1.1 shows a schematic of this crystallization pathway in several stages. Stage 1 refers to the body fluid surrounding the cell that forms the mineralized tissue. The fluid is supersaturated with ions such as calcium and phosphate that are the constituents of the mineral to be formed. Next, ions are transported into the cell cytoplasm either through a passive concentration gradient mechanism or with the aid of ion exchangers and channels located on the cell membrane (stages 2 and 3). Stage 4 shows the specialized vesicles within which the first deposits of mineral maybe produced (Anderson 2003; Golub 2009). These highly disordered minerals may then be exocytosed to the mineralization front or may be re-dissolved and transported though the cell wall in ionic form. Step 6 depicts the extracellular organic matrix containing different mineralization regulatory proteins that have been processed within the cell and secreted outside. This matrix is the framework onto which the first mineral deposition occurs. Final stages of biomineralization involve further growth and maturation of minerals (stages 7 and 8). Increasing evidence in recent years indicate that the early forming mineral deposits in the extracellular space have a short-range order amorphous nature (Beniash et al., 2009; Mahamid et al., 2008). In later stages of mineralization, the
amorphous calcium phosphate phase transforms into more ordered phases and will eventually crystallize into apatite. This process has also been observed for other mineralized structures such as calcium carbonates in marine organisms (Politi et al., 2004).

![Figure 1.1](image)

Figure 1.1 Mineralization of extracellular matrix. Adapted from Weiner and Addadi, *Annu. Rev. Mater. Res.* 2011.

Regardless of the route that nature has used to produce the mineralized tissue, the final product has unique composition and organizational structure that is meticulously tuned to meet the functional purpose. One famous example is the intricate hierarchical organization of bone from the molecular to macroscopic levels (Weiner and Wagner 1998). Carbonated apatite as the mineral component, collagen type 1 as the organic framework for mineralization, and non-collagenous extracellular matrix proteins as mineralization regulatory agents constitute bone at the molecular level. Collagen fibers from bone of different parts of body however, are organized in different patterns and alignments which, strengthened by embedded HA crystals, generate different mechanical properties for bone designed for the vast range of stress that is experienced across the body. Mammalian teeth are another example of hierarchical complexity. Tooth is a composite material composed of layers of different mineral composition. The pulp in the center
of tooth is entirely made of soft connective tissue, whereas cementum covering the root has approximately 45% by weight mineral (Nanci and Ten Cate 2008). Dentin and enamel, the tissues surrounding the pulp, are respectively 70% and 95% by weight mineralized. The harder tissues closer to the surface of tooth provide mechanical resilience whereas the softer components in underneath layers provide flexibility and toughness. The multilayered tooth tissue as a whole resists extreme forces of mastication and constant changes in pH and temperature in the oral cavity for decades.

In recent years the field of biomineralization has attracted much interest for two main reasons: 1) to mimic nature’s models to design materials with superior structural and mechanical properties; and 2) to unravel pathways and find treatment for calcified tissue disorder and disease. None of these are possible without understanding the molecular mechanisms behind biomineralization.

### 1.2. Enamel: a unique model to study biomineralization

Dental enamel is the hardest tissue in mammals. It is indispensable for tooth function as it helps to deliver and withstand the enormous mechanical forces of mastication. A composition of over 95 wt. % carbonated hydroxyapatite (HA) mineral and small percentage of water and organic material gives mature enamel striking hardness as well as outstanding flexural strength at the same time. Three distinct features distinguish enamel from other mineralized tissues of body such as bone, dentin, and cartilage and make it a unique model system to study biomineralization: 1) The highly mineralized structure of enamel is produced from a non-collagenous organic matrix; 2) Unlike other mineralized tissues in body that are of mesenchymal origin, ameloblasts, the cells that form enamel, are epithelial derived; 3) If damaged or lost, the enamel tissue cannot be regenerated. Unravelling the molecular details of enamel formation would not only make it possible to devise strategies for tissue restoration, but would also provide
new insights for optimum design of calcium phosphate based biomaterials for dental and orthopedic applications.

Similar to many other biominerals, enamel possesses several hierarchical organizations from atomic to macroscopic levels. At the nanoscale, needle-like crystallites of non-stoichiometric carbonated apatite constitute the inorganic phase of enamel. Bundles of HA crystallites together comprise the main structural units of enamel called rods (or prisms, used interchangeably in the literature) and interrods with a cross-section of 3-6 µm in diameter (Figure 1.2). Interrods surround the rods and contain HA crystallites that have a different orientation from those of rods (red arrows in Figure 1.2).

At the microscopic scale, enamel consists of four different morphological patterns (Lyngstadaas, Møinichen, and Risnes 1998; Møinichen, Petter Lyngstadaas, and Risnes 1996; Warshawsky et al., 1981). While the closest enamel layer to the dentin, the dentino-enamel junction (DEJ), contains no rods, the bulk of enamel consists of highly organized arrays of intertwoven rods with alternating directions. Enamel bulk is followed by the outer layer of enamel where HA crystals have a parallel arrangement with each other and are perpendicular to the surface. The final enamel layer at the surface also lacks the rod micromorphology and is highly compact (Figure 1.3).
The different microstructures as well as slight changes in chemical composition give the enamel sub-layers a range of mechanical properties. For example (Cuy et al., 2002), the DEJ of human molar enamel has been reported to have hardness and elastic modulus values of greater than 3 GPa and 70 GPa, respectively. These values for surface layer exceed 6 GPa and 115 GPa making it the hardest layer of enamel. The water and proteins residing around the mineral rods and known as the “rod sheath” add an additional level of toughness to the tissue and help it dissipate the mastication forces and prevent it from fracture.
Figure 1.3 Scanning electron micrograph of enamel microstructure in a transverse section of the mouse mandibular incisor.
1.3. Amelogenesis

The complex enamel structure is the result of an orchestrated mineralization process guided by a single layer of highly specialized cells known as ameloblasts. The enamel biomineralization is therefore also referred to as “amelogenesis”. Amelogenesis can be divided into three main stages: presecretory, secretory, and maturation. During these steps, ameloblasts undergo several phenotypic changes and secrete different matrix proteins onto the mineralization front (Figure 1.4). Following the biomineralization model in Figure 1.1, ameloblasts deposit an organic matrix containing various proteins that provide a template for formation of early mineral ribbons and development of enamel rods. Under the cooperative regulation of matrix proteins the four layers of enamel are formed during the secretory stage. The composition of enamel at this stage is mainly organic (66 wt. %) and is partially mineralized (~30 wt. %). Once the enamel thickness is established, in the final stage the tissue matures into a 95% mineralized structure facilitated by the removal of secretory-stage matrix proteins through specific proteases and further growth of the enamel rods in thickness.
1.3.1. Pre-secretory stage

A series of interactions between oral epithelium and the mesenchyme lead to tooth development in four main successive stages of lamina, bud, cap, and bell (reviewed by Lesot and Brook 2009)). During the early bell stage, the epithelial histogenesis results in the formation of an outer oral epithelia, two intermediate cell layers known as stellate reticulum (SR) and stratum intermedium (SI), and finally an inner oral epithelial layer that will later differentiate into ameloblasts. The inner epithelium is separated from the mesenchyme-derived dental papilla by a
basement membrane that is rich in type IV collagen and laminin. The dental papilla will eventually form the dentin and pulp tissues. The inner epithelial cells at this stage are cuboidal shaped with their nucleus in the center (Figure 1.4 top panel #1). The process of ameloblast differentiation starts with the formation of an unmineralized predentin layer beneath the basement membrane. As the differentiation continues, the oral epithelial cells elongate and become polarized (Figure 1.4 #2). The developing ameloblasts are tightly aligned and held together by structures known as “junctional complexes” which regulate the passage of molecules and ions from and to the cells. The basement membrane begins to degrade while differentiating ameloblasts extend cytoplasmic projections through the gap regions within degrading basement membrane (Figure 1.4 #3). The cells secrete patches of the early enamel matrix proteins through their developing cytoplasmic extensions onto the mantle predentin layer. The main enamel matrix protein, amelogenin (AMEL) has been detected by immunogold labelling in the early matrix secreted by the differentiating epithelial cells even before the basement membrane begins to fragment (Nanci and Ten Cate 2008). The matrix proteins develop the first layer of enamel on the predentin matrix establishing the dentino-enamel junction (DEJ).

1.3.2. Secretory stage

The DEJ minerals intertwine with the collagen fibers and crystallites of mantle dentin, the first layer of dentin formed near enamel, and form an irregular mineralized structure with no identifiable rod-like morphology. As the basement membrane is removed and matrix proteins are released onto the mineralizing DEJ, the ameloblasts continue to differentiate and expand their cytoplasmic extensions at their distal extremity to more complex secretory structures with a picket-fence patterning known as the “Tomes’ process” (Figure 1.4 #4 and Figure 1.5). Matrix proteins, mainly amelogenin, ameloblastin, and enamelin as well as the protease matrix
Metalloproteinase-20 (MMP-20), are constantly released against the Tomes’ process onto the mineralization front throughout the secretory stage. The respective messenger RNAs are first translated into the proteins by ribosomes in the extensive rough endoplasmic reticulum of secretory ameloblasts and then post-translationally modified in the Golgi complex inside the cell. The proteins are then packaged in vesicles known as the “secretory granules” and translocated to the Tomes’ process at the secretory face of the cell. The secretory granules release their content at the mineral growth sites associated with cellular membrane infoldings on the Tomes’ process (Figure 1.5 A). Matrix proteins and their fragments produced by the proteolytic cleavage of full-length molecules by MMP-20, cooperatively control and regulate the formation and development of enamel rods. As the proteins are discharged against the matrix and mineral formation progresses, ameloblasts move away from the DEJ extending their Tomes’ process from the DEJ to their distal surface. The continuous motion of ameloblasts occurs at an angle almost perpendicular to the DEJ while enamel rods develop along the sides of the Tomes’ process picket-fence morphology. Every rod forms and elongates following a single ameloblast and eventually extends across the entire enamel bulk. It is believed that the development of rods at the proximal and distal sides of the Tomes’ process in conjunction with the non-linear and winding movement of the cells creates the decussating pattern of enamel bulk (Hu et al., 2007; Nanci and Ten Cate 2008; Simmer et al., 2012) (Figure 1.3). The area surrounding the rods is filled by developing interrods (Figure 1.5). Once the final enamel layer is formed, ameloblasts retract their Tomes’ process and form the compact enamel surface layer to complete the secretion of the entire enamel thickness (Figure 1.4 #5). As the Tomes’ process does not exist during the formation of the surface enamel layer, similar to the DEJ, this layer is also aprismatic (i.e. contains no rods).
Figure 1.5 A) Secretion of matrix proteins in secretory granules (sg) against the distal portion and proximal portion of Tomes’ process (dpTP and ppTP) leads to the formation and mineralization of rods (R) and interrods (IR). Membrane infoldings (im) are associated with rod (RGS) and interrod (IGS) growth sites. B) A schematic of a longitudinal section of ameloblast cells. Ameloblasts are elongated and polarized with their nuclei directed towards the Stratum Intermedium. The Tomes’ process resembling picket-fence structures are represented at the distal side of the cells. Pictures are adapted from Nanci and Ten Cate, Ten Cate's oral histology: development, structure, and function. 2008.
1.3.3. Maturation stage

After losing the Tomes’ process as the main secretory apparatus ameloblasts experience a short transition phase by restructuring and changes in morphology and gene expression pattern while about 25% of the cells undergo apoptosis (programmed cell death). Ameloblasts convert from essentially secretory cells (evidenced by the presence of numerous secretory vesicles) to transport cells with shortened cell bodies and loss of Tomes’ process. The cells continue to secrete organic matrix molecules but to a much lesser degree and with a different composition than the secretory stage (Figure 1.4). At the basal end of the cells, the closest cell layer to the ameloblasts, the stratum intermedium, integrates with the adjacent cell layers, the stellate reticulum and outer oral epithelia, to form a new layer called the papillary layer (PL). The PL contains extensive mitochondria organelles for energy production and blood vessels at the proximity of the bases of the ameloblasts which are believed to participate in ion transport to and from the cells during the maturation phase (Josephsen et al., 2010).

The final stage of enamel formation begins with cells entering a modulation cycle between two different ruffle-ended (RA) and smooth-ended (SA) morphologies of the apical membrane. The total number of ameloblasts reduces to 50% as the cell apoptosis continues during the maturation stage. A new basal lamina-like structure forms at the interface of the apical side of the cells and the enamel surface and is attached to the cells via special cell adhesion structures called the hemidesmosomes (Nanci and Ten Cate 2008). The interface between cells and mineral is rich in glycoproteins such as laminin-5 but does not contain collagen type IV and is therefore considered as a basal lamina-like layer. It also contains matrix proteins AMTN and ODAM (Figure 1.7) (Dos Santos Neves et al., 2012). The role of the newly-structured ameloblasts is to mineralize and harden the immature enamel by removing the residual organic matrix and filling
the space with new crystals by adding mineral ions to the sides of already-formed enamel rods. Ion transport to and from the enamel as well as organic matrix removal from the enamel space is regulated by the two cyclic RA and SA phases of the ameloblasts in the maturation stage. The cells spend 80% of the cycle in the RA state and 20% in the SA state. The RAs are known to have tight junctions close to their distal extremity (i.e. close to their ruffled border at the enamel side) and therefore, ions and other molecules actively pass through their proximal side (close to the PL layer). On the contrary, the tight junctions of the SAs are situated close to the proximal side and free passage of molecules occurs through their distal side (Figure 1.4 and Figure 1.6).
Another important difference between the two phases is the pH of enamel space. During the RA phase the enamel pH is slightly acidic whereas it is close to neutral during the SA phase. The maturation stage events have been summarized in Figure 1.7 (Simmer et al., 2010). Numerous ion exchangers as well as membrane associated and cytosolic proteins involved in transport of molecules and endocytosis during enamel maturation have been identified in recent years (Lacruz et al., 2010; Lacruz et al., 2013a). In order for new crystals of HA to form Ca\(^{2+}\), PO\(_4^{3-}\), and hydroxyl ions need to be transported to the enamel space and deposited to the sides of existing crystals (Figure 1.7 A). One unit cell of HA has a chemical formula of (Ca\(_{10}\)(PO\(_4^{3-}\))\(_6\)(OH\(^-\))\(_2\). Assuming a pH of around 7.2 in the beginning of RA phase, the phosphate ions will be roughly 50% in H\(_2\)PO\(_4^-\) and 50% in HPO\(_4^{2-}\) forms (The second ionization product of phosphoric acid at 25°C, pK\(_{a2}\) = 6.31×10\(^{-8}\)). Three H\(_2\)PO\(_4^-\) and three HPO\(_4^{2-}\) are therefore consumed to generate six PO\(_4^{3-}\) ions which will in turn, result in the release of nine protons. This number will rise to eleven from the decomposition of two molecules of water to generate two hydroxyl ions and two protons (Simmer et al., 2010). The acid production in high levels lowers the pH of the enamel in the RA phase and is neutralized by bicarbonate ions to raise the pH of enamel to close to neutral to prevent the newly deposited minerals from dissolution back to the ionic form. A recent genome-wide transcript profiling of developing rat incisor enamel revealed the up-regulation of several calcium transport genes during the maturation stage (Lacruz et al., 2012b). These include a transmembrane protein called Stim1 that is associated with endoplasmic reticulum-related calcium stores as well as a sodium-calcium-potassium exchanger named NCKX4 (coded by gene Slc24a4) that is expressed at the distal side of the mid-late maturation stage ameloblasts in mice (Hu et al., 2012). A sodium/phosphate co-transporter (solute carrier 34, SLC34) has also been proposed as one of the possible molecules involved in phosphate transport (Lacruz et al., 2012b). The secretory matrix proteins are simultaneously degraded by
proteases such as kallikrein (KLK4) (Smith et al., 2011) and absorbed and transported out of the cells to provide space for the growing crystals (Figure 1.7 B). Several endocytosis related genes including plasma membrane adaptor protein complex 2 (AP2) have been newly reported and confirmed by immunolocalization to be associated with the apical poles of the ameloblasts (Lacruz et al., 2013b). WDR72 is another protein involved in endocytosis vesicle trafficking. Mutations in WDR72 gene cause a hypomaturation phenotype of Amelogenesis imperfecta (AI) in humans (Katsura et al., 2014). Figure 1.7 C shows the removal of magnesium ions from the enamel matrix possibly by the aid of a putative metal transporter CNNM4 that is present in transition and maturation stage ameloblasts (Parry et al., 2009). Mutations in this gene result in amelogenesis imperfecta in humans characterized by low mineral density, retention of the organic matrix, and possibly stabilized amorphous phase caused by the presence of magnesium ions in the enamel structure (Parry et al., 2009). Carbonic anhydrases, mainly CA2 and CA6, play a major role in transport of bicarbonate ions through the cells and onto the enamel matrix (Figure 1.7 D). HCO$_3^-$ ions are produced by the reaction of a carbon dioxide molecule and water in the cell cytoplasm catalyzed by the enzyme carbonic anhydrase 2 and zinc ion as the reaction cofactor (Toyosawa et al., 1996). The generated proton from the reaction leaves the cell at the basal side facilitated by Sodium/hydrogen exchanger (NHE). HCO$_3^-$ ions may also enter the cell together with Na$^+$ from the basal side of the cell and with the aid of sodium/bicarbonate co-transporter (NBCe1) (Lacruz et al., 2012c). The ion exchanger 2 (AE2) will then transport the bicarbonate ions to the enamel by exchanging them with chloride ions at the proximity of enamel (Lacruz et al., 2012c; Lyaruu et al., 2008). The chloride ions have been carried to the enamel from the cell cytoplasm by cystic fibrosis transmembrane conductance regulator (CFTR) to be later exchanged with bicarbonate ions (Figure 1.7 D). Mutations in this gene in pigs result in hypomineralized enamel with abnormal crystal growth (Lacruz et al., 2012c). Once the HCO$_3^-$
ions reach the enamel space, they combine with the hydrogen ions produced by the formation of new minerals to yield water and carbon dioxide (Figure 1.7 E). This reaction is catalyzed by CA6, a secretory form of carbonic anhydrase family (Lacruz et al., 2012c; Smith, Nanci, and Moffatt 2006). By the end of the maturation stage, the enamel rods have reached a fully crystalline HA structure, are significantly thicker, and are organized in a packed and interwoven alignment to yield a highly mineralized and compact enamel material.
Figure 1.7 A schematic representation of the maturation stage of amelogenesis. A) Transport of calcium and phosphate ions to the enamel matrix; B) degradation of the organic matrix by KLK4 and endocytosis by WDR72; C) removal of Mg$^{2+}$ ions from the enamel space by CNNM4; D) generation and transport of HCO$_3^-$ to the enamel; E) conversion of HCO$_3^-$ to CO$_2$ and water catalyzed by CA6. The maturation-stage matrix proteins AMTN and ODAM are present in the basement membrane at the interface of the enamel surface and apical pole of ameloblasts. Adapted from Simmer, et al. J. Dent. Res. 2010.
1.4. Enamel Matrix Proteins and Biomineralization

Matrix proteins shape the enamel rods and guide their development at every step of the amelogenesis process. The exact temporo-spatial expression profiling of these proteins is tightly controlled by the ameloblast cells. *In vitro* mineralization studies as well as transgenic animal models focusing on enamel matrix proteins have shed light on the importance of these molecules in the formation of fully functional enamel tissue (Moradian-Oldak 2012). The major enamel matrix protein is amelogenin (AMEL). Others include ameloblastin (AMBN), enamelin (ENAM), amelotin (AMTN), odontogenic ameloblast associated (ODAM/APIN), as well as two major proteases matrix metalloproteinase-20 (MMP-20) and kallikrein-4 (KLK4). Evolutionary analysis investigations have shown that genes encoding these proteins are part of the large secretory calcium-binding phosphoprotein (SCPP) family. Except for AMEL which is located on sex chromosomes, other enamel matrix proteins are all clustered on human chromosome 4 (4q13) together with some salivary proteins and milk caseins (Figure 1.8). At approximately 15Mb from the enamel genes, the small integrin-binding ligand N-linked glycoprotein (SIBLING) gene family encoding several bone and dentin matrix proteins is also clustered on the same chromosome. This gene family together with milk caseins, saliva proteins as well as ameloblast genes all share a common SCPP ancestral gene and are mostly involved in calcium phosphate regulation activities in the extracellular environment (Kawasaki and Weiss 2003). They all have similar structural features and most contain a conserved phosphorylation SXE motif suggesting calcium binding properties (Sire *et al.*, 2007). Throughout the course of evolution, enamel genes have evolved to serve the specific function of developing a tissue with the highest level of mineralization in the vertebrate body.
Figure 1.8 The SCPP gene family. The SCPP ancestor gene was duplicated to SIBLING ancestor and ENAM ancestor. These genes in turn evolved to form the dentin and bone SIBLING family as well as enamel proteins, milk caseins and some salivary proteins, all located on chromosome 4. AMEL was translocated to chromosomes X/Y. Adapted from Sire, et al. Cells Tissues Organs. 2007.

1.4.1. Amelogenin (AMEL)

The predominant protein of enamel matrix is AMEL which together with its cleavage products comprise over 90% of the organic matrix. The expression of AMEL starts in the pre-secretory stage of amelogenesis when the DEJ layer is established (Nanci and Ten Cate 2008). Shorter forms of AMEL are also expressed by preodontoblasts in low levels before the initiation of amelogenesis during the signaling events between the mesenchymal cells and inner epithelial cells and are proposed to have roles in determining the fate of the cells and their differentiation to enamel and dentin forming cells (Veis 2003). AMEL expression continues throughout the pre-secretory and secretory stages of amelogenesis (Figure 1.4). The full-length protein contains highly conserved N-terminal and C-terminal domains that have remained unchanged for 250 million years (Sire et al., 2005). Evolutionary analysis reports suggest that AMEL is a
descendant of an AMBN ancestor which itself arose from the ENAM ancestor gene (Figure 1.8) (Sire et al., 2005; Sire et al., 2007). AMEL has several splice variants and is also cleaved to shorter forms during the secretory stage mainly by the protease MMP-20 (Yang et al., 2011a). AMEL and its cleavage products have an important role in the development of enamel crystals during amelogenesis. Mutations in X-chromosomal AMEL result in X-linked AI in humans (Wright et al., 2008) and deletion of the conserved N-terminal or C-terminal domains lead to defects in structural organization of enamel in mice (Paine et al., 2000; Paine et al., 2003a). AMEL<sup>−/−</sup> mice have hypoplastic enamel with chalky white appearance and disorganized structure that lacks the aligned rod micromorphology (Gibson et al., 2001; Wright et al., 2008). It is therefore believed that AMEL molecules serve as a template for developing enamel rods and guide their shape and structural organization. AMEL is a bipolar molecule with most of its charged residues concentrating on the conserved hydrophilic C tail (Figure 1.9). The rest of the sequence is non-polar with several prolines (Pro), glutamines (Glu), and histidines (His) in the middle region of the molecule. The N terminus is tyrosine-rich and contains a conserved serine16 amino acid that is predicted to be phosphorylated (Figure 1.9). This domain is therefore also called TRAP (tyrosine-rich amelogenin peptide). An important splice variant of AMEL existing in the enamel matrix contains only the N- and C- termini and is called LRAP (Leucine-rich amelogenin peptide). AMEL is an intrinsically disordered protein, but possesses some local structured motifs such as β-sheets and β-strands mostly in its N-terminal TRAP region and random coils, polyproline type II (PPII) helix, and β-turns in its Pro-rich middle region (Delak et al., 2009; Margolis, Beniash, and Fowler 2006). The fascinating structural feature of AMEL is its ability to self-assemble to multimeric forms as a function of pH (Margolis, Beniash, and Fowler 2006). At pH 3.0 AMEL exists as a monomer (Beniash, Simmer, and Margolis 2012). As the pH rises the monomers join to form oligomers of several monomers such as dimers, trimers,
tetramers, and dodecamers. Several oligomers join to form “nanospheres” 15-20 nm in diameter in pH values close to neutral and larger sizes at pH8.0 (Du et al., 2005; Fang et al., 2011; Margolis, Beniash, and Fowler 2006; Wiedemann-Bidlack et al., 2007).

**Figure 1.9** The primary sequence of secreted human AMEL protein (without the signal sequence) taken from Ensembl Genome Browser ([http://www.ensembl.org/](http://www.ensembl.org/)). The conserved residues are in bold letters. The colored bars indicate the N-terminus, midsection, and C-terminus of the molecule.

This phenomenon has been observed for both *in vitro* produced recombinant and *in vivo* native forms of AMEL (Fincham *et al.*, 1995; Wiedemann-Bidlack *et al.*, 2007). The flexibility of AMEL is the key to the formation of higher molecular weight assemblies facilitated by dynamic conformational changes in the secondary structure of the molecule (Beniash, Simmer, and Margolis 2012; Zhang *et al.*, 2011). Recent data also suggest changes in local structural motifs on the AMEL backbone when the molecule is in contact with the calcium phosphate minerals to adapt protein-mineral molecular interactions (Beniash, Simmer, and Margolis 2012; Lu *et al.*, 2013). The AMEL nanosphere assembly has a crucial role in the development of enamel crystals. At physiologic pH AMEL nanospheres form aligned bead-like chains detected among the forming enamel crystal bundles and in parallel with their crystallographic C-axis (Fincham *et al.*, 1995). Numerous *in vitro* mineralization studies in supersaturated calcium phosphate solutions and characterized by transmission electron microscopy (TEM) also demonstrated that AMEL
nanosphere chains guide the formation of oriented bundles of amorphous calcium phosphate (ACP) minerals that will later transform to aligned HA crystallites (Du et al., 2005; Fang et al., 2011; Kwak et al., 2011; Wang et al., 2008; Yang et al., 2010b). The proposed mechanism is shown in Figure 1.10. AMEL nanospheres are suggested to stabilize pre-nucleation clusters with calcium and phosphate ions. These nanocluster composites are further transformed into parallel arrays of ACP directed by AMEL nanosphere chains. These nanocomposite assemblies further grow and crystallize to elongated bundles of HA rods. In the absence of AMEL, the random formation of pre-nucleation clusters leads to bulk ACP minerals that will later crystallize to HA crystals with no preferred orientation (Figure 1.10 top panel). An alternative proposal emphasizes the simultaneous self-assembly of AMEL molecules and the onset of spontaneous mineralization (Beniash, Simmer, and Margolis 2005). Based on this mechanism, aligned mineral crystals are developed by assembling AMEL molecules, not by the pre-assembled template of AMEL nanospheres. It has been shown that the hydrophilic C-terminal telopeptide on the AMEL sequence is critical in aligning the CaP minerals. A truncated version of recombinant AMEL lacking the C-terminus fails to orient the minerals into parallel ribbons. In the presence of recombinant AMEL minus the C-terminus the amorphous CaP precipitates eventually convert to needle-like HA crystals of random orientation whereas recombinant full-length AMEL guides the CaP minerals to aligned crystals (Beniash, Simmer, and Margolis 2005; Fang et al., 2011; Kwak et al., 2009; Kwak et al., 2011). The native protein however, phosphorylated on the serine16 residue, has a different effect on mineralization. Revealed by in vitro TEM studies, both native full-length AMEL and the most abundant cleavage product of AMEL lacking the C-terminus, delay the conversion of ACP to HA crystals. In the presence of native AMEL products, ACP minerals are stable even after a day (Kwak et al., 2009; Kwak et al., 2011; Wiedemann-Bidlack et al., 2011). Stable ACP material for longer periods allows for
enamel minerals to align and shape into final hierarchical intertwined rod/interrod organization in an organic-amorphous CaP matrix before conversion to HA crystals in the later stages of amelogenesis in vivo (Beniash et al., 2009).

![Diagram of aggregation and post-nucleation growth](image)

**Figure 1.10** The proposed mechanism for the formation of aligned bundles of HA crystals guided by AMEL nanospheres; Adapted from Yang, et al. *J. Phys. Chem.* 2010.

AMEL has been used in *ex vivo* studies on etched enamel surfaces in biomimetic attempts to restore the lost enamel caused by dental caries. A combination of 30-40 µg/ml AMEL and 1mg/L fluoride dissolved in supersaturated remineralization buffers result in the formation of packed aligned fluoridated apatite crystals similar to bulk enamel microstructure (Fan, Sun, and Moradian-Oldak 2009; Fan *et al.*, 2011). Control buffer or containing AMEL resulted in plate-
shaped octacalcium phosphate (OCP) deposits (Fan, Sun, and Moradian-Oldak 2009). In a recent study, AMEL containing chitosan hydrogels were employed for enamel regeneration leading to the formation of a second enamel-like layer with aligned HA crystals, firm attachment to the natural enamel layer underneath, and improved anti-microbial properties (Ruan et al., 2013). The hardness and elastic modulus of the new material had improved compared to the etched enamel, but was much lower than the healthy enamel (Ruan et al., 2013).

1.4.2. Ameloblastin (AMBN)

AMBN is the second most abundant enamel matrix protein comprising approximately 5% of the total secreted matrix (Moradian-Oldak 2012). The expression of AMBN starts from the differentiating inner oral epithelial cells during the pre-secretory stage and continues throughout the secretory stage and into the maturation stage of amelogenesis (Figure 1.4) (Krebsbach et al., 1996). The full-length protein contains several prolines (Pro), leucines (Leu), and glycines (Gly) and has an O-linked glycosylation, a proline hydroxylation, and phosphorylation sites. Immunogold labelling analysis has detected AMBN molecules co-localized with AMEL molecules in the Golgi complexes within the cells and in the secretory granules in the enamel matrix (Nanci and Ten Cate 2008). Upon release into the matrix during the secretory stage, AMBN undergoes proteolytic cleavage at multiple sites by the secretory protease MMP-20 (Chun et al., 2010; Iwata et al., 2007). The C-terminal fragments are degraded while the N-terminal fragments are detected across the enamel matrix (Uchida et al., 1997). AMBN interacts with AMEL (Holcroft and Ganss 2011; Ravindranath et al., 2004) and has high affinity for calcium at the C-terminus (Yamakoshi et al., 2001) and therefore, has been suggested to cooperate with AMEL in developing and maintaining rod/interrod integrity (Vymetal et al., 2008). Transgenic mice overexpressing AMBN exhibited defects in their enamel crystallite
morphology and habit emphasizing a critical role for AMBN in the proper formation of enamel rod structure (Paine et al., 2003b). Expression of a truncated version of AMBN lacking exons 5 and 6 resulted in thin dysplastic enamel with abnormal calcified regions that detached from the dentin (Wazen et al., 2009). The ameloblasts in these animals lost their polarity, detached from the matrix and proliferated into multi cell layers (Fukumoto et al., 2004). In some mutant animals odontogenic tumors developed, which showed positive immunohistochemical staining for enamel matrix proteins, suggesting an epithelial origin of these tumors (Fukumoto et al., 2004). These results together with cell-binding assays showing strong AMBN attachment to differentiated ameloblasts demonstrated that AMBN is a cell adhesion molecule promoting differentiation by binding to the cells and preventing them from proliferation (Fukumoto et al., 2004; Wazen et al., 2009).

1.4.3. Enamelin (ENAM)

Native ENAM is a large glycoprotein of 186kDa in size which is expressed and secreted exclusively by ameloblasts almost simultaneously with AMEL and AMBN during the establishment of DEJ and throughout the secretory stage and into the early maturation stage (Hu et al., 1997; Hu et al., 2001). The secreted protein is cleaved to multiple peptide products including an N-terminal 89kDa fragment that is later cleaved to generate a conserved acidic 32kDa peptide (Uchida et al., 1991). In vitro investigations on the 32kDa ENAM fragment have shown that it interacts with rP148 (C-terminally truncated recombinant porcine AMEL) and alters the self-assembly of AMEL molecules from nanospheres to oligomers and nanospheres of smaller hydrodynamic radii (Yang et al., 2011b). In a gelatin gel diffusion system mixed with native porcine amelogenins the induction time for HA formation is reduced with the addition of 32kDa ENAM peptide (Bouropoulos and Moradian-Oldak 2004). The same ENAM fragment
added to rP148 in a dual membrane system altered the crystallinity of growing OCP crystals and increased their aspect ratio in a dose dependent manner (Iijima et al., 2010). These observations indicate that ENAM and AMEL interactions are also important for proper enamel formation.

Similar to other matrix proteins mutations in the ENAM gene result in AI (Rajpar et al., 2001; Wright et al., 2008). In the absence of ENAM true enamel does not form. Mice lacking ENAM develop enamel composed of organic matrix but almost devoid of any mineral except for some mineralization foci close to the DEJ (Hu 2008). The erupted teeth in these animals showed a highly irregular thin enamel layer on top of a normal dentin layer. The in vitro and in vivo data so far suggest that ENAM is crucial for the formation of enamel and it possibly cooperates with AMEL to regulate enamel crystal elongated growth and development (Moradian-Oldak 2012).

1.4.4. Matrix Metalloproteinase-20 (MMP-20)

MMP-20 or “enamelysin” is a member of the MMP family that is expressed in a tooth-specific manner (Bartlett et al., 1996). It is the main protease of secretory stage amelogenesis and cleaves the main enamel matrix proteins during this stage to shorter peptides which together with full-length proteins guide the development of enamel rods (Lu et al., 2008). In vitro, the proteolytic cleavage of recombinant AMEL by MMP-20 and co-assembly of full-length AMEL and its cleavage products in different ratios resulted in the hierarchical assembly of AMEL microstructures ranging from nanorods to aggregates (Yang et al., 2011a). Pigmented hypomature AI has been reported in humans caused by mutations in MMP-20 gene (Kim et al., 2005). Due to the retention of proteins in the matrix and deficient proteolytic cleavage the MMP-20 null mice develop a thin enamel layer that is structurally abnormal and hypomineralized (Smith et al., 2011). The mineral content and hardness of enamel in these animals are reduced by 50% and 37%, respectively (Bartlett et al., 2004). The pH regulation in the maturation stage of
amelogenesis in MMP-20<sup>−/−</sup> mice is affected and less acidity is detected in the enamel matrix (Tye <i>et al.</i>, 2010). Several ion transporters including CA6 and CFTR are also down-regulated (Tye <i>et al.</i>, 2010). The mineralization of mantle dentin in MMP-20<sup>−/−</sup> mice is delayed and the DEJ is hypomineralized explaining the weak mechanical properties of enamel in these animals denoting the importance of MMP-20 from the early stages of enamel mineralization (Beniash, Skobe, and Bartlett 2006; Smith <i>et al.</i>, 2011).

1.4.5. <b>Kallikrein-4 (KLK4)</b>

KLK4 is the second important enamel protease that is specifically expressed during the maturation stage and is essential for degrading the secretory proteins in the matrix and proper enamel maturation (Lu <i>et al.</i>, 2008). The expression of KLK4 has also been reported in prostate, breast, ovaries, and associated tumors (Dong <i>et al.</i>, 2001). Certain proteolytic cleavage products of parent proteins are stable in the enamel matrix during the secretory stage and are only degraded by KLK4 in the maturation stage once their purpose in developing enamel rods has been fulfilled. It has been demonstrated <i>in vitro</i> that while native AMEL is cleaved by both MMP-20 and KLK4, albeit at different cleavage sites, the 32kDa fragment of native enamelin is not processed by MMP-20 but is cleaved readily by KLK4 giving multiple cleavage products (Yamakoshi <i>et al.</i>, 2006). Unlike MMP-20 deficient mice, the enamel in KLK4<sup>−/−</sup> animals has a normal thickness. However, due to the incomplete processing of matrix proteins and their retention in the enamel space during the maturation stage, the HA rods are not able to grow to tightly-packed arrays and do not interlock (Simmer <i>et al.</i>, 2009). Although well-organized, individual crystallites within enamel rods are discernible from one another and the mineral density in mature enamel is lowered to 85% by weight (Smith <i>et al.</i>, 2011). Similar to mice lacking MMP-20, structural defects in the DEJ are also evident in KLK4 knock-out mice (Smith...
et al., 2011). Mutations in the KLK4 gene cause autosomal recessive hypomaturation AI in humans with enamel rods that have normal length but have not reached full thickness (Hart et al., 2004).
The following subsections on the maturation-stage enamel proteins contain parts of the review article (Ganss and Abbarin 2014):


1.4.6. Amelotin (AMTN)

AMTN was originally discovered as an enamel-specific gene by differential display analysis of mRNA expression from dental tissues in mice (Iwasaki *et al.*, 2005). An expressed fragment of AMTN was also found as EO-063 in a signal trap screening approach in rat enamel (Moffatt *et al.*, 2006a). The AMTN protein contains an N-terminal signal sequence and is secreted to the enamel matrix. It consists of nine exons and the mature, secreted AMTN protein (after cleavage of the N-terminal signal sequence) is 20.4kDa in size with a pI of 5.88. It is rich in proline, leucine, threonine, glutamine and glycine and is predicted to be modified by serine, threonine and tyrosine phosphorylation and O-glycosylation (Figure 1.11). AMTN expression seems to be restricted to maturation stage ameloblasts and the junctional epithelium, although the application of increasingly sensitive techniques has shown AMTN transcripts in calvaria-derived osteoblasts (Atsawasuwan *et al.*, 2013). It remains to be determined whether this low level expression has any functional implication *in vivo*. 
32

MRSTILLFCLLGSTRSLPQLKPALGLPPTKLPDQGTLPNQQSNQVFPSLSLPLTQML
TLGPDLLNHPPAGMTPGTQTHPLTGGLNVQQQLHPHVLPFVTQLGAQGTTLSSEELP
QIFTSIIHSLFGGILPTSQAGANPDVQDGSLPAGGAGVNPATQGTPAGRLPTPSGTDD
DFAVTPAGIQRSTHAIEEATTESANGIQ

Figure 1.11 Human AMTN sequence including the signal peptide (underlined). The bold letters indicate the well-conserved amino acids by alignment of 10 complete mammalian AMTN sequences and a putative ancestral sequence (Sire et al., 2007). The IPLT motif shown in the green box is a predicted O-glycosylated site. The red box shows the putative serine phosphorylation site.

AMTN is, based on lack of expression in other tissues, considered to be specific to the ameloblast lineage. Evolutionary analyses have provided further evidence for AMTN as an enamel-specific protein (Gasse, Silvent, and Sire 2012). The expression of mouse amelotin was found by several independent groups to be rapidly and dramatically up-regulated in ameloblasts at the transition from secretory to maturation stage, and this expression profile is very different from that of the more established enamel proteins AMEL, AMBN, and ENAM (Iwasaki et al., 2005; Moffatt et al., 2006b; Trueb et al., 2007). In contrast, a single report using non-affinity-purified antibodies has shown AMTN expression in secretory stage enamel matrix of the mouse (Gao et al., 2010). The secreted AMTN protein accumulates at the interface between cells and enamel mineral and appears to be part of the specialized basal lamina-like layer that reappears at the onset of the maturation stage (Dos Santos Neves et al., 2012; Somogyi-Ganss et al., 2012). This restricted and specific localization of AMTN suggests functional roles in cell adhesion and/or surface enamel mineralization. Since AMTN can form multimeric aggregates in solution (Holcroft and Ganss 2011) it is also possible that it is part of a basal lamina-like structure that may be involved in transport control in and out of the maturing enamel. Our laboratory has described a simple cell adhesion experiment where bacterially produced, recombinant (and
therefore lacking post-translational modifications) AMTN did not mediate any cell attachment (Somogyi-Ganss et al., 2012). However, since AMTN is predicted to contain P-serine and potentially O-glycosylations, these modifications may alter the adhesive properties of the protein. It may also be possible that AMTN requires other interacting proteins to mediate cell adhesion, and in this context it is particularly interesting that AMTN has been shown to interact with ODAM (Holcroft and Ganss 2011).

The expression of AMTN coincides with the establishment of the dense, highly mineralized, aprismatic surface enamel layer (Møinichen, Petter Lyngstadaas, and Risnes 1996) and it is conceivable that the protein is involved in the establishment of this layer. Our laboratory has recently reported transgenic mice engineered to overexpress AMTN under the AMEL gene promoter (Lacruz et al., 2012a). These mice express AMTN not only at higher amounts, but also earlier during amelogenesis and as a result show a hypoplastic yet densely mineralized irregular enamel layer that does not show any organized rod and interrod microstructure, indicating that AMTN may have an accelerating role in the controlled mineralization of hydroxyapatite (Figure 1.12 left panel). The formation of Tomes’ process in these animals was completely disrupted. Our group has also recently developed AMTN-deficient animals which, in contrast to the AMTN-overexpressed mice, have hypomineralized enamel with structural defects in outer and surface enamel layers. Mineralization in these mice is delayed and residual organic matrix is detected especially in the final enamel layer at the interface with the ameloblast cells (Figure 1.12 right panel). The enamel surface in AMTN knockout mice is rough compared to the wild-type animals and shows higher levels of attrition. In the absence of AMTN, ameloblast morphology as well as the expression pattern of other matrix proteins was essentially unaffected.
As teeth erupt into the oral cavity, the reduced enamel epithelium fuses with the oral epithelium and is then slowly converted into junctional epithelium (JE) in a coronal-to-apical direction during and after tooth eruption. The JE ultimately covers the teeth and provides the “seal” around teeth to prevent invasion of oral microorganisms. AMTN protein is continuously detected at the ameloblast-enamel interface from the maturation through the reduced enamel epithelium to the junctional epithelium in mice (Somogyi-Ganss et al., 2012) and rats (Moffatt et al., 2006b; Nishio et al., 2013). The presence of AMTN during formation of the primary JE has only been detected by immunohistochemistry, but not by in situ hybridization, which either indicates insufficient sensitivity of the technique to detect low abundance AMTN mRNA transcripts, or that the AMTN protein found in the JE is residual from production by the reduced enamel epithelium (Sawada et al., 2014). AMTN expression has also been detected in certain types of odontogenic tumors (Crivelini et al., 2012; Ren et al., 2011) and is often associated with sites of mineral deposition (Stolf et al., 2013).
Figure 1.12 Transgenic mice overexpressing AMTN (left panel) or expressing no AMTN (right panel). The enamel in AMTN overexpressor animals (tg57) was compact and disorganized and showed significant reduction in thickness compared to the wild-type (WT) littermates; pictures adapted from Lacruz, et al. PLoS ONE. 2012. The absence of AMTN in AMTN knockout mice on the other hand, resulted in hypomineralized enamel with residual organic matrix especially at the surface, and a brittle surface. The red ovals show the rough fractured surface in these animals in comparison with WT enamel; pictures adapted from Nakayama, et al. J. Dent. Res. 2015.

1.4.7. Odontogenic Ameloblast-associated (ODAM)

ODAM was initially identified as the major protein component in the amyloid deposits of calcifying epithelial odontogenic (Pindborg) tumors (Solomon et al., 2003) and was named APin (for Amyloid in Pindborg tumors). It was also found as EO-009 in the signal trap screening approach mentioned above (Moffatt et al., 2006a) in rat incisor enamel. Due to its high expression in enamel-associated epithelia (Moffatt 2008; Park et al., 2007) it has been re-named ODAM (for Odontogenic, Ameloblast-associated). The ODAM gene consists of 12 exons and
the protein is 28.3kDa in size with a pI of 4.83. It is rich in glutamine and proline and predicted to be phosphorylated at multiple serine and threonine residues and O-glycosylated at multiple positions. ODAM expression is highest in maturation stage ameloblasts and JE, but it is present in other tissues including nasal and salivary glands (Moffatt 2008), indicating a broader biological role.

ODAM is expressed in a pattern very similar to AMTN during enamel formation. The first published report that indicated an involvement of ODAM in enamel formation showed high expression during amelogenesis in the rat incisor (Park et al., 2007) using immunohistochemistry and in situ hybridization. The authors further postulated that ODAM could exert its function during enamel formation and mineralization by modulating the expression of the enamel protease MMP-20. The onset of ODAM expression is slightly earlier (late secretory stage) than AMTN, but both proteins are localized in the basal lamina-like layer at the ameloblast-enamel interface. There appears to be a subtle difference in ODAM/AMTN localization with ODAM closer to the cell surface and AMTN closer to the enamel surface (Dos Santos Neves et al., 2012), but whether this bears any functional significance is not known. ODAM knockout mice have not been described in the literature to date. Further studies on the molecular mechanism of ODAM-mediated regulation of enamel mineralization have postulated that an intracellular form of ODAM is phosphorylated by the bone morphogenetic protein receptor type IB (BMPR-IB)-mediated action of BMP-2 and thus modulates the signaling pathways involved in ameloblast differentiation (Lee et al., 2012a). The same group has further shown that intracellular ODAM cooperates with the runt domain transcription factor Runx2 and thus regulates the expression of MMP-20. This modulation of MMP-20 by ODAM was interpreted as a mechanism by which enamel matrix maturation is regulated by ODAM (Lee et al., 2010). While the notion of an
intracellular form of ODAM is interesting and not unprecedented – as shown for the SIBLING protein osteopontin (OPN) (Zohar et al., 1997; Zohar et al., 2000) – it remains to be explained how and why the ODAM protein is retained or accumulates in the cytoplasm and the nucleus of ameloblasts in vivo and in culture. These findings contrast the identification of ODAM from rat ameloblasts by a signal trap screening approach (Moffatt et al., 2006a), which is designed to target secreted proteins. Interestingly, a direct inductive effect on dentin mineralization was observed when recombinant ODAM protein was applied to odontoblast cells in culture and in a dental pulp capping experiments in rats in vivo (Yang et al., 2010a).

While the localization of AMTN in the JE is restricted to the cell/mineral interface, ODAM is localized in a pericellular fashion in the JE. Both AMTN and ODAM are re-expressed in the JE after gingivectomy and regain their normal expression pattern after the gingival tissue has regenerated (Nishio 2010). Notably, ODAM expression is induced in epithelial rests of Malassez (ERM) after experimental periodontal detachment and ODAM is re-expressed during orthodontic tooth movement (Jue et al., 2014; Nishio et al., 2010), indicating that ODAM may play a role in periodontal regeneration. However, the work to date is largely descriptive and functional studies have not been published. ODAM was initially discovered in Pindborg tumors (Murphy et al., 2008; Solomon et al., 2003) and has further been found in other types of lung and breast tumors of epithelial origin and has been suggested as a prognostic marker for such neoplasms (Kestler et al., 2008), particularly human breast cancer (Siddiqui et al., 2009). Similar to AMTN, the expression of ODAM in some types of odontogenic tumors has also been confirmed (Crivelini et al., 2012; Ren et al., 2011). ODAM expression has further been described in odontoblasts, osteoblasts and various cancer cells (Lee et al., 2012b). The first study looking at functional aspects of the expression of ODAM in tumors has shown that it can inhibit tumorigenic
characteristics in the human breast cancer cell line MDA-MB-231 in vitro and the transplantation of ODAM-expressing tumor cells into mice leads to significantly reduced tumor growth and their inability to metastasize, compared to control cells that did not express ODAM (Kestler et al., 2011). Further dissection of the molecular pathways affected by ODAM revealed that it can act through elevation of the tumor suppressor phosphatase and tensin homologue (PTEN) and inhibition of the apoptosis-blocking PI3 kinase/AKT pathway (Foster et al., 2013).

1.4.8. Other Enamel Proteins

In addition to the enamel and SIBLING gene cluster, which contain genes involved in mineralized tissue formation, as well as other calcium binding phosphoproteins such as the casein genes and salivary protein genes (statherin etc.), a number of newly characterized or uncharacterized annotated genes are also found in this genomic region. Secretory calcium-binding phosphoprotein-proline-glutamine-rich1 (SCPPPQ1) is a new enamel matrix protein that has been identified as EO-463 by screening for secreted proteins in rat enamel (Moffatt et al., 2006a) and as a novel gene in the secreted calcium-binding phosphoprotein (SCPP) gene cluster in mammals (Kawasaki, Lafont, and Sire 2011), characterized by its high content of proline and glutamine (PQ). The SCPPPQ1 gene (annotated as Gm17660 in the mouse) consists of ten exons and the protein is 8.3kDa in size with a pI of 4.68, rich in proline and leucine. The protein is predicted to be phosphorylated at two serine residues at the N-terminus, but not glycosylated. A recent report of northern blotting of rat tissues demonstrated that SCPPPQ1 is tooth-specific (Moffatt et al., 2014). Immunohistochemical analysis also detected the protein during the late maturation stage of amelogenesis and in the junctional epithelium at the cell-tooth interface (Moffatt et al., 2014). Chymotrypsin C (Caldecrin-Ctrc) is a serine protease that was recently found to be up-regulated during enamel maturation in a whole-genome array analysis of rat
incisor enamel organ cells (Lacruz et al., 2011). Originally thought to be pancreas-specific, the expression profiling of Ctrc in enamel is similar to KLK4 but in lower levels which has been confirmed by quantitative PCR and western blot analysis (Lacruz et al., 2011). Other uncharacterized, protein-coding genes are currently annotated in the human genome and include C4orf40, C4orf26, C4orf22, and C4orf36, all within the enamel/SIBLING gene cluster. C4orf26 has recently been described as a candidate gene for AI, and the protein apparently possesses hydroxyapatite nucleation and growth activity (Parry et al., 2012).

1.5. Rationale, Hypothesis and Objectives

Our laboratory was the first to characterize the AMTN protein (Iwasaki et al., 2005) and develop transgenic mice to elucidate its possible functions in tooth formation. Our recent findings on AMTN in wild type (Somogyi-Ganss et al., 2012), knockout (Nakayama, Holcroft, and Ganss 2015), and AMTN overexpressors (Lacruz et al., 2012a) all hint at a direct regulatory role for AMTN in mineralization of enamel (Bartlett and Simmer 2015). My PhD project was designed to address this question by investigating the behavior of the protein in various mineralization systems.

The central hypothesis of this thesis is therefore as follows:

“The Enamel protein amelotin is a direct regulator of hydroxyapatite mineralization”.

The following specific objectives were defined to test this hypothesis:

Objective I: Examine the effect of AMTN on calcium phosphate mineralization in vitro in a simulated body fluid mimicking the forming enamel environment (chapter 2)

Objective II: Identify the mineralization regulatory site(s) within the primary sequence of AMTN (chapter 2)

Objective III: Study the influence of AMTN on biomineralization in an established mineralizing cell culture-based system (chapters 2, 3, & 4)

Objective IV: Investigate the mineralization-inducing effect of AMTN in collagen-based mineralizing systems (chapters 2 & 3)
Chapter 2

2. The Enamel Protein Amelotin is a Promoter of Hydroxyapatite Mineralization

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Langmuir studies, *in vitro* mineralization and light scattering data, expression and purification of recombinant proteins, SEM imaging, as well as the generation of figures and data analysis were performed by me (NA). The MC3T3-E1 cell culture experiments (Figure 2.6), and TEM imaging of early secretory enamel matrix of wild-type and transgenic mice (Figure 2.7) were performed by KI and SSM, respectively. Site-directed mutagenesis of AMTN was conducted by JH, who also contributed to the production of recombinant proteins. The mineralization experiment that resulted in minerals used in Figure 2.5 was performed by me and characterized using the immunogold labeling technique by SSM. The TEM imaging of mineral deposits was performed by Douglas Holmyard at the Advanced Bioimaging Centre, Mount Sinai Hospital. Authors’ roles are also described in the acknowledgements section (2.7)
Abstract

Amelotin (AMTN) is a recently discovered protein that is specifically expressed during the maturation stage of dental enamel formation. It is localized at the interface between the enamel surface and the apical surface of ameloblasts. AMTN knock-out mice have hypomineralized enamel whereas transgenic mice overexpressing AMTN have a compact but disorganized enamel hydroxyapatite (HA) microstructure indicating a possible involvement of AMTN in regulating HA mineralization directly. In this study, we demonstrated that recombinant human (rh) AMTN dissolved in a metastable buffer system, based on light scattering measurements, promotes HA precipitation. The mineral precipitates were characterized by scanning and transmission electron microscopy and electron diffraction. Colloidal gold immunolabeling of AMTN in the mineral deposits showed that protein molecules were associated with HA crystals. The binding affinity of rh-AMTN to HA was found to be comparable to that of amelogenin, the major protein of the forming enamel matrix. Overexpression of AMTN in mouse calvaria cells also increased the formation of calcium deposits in the culture medium. Overexpression of AMTN during the secretory stage of enamel formation in vivo resulted in rapid and uncontrolled enamel mineralization. Site-specific mutagenesis of the potential serine phosphorylation motif SSEEL reduced the in vitro mineral precipitation to less than 25%, revealing that this motif is important for the HA mineralizing function of the protein. A synthetic short peptide containing the SSEEL motif was only able to facilitate mineralization in its phosphorylated form (P$^S_p$SEEL), indicating that this motif is necessary, but not sufficient for the mineralizing properties of AMTN. These findings demonstrate that AMTN has a direct influence on biomineralization by promoting HA mineralization and suggest a critical role for AMTN in the formation of the compact aprismatic enamel surface layer during the maturation stage of amelogenesis.
2.1. Introduction

Dental enamel is the hardest tissue in mammals. Mature enamel comprises approximately 96% carbonated hydroxyapatite (HA) mineral, giving enamel outstanding hardness and flexural strength. Enamel is formed in a biomineralization process under the guidance of matrix proteins secreted by oral epithelial derived ameloblasts in three different stages (Moradian-Oldak 2012; Nanci and Ten Cate 2008). In the presecretory stage, epithelial cells differentiate into ameloblasts and establish the dentino-enamel junction by depositing the initial mineralizing enamel layer on a predentin matrix. In the secretory stage, ameloblasts release specific proteins into the enamel matrix through a cytoplasmic extension at their distal extremity called Tomes’ process. This matrix eventually mineralizes and forms individual, intertwined hydroxyapatite “rods” or “prisms” in the wake of the ameloblasts, which move away from the denino-enamel junction (DEJ) as enamel thickens. During the final maturation stage, enamel crystals grow in thickness to replace the organic matrix, which is removed by specific proteases. Ameloblasts undergo a functional transition from secretory to smooth and ruffle-ended cells (Hu et al., 2007), which are now involved in ion and peptide transport and pH regulation (Lacruz et al., 2013a; Simmer et al., 2010). The most extensively studied proteins produced by secretory ameloblasts are amelogenin (AMEL), ameloblastin (AMBN) and enamelin (ENAM). AMEL, which constitutes over 90% of the organic matrix, is believed to provide a framework for the development of the organized rod or prism pattern, and to regulate apatite crystal morphology and thickness (Margolis, Beniash, and Fowler 2006; Moradian-Oldak 2012). AMBN likely regulates ameloblast cell proliferation (Fukumoto et al., 2004) and ENAM regulates HA crystal nucleation and growth (Hu 2008). The enamel proteins are degraded by two main proteases, MMP-20 and KLK4, which are active during the secretory and maturation stages, respectively.

*In vivo* results demonstrate that the absence of any of these matrix proteins or proteolytic
enzymes in transgenic mice leads to structural deficiencies of enamel (Fukumoto et al., 2004; Gibson et al., 2001; Hu 2008; Simmer et al., 2009; Smith et al., 2011; Wazen et al., 2009; Wright et al., 2008). Recently, two new enamel matrix proteins, Odontogenic ameloblast-associated (ODAM) (Moffatt et al., 2006a) and AMTN (Iwasaki et al., 2005) have been identified, but their roles during enamel formation remain to be elucidated.

The result of cooperative interplay of matrix biomolecules is mature enamel in four distinct layers (Lyngstadaas, Møinichen, and Risnes 1998; Møinichen, Petter Lyngstadaas, and Risnes 1996): an initial aprismatic enamel layer at the dentino-enamel junction, bulk enamel composed of a highly orchestrated array of interwoven rods or prisms, Outer enamel consisting of parallel HA crystallites, and lastly a compact aprismatic enamel surface layer. Previous studies on enamel matrix proteins highlight some of the functions of these biomolecules including a critical role in biomineralization, and possible interaction with one another and with the inorganic phase to form the complex hierarchical structure of bulk enamel (Holcroft and Ganss 2011; Smith et al., 2011; Yang et al., 2010b). Despite extensive research however (mostly on AMEL (Moradian-Oldak 2012)), the details of how the compact enamel surface layer is formed remain largely unknown. The surface enamel is the critical interface with the oral cavity where caries and erosion, the most prevalent human disease (Selwitz, Ismail, and Pitts 2007; Ten Cate 2012), are initiated and propagate. Amelotin (AMTN) (Iwasaki et al., 2005; Moffatt et al., 2006a) is predominantly found in dental enamel during the maturation stage of amelogenesis (Moffatt 2008; Somogyi-Ganss et al., 2012) and at relatively low levels in the gingival junctional epithelium (Nishio 2010) after tooth eruption. The secreted AMTN protein is localized at the interface between the enamel surface and ameloblasts, the enamel forming cells, at the maturation stage (Moffatt et al., 2006a; Somogyi-Ganss et al., 2012). Overexpression of AMTN
under the AMEL gene promoter in a transgenic mouse model resulted not only in expression of AMTN at higher levels than normal but at an earlier stage than the maturation stage (i.e. secretory stage) of amelogenesis (Lacruz et al., 2012a). This led to a disorganized but compact enamel structure and disruption of the Tomes’ process (Nanci and Ten Cate 2008). The absence of AMTN in AMTN-knockout mice on the other hand, resulted in a hypomineralized enamel with an irregular surface (Nakayama et al., submitted). AMTN has also been localized in areas of dystrophic calcification in calcifying cystic odontogenic tumors (Stolf et al., 2013). Our recent findings on AMTN in mice (wild type (Somogyi-Ganss et al., 2012), knockout, and transgenic (Lacruz et al., 2012a) suggest a direct influence of AMTN on the formation of surface enamel layer which has a compact aprismatic texture different from that of bulk enamel. In addition, AMTN was found to be localized closer to enamel than ameloblasts (Dos Santos Neves et al., 2012), suggesting an involvement in mineralization rather than direct cell adhesion. The present study therefore, explored the effect of AMTN protein on calcium phosphate mineralization in vitro. The effect of AMTN on mineralization was further assessed in a cell culture study where the protein was overexpressed in mouse calvaria cells. Focused investigation of early secretory enamel matrix in transgenic mice overexpressing AMTN (Lacruz et al., 2012a) was also pursued using transmission electron microscopy to assess the influence of AMTN on the structure and organization of forming enamel minerals.

2.2. Materials and Methods

2.2.1. Recombinant human protein production

The N-terminally 6×His tagged recombinant human (rh) AMTN protein was expressed in E. coli (pET-15b expression system- Novagen, Merck KGaA, Darmstadt, Germany) and affinity purified on a Ni-NTA Agarose column (Qiagen, Valencia, CA, USA) to near homogeneity.
(Figure 2.1 A). The protein elute from the column was then dialysed against 50mM NH$_4$HCO$_3$ (Mallinckrodt, Mississauga, ON, Canada), freeze dried (Thermo Savant ModulyoD, Holbrook, NY, USA), and stored at -80°C. The 6×His tag was cleaved from the protein N-terminus using a thrombin cleavage kit (CleanCleave™, Sigma-Aldrich, St. Louis, MO, USA). The protein was then re-dialysed against 50mM NH$_4$HCO$_3$ to remove the remaining calcium salt from the thrombin cleavage buffer (About 5ml of protein solution in 4L of dialysis buffer), freeze dried, and stored at -80°C. Two mutated versions of rh-AMTN were also generated in which a highly conserved SSEEL motif (Gasse, Silvent, and Sire 2012; Sire et al., 2007) was altered. Rh-AMTN mutants were amplified from AMTN cDNA with specific primers and inserted into pET-15b vector. Similar to the full-length protein, the AMTN mutants were then expressed in E. coli bacteria and affinity purified. In rh-AMTN$_{S\rightarrow A}$ mutant the serine residues were replaced by alanines whereas, in rh-AMTN$_{DEL}$ mutant, the entire conserved motif was omitted from the sequence (Figure 2.1 B).

2.2.2. AMTN short peptides

Two short peptides, with and without phosphorylated serines in the conserved SSEEL motif, were synthetized (GenicBio Limited, Shanghai, China). Purity and sequence of these peptides was determined by HPLC and mass spectrometry, respectively (Figure 2.1 B and Figure 2.10).
2.2.3. Protein-hydroxyapatite binding

About 5 mg of synthetic hydroxyapatite powder (Berkeley Advanced Biomaterials Inc., Berkeley, CA, USA) with a specific surface area of 35 ± 5 m²/g (determined using BET method (Brunauer, Emmett, and Teller 1938); micromeritics®, Norcross, GA, USA) was added to increasing concentrations of protein (400-1500 μg/ml dissolved in 10 mM Tris-HCl pH 7.2 at 37°C in a total volume of 280 μl). Samples containing the HA powder and protein rotated at 37°C overnight. The HA powder was then sedimented by centrifugation at 14000 rpm for 10 minutes. The concentrations of the protein in solution before and after incubation with HA powder was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Thermo...
Scientific, Rockford, IL, USA). Using these parameters, the amount of protein bound to the unit surface area of HA powder was obtained. Linear adsorption isotherms (Kresak et al., 1977) were plotted using the Langmuir equation (Equation 1). The maximum number of adsorption sites available for the protein per unit of HA surface area and the affinity of the protein molecules for HA adsorption sites were obtained from the resulting best-fit lines:

\[
\frac{C_{eq}}{Q} = \frac{1}{NK} + \frac{C_{eq}}{N} \quad \text{(Equation 1)}
\]

\(C_{eq}\) = equilibrium protein concentration after incubation with HA (mol/ml)

\(Q\) = amount of protein bound to the unit surface area of HA powder (mol/m²)

\(N\) = the maximum number of adsorption sites per unit of HA surface area (mol/m²)

\(K\) = affinity of the protein molecules for HA adsorption sites (M⁻¹)

In addition to rh-AMTN produced as described above, osteopontin from bovine milk (OPN; a kind gift from Dr. E. Sørensen), a known strong binder to HA (Addison et al., 2007; Boskey et al., 1993; Goldberg et al., 2001; Sodek, Ganss, and McKee 2000), and myoglobin from equine heart (Myo; Sigma M1882), a protein that is not associated with mineralized tissues and has been found to bind to HA with low affinity (Iafisco et al., 2008), were used for comparison. The native milk OPN used in this study contained ~20% full-length protein and ~80% N-terminal fragment consisting of residues 1-147 (Addison et al., 2007; Boskey et al., 2012; Sørensen and Petersen 1993).

2.2.4. In vitro mineralization assay

A modified simulated body fluid (SBF) buffer (Oyane et al., 2003) with ion concentrations similar to those found in the enamel fluid (Aoba and Moreno 1987) was prepared for the in vitro
mineralization study. The buffer contained 15mM K\(^+\) (KCl 99.5% AnaLaR BDH Inc., Toronto, ON, Canada), 0.8mM Mg\(^{2+}\) (MgCl\(_2\)×6H\(_2\)O Sigma), 10mM HCO\(_3^-\) (NaHCO\(_3\) Sigma), 2.8mM Ca\(^{2+}\) (CaCl\(_2\)×2H\(_2\)O analytical reagent AnaLaR), 1.67mM phosphate (Na\(_2\)HPO\(_4\) Sigma S7907), 50mM HEPES (Biotechnology grade Bioshop Canada Inc., Burlington, ON, Canada), and 140mM Na\(^+\) (NaCl reagent grade Bioshop, NaHCO\(_3\) Sigma). After adjusting the pH to 7.2 at 37°C with 1N NaOH (Mohan 2003), the buffer was filtered (0.2µm, Acrodisc\(^®\), Sigma). Rh-AMTN protein, and/or equimolar concentrations of other proteins or peptides used in this study, was dissolved in the mineralization buffer. The buffer was added to wells of a sterile non-tissue culture 96-well polystyrene plate (300µl per well) and incubated at 37°C in a humidified incubator to prevent evaporation. In order to monitor the kinetics of calcium phosphate mineralization, a light scattering assay was conducted in which the clarity versus turbidity of the solution indicated the onset and extent of precipitation from the mineralization buffer influenced by the dissolved protein compared to the SBF buffer alone. The light scattering measurements were performed in a microplate reader (Titertek Multiskan MCC/340 Labsystems, Finland) at 540 nm at different time points during the mineralization experiments. Statistical analysis on turbidity results was conducted using one-way ANOVA analysis and significance was assigned at \(p < 0.05\). At the end of the incubation period, the liquid was carefully aspirated; wells containing calcium phosphate precipitates were briefly washed with deionized water and air-dried in a fume hood.

2.2.5. Characterization of the Calcium Phosphate Precipitates

The \textit{in vitro} mineralization samples were gold coated (Desk II sputter coater, Denton Vacuum Inc., Moorestown, NJ, USA) and imaged using scanning electron microscopy (FEI XL30 ESEM, Hillsboro, OR, USA) with a secondary electron detector and at 20kV accelerating voltage. The
morphologies and structural phase of the formed calcium phosphate particles were further characterized using transmission electron microscopy (TEM, FEI Tecnai™). The formed minerals were collected and embedded in Quetol-Spurr mixture. 150nm sections were cut from the plastic with a diamond knife (JUMDI, Canemco & Marivac, Gore, QC, Canada) on an ultramicrotome (RMC MT6000, Boeckeler Instruments Inc., Tucson, AZ, USA) and placed on 200 mesh copper grids (Veco, Canemco & Marivac) for microscopy. Images were obtained at 200kV using bright field and selected area electron diffraction (SAED) modes.

### 2.2.6. Colloidal gold immunolabeling

The mineral precipitates from the mineralization buffer containing 200µg/ml rH-AMTN protein were collected and fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in Hanks’ balanced salt solution (Lonza, Walkersville, MD, USA) at pH 7.3 for two hours. The sample was then centrifuged at 14000 rpm for 10 minutes and the supernatant carefully aspirated. The mineral pellet was dehydrated in 100% ethanol and embedded in LR white acrylic resin (London Resin Company, Reading, UK). The resin block was cut into 100 nm sections with a diamond knife (JUMDI, Canemco & Marivac) using an ultra-microtome (RMC MT6000, Boeckeler Instruments Inc.). Sections were placed on Formvar™ supported carbon-coated nickel grids (100-mesh Veco Hexagonal, Canemco & Marivac). The sections mounted on the grids were then incubated with primary anti-mouse AMTN antibody (produced in our laboratory (Somogyi-Ganss et al., 2012), 1:10 dilution) and secondary protein A-gold complex (15-nm-diameter gold particles; Dr. G. Posthuma, University of Utrecht, Utrecht, The Netherlands), as reported in detail(Somogyi-Ganss et al., 2012). Sections were stained with uranyl acetate (Canemco & Marivac) and analysed by TEM (FEI Tecnai™). In the control experiments, sections were not treated with the primary AMTN antibody.
2.2.7. Cell culture assay

For transient overexpression of murine AMTN, the full-length cDNA was cloned into the mammalian expression vector using the pcDNA™ 3.1 Directional TOPO® Expression Kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The mouse osteogenic cell line MC3T3-E1 (subclone 14; ATCC # CRL-2594) was grown as recommended to 80% confluence in 24 well tissue culture plates, which was arbitrarily set as day zero. 1 µg of expression vector or empty vector was transfected into MC3T3-E1 cells (Ruan et al., 2013) using LipofectAmine 2000 (Invitrogen) and Opti-MEM (Invitrogen). 24 hours after transfection, culture medium was changed to α MEM containing the following additives: 10 nM dexamethasone (DEX; Sigma D4902), 10 mM β-glycerophosphate (BGP; Sigma G9891) and 50 μg/ml ascorbic acid (AA; Sigma A4403). The culture medium was changed every 3 days. At the end of the culture period cells were stained with Alizarin red and photographed. The number of calcified nodules was quantified using Image J software automated counting.

Total RNA was extracted from separate wells at day 7 and at day 14. RT-PCR for AMTN, alkaline phosphatase (ALP) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; control) were then performed using SuperScript 3 reverse transcriptase (Invitrogen), advantage 2 PCR kit (Clontech, Mountain View, CA, USA), and specific primers.

2.2.8. TEM analysis of transgenic mice

Transgenic mice that overexpress AMTN under AMEL gene promoter (line tg57) have been generated and characterized previously (Lacruz et al., 2012a). In this study, the early- and mid-secretory stage enamel matrix in non-demineralized, transverse tissue sections of mandibular incisors were analysed in further detail by TEM. As described previously (Lacruz et al., 2012a), tg57 and wild-type (WT) mice were sacrificed by cardiac perfusion. Hemi-mandibles were
dissected, fixed, and stained with 2% uranyl acetate. Samples were then dehydrated in graded concentrations of ethanol and propylene oxide (Acros, Fair Lawn, NJ, USA) infiltrated and embedded in Jembred 812 resin (Canemco & Marivac), and cut into 100nm thick sections. After post-staining with 2% uranyl acetate, the sections were mounted on Cu TEM grids (100-mesh Veco Hexagonal) and analysed with bright field and selected area electron diffraction (SAED) modes (FEI Tecnai™).

2.3. Results

2.3.1. Adsorption Isotherms

The Langmuir adsorption theory, known to model the coverage of protein molecules on solid surfaces (Bouropoulos and Moradian-Oldak 2003; Iafisco et al., 2010), was employed to obtain the binding affinity of AMTN to hydroxyapatite. The adsorption isotherms of the proteins used in this study fit the Langmuir model well ($R^2$ values $> 0.96$; Figure 2.2). The muscle protein Myo had the lowest binding affinity to apatite ($1.4 \times 10^5$ M$^{-1}$). Milk OPN was found to have a very high K value ($8.3 \times 10^6$ M$^{-1}$; Figure 2.2). The binding affinity of AMTN protein for HA adsorption sites ($3.86 \times 10^5$ M$^{-1}$) was lower than that of OPN, but higher than that of Myo. Maximum number of adsorption sites per unit of surface area on HA for binding to AMTN molecules was $5.98 \times 10^8$ mol/m$^2$ which was three times higher compared to the other molecules. Regardless of the significant difference between HA binding affinities for OPN and Myo, their N values were similar (Figure 2.2).
<table>
<thead>
<tr>
<th>Protein</th>
<th>N (mol/m²)</th>
<th>K (M⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteopontin</td>
<td>1.97×10⁻⁸</td>
<td>8.3 × 10⁶</td>
<td>0.9998</td>
</tr>
<tr>
<td>Amelotin</td>
<td>5.98×10⁻⁸</td>
<td>3.86 × 10⁵</td>
<td>0.9736</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>2.10×10⁻⁸</td>
<td>1.4 × 10⁵</td>
<td>0.9697</td>
</tr>
</tbody>
</table>
**Figure 2.2** Linear adsorption isotherms of amelotin, osteopontin, and myoglobin. Protein-HA adsorption data fit the Langmuir model well. Equation 1 was employed to obtain the best-fit linear plots for each protein. Using the slope and Y-intercept of each plot, affinity of the protein molecules for HA adsorption sites (K) and the maximum number of adsorption sites per unit of HA surface area (N) were calculated. Summary of adsorption isotherm results is shown above. \( R^2 \) is the correlation coefficient obtained for linear adsorption isotherms.

### 2.3.2. Rh-AMTN promotes HA mineralization *in vitro*

To investigate the effect of rh-AMTN on calcium phosphate mineralization the protein was dissolved in modified SBF, as described above. The buffer containing 100µg/ml rh-AMTN resulted in extensive calcium phosphate precipitation from the solution after 48 hours of incubation at 37°C (Figure 2.3). Transmission electron micrographs of the sectioned precipitates showed needle-shape crystals of hydroxyapatite as confirmed by SAED analysis which contained characteristic diffraction patterns in (002) and (211) planes (Figure 2.3 C, D). The HA mineralizing activity of AMTN was further confirmed by light scattering measurements. Higher concentrations of AMTN resulted in higher light scattering values after about two days indicating HA mineralization in a dose-dependent manner (Figure 2.4 A). Control experiments using only the mineralization buffer or equimolar concentrations of OPN or Myo in the buffer did not show any signs of mineral precipitation (Figure 2.4 B).

Immunohistochemical TEM imaging of AMTN in thin sections of HA minerals formed in the buffer containing 200µg/ml rh-AMTN revealed the presence of numerous gold particles incorporated randomly within the HA crystals (Figure 2.5 A). Control samples that had only been processed with the secondary protein A-gold complex did not show any gold particles in these experiments (Figure 2.5 B).
Figure 2.3 Mineral precipitates from the mineralization buffer containing 100 μg/ml rh-AMTN after 48 hours of incubation at 37°C. (A) and (B) SEM images. (C) TEM imaging of the mineral structure consisting of needle-like crystallites. (D) SAED analysis of the crystals with discrete concentric rings corresponding to (002) and (211) crystallographic planes of hydroxyapatite.
Figure 2.4 Light scattering plots of mineralization buffers containing (A) 0-40μg/ml AMTN and (B) AMTN, Myo, and OPN in equimolar concentrations, up to 7 days. The mineral promoting effect of rh-AMTN was dose-dependent whereas, mineralization buffer alone or buffer containing OPN or Myo remained clear. Data is presented as mean ± standard deviation; (*): mean significantly different from other groups at every time point (* p < 0.001, ** p < 0.05, sample size (n) = 8 per group).
Figure 2.5 TEM images of HA minerals precipitated from the mineralization buffer containing 200µg/ml rh-AMTN (A) immunolabeled with primary AMTN antibody and secondary protein A-gold complex (black round particles); or (B) only treated with protein A-gold complex (control). HA crystals contained numerous immunolabeled AMTN molecules that were homogenously distributed throughout the mineral (A).

2.3.3. AMTN overexpression in mouse calvaria cells promotes mineralization

To assess whether AMTN overexpression would affect mineralization in an established cell culture system of osteoblast mediated hydroxyapatite formation we used the mouse calvaria cell line MC3T3-E1. We found that AMTN overexpression significantly increased the amount of alizarin red-positive cell nodules (Figure 2.6 A, B). The expression level of the osteoblast differentiation marker alkaline phosphatase (ALP) (Hoemann, El-Gabalawy, and McKee 2009) was apparently unaffected by AMTN overexpression (Figure 2.6 C).
Figure 2.6 Transfection of mouse calvaria cell line MC3T3-E1 with murine AMTN. (A) Alizarin red staining of cells showed higher levels of staining for AMTN-transfected cultures (+AMTN) than control cultures (-AMTN). (B) Quantification of calcified nodules in control (n=4) versus AMTN-transfected samples (n=3). Data is presented as mean ± standard deviation; (*) indicates a significant difference; * p < 0.00001 (C) RT-PCR analysis of total RNA from 14-day cultured cells overexpressing AMTN (shown by a + sign) or control cells containing no AMTN (shown by a – sign). The left, middle, and right panels show the results for ALP, AMTN, and GAPDH expression, respectively. Overexpression of AMTN in these cells did not change the expression level of ALP or GAPDH.

2.3.4. Rapid enamel mineralization in transgenic mice

To obtain detailed information about the structure and organization of forming minerals under the influence of overexpressed AMTN by secretory ameloblasts, the enamel matrix in tg57 mice was characterized. In WT animals, mineral bundles in organized arrays were detected in the extracellular matrix at the early secretory stage (Figure 2.7 B, C). In tg57 mice on the other hand, a uniformly dense but disorganized mineralization front was observed in close proximity of the secretory surface of ameloblasts (Figure 2.7 D, E). The SAED analysis of these newly formed minerals in both phenotypes revealed a pattern of diffuse rings indicating a largely amorphous structure (Figure 2.7 A, F).
Figure 2.7 TEM images of early secretory enamel matrix in WT (B, C) and tg 57 mice overexpressing AMTN (D, E). Unlike the organized patterns of forming minerals seen in WT mice (arrows in C), mineralization in tg57 mice was massive and disordered (arrows in E). SAED analyses of the early mineral deposits showed amorphous structures in both phenotypes (A, F).

2.3.5. The conserved SSEEL motif regulates the HA mineralizing ability of rh-AMTN

In search of mineral interacting sites within the primary sequence of AMTN, two mutants of rh-AMTN with altered SSEEL motifs were generated and examined using the in vitro crystallization assay. Mineral precipitation from buffers containing equimolar concentrations of rh-AMTN$_{S\rightarrow A}$ or rh-AMTN$_{DEL}$ (Figure 2.1) was significantly reduced in comparison with full-length rh-AMTN but not completely abrogated (Figure 2.8). Replacing SS by AA in rh-
AMTN$_{S\rightarrow A}$ led to a similar effect as deleting the entire conserved region in rh-AMTN$_{DEL}$ signifying that serine residues are essential for the role of AMTN in mineralization.

**Figure 2.8** Light scattering results of mineralization buffers containing 100µg/ml rh-AMTN or equimolar concentrations of AMTN mutants after 7 days of incubation at 37°C. The corresponding SEM images of the precipitates are shown above (a-e). Data is shown as mean ± standard deviation; (*) mean significantly different from other groups (* p < 0.001, ** p < 0.0001, n=8 per group). Replacing SS by AA in rh-AMTN$_{S\rightarrow A}$ or deletion of the entire SSEEL motif in rh-AMTN$_{DEL}$ resulted in significant reduction in mineralizing ability of AMTN protein. In AMTN mutant samples (c and d), disperse mineral particles were observed that were larger in size than the mineral precipitates from the AMTN sample (b). Bar scales = 50µm. The crystalline structure of deposits from the AMTN mutant samples was hydroxyapatite (SAED pattern in e).
2.3.6. The effect of phosphorylation

In these experiments we examined whether phosphorylation of the serine residues in the functional SSEEL motif influences the HA mineralizing activity of AMTN. Similar to full-length AMTN, the short AMTN phosphopeptide promoted HA precipitation significantly (Figure 2.9). The extent of mineral precipitation however, was considerably lower compared to the full-length protein. In addition, the short AMTN peptide containing the non-phosphorylated conserved SSEEL motif did not facilitate mineralization above control levels. Minerals that precipitated from the buffer containing AMTN phosphopeptide were much smaller and had irregular morphology compared to the larger spherical-shaped precipitates from the full-length AMTN samples (Figure 2.9; SEM images a-d). The crystalline structure of these calcium phosphate deposits however, remained hydroxyapatite as shown by SAED analysis (Figure 2.9 e).

2.4. Discussion

The intricate Enamel microstructure is formed through a tightly controlled biomineralization process guided by enamel matrix proteins. The role of each protein individually and together with other proteins expressed at every stage of the process is under investigation. In this study we focused on the maturation-stage protein AMTN and tested its effect on mineralization using a combination of model systems.

We initially performed Langmuir adsorption isotherm experiments to determine the binding affinity of AMTN to HA mineral to be $3.86 \times 10^5$ M$^{-1}$, a value in the same order of magnitude ($6.38 \times 10^5$ M$^{-1}$) as previously reported for recombinant AMEL protein (Sun et al., 2008). As the N value for AMTN is three times higher than the other two proteins, larger amounts of AMTN molecules are required to adhere to and saturate all the binding sites per unit of surface area in apatite. This result suggests a preferential HA binding motif on AMTN compared to OPN or
Figure 2.9 Light scattering results of mineralization buffers containing 100µg/ml rh-AMTN or equimolar concentrations of AMTN short peptides after 7 days of incubation at 37°C. The corresponding SEM images of the precipitates are shown above (a-e). Data is shown as mean ± standard deviation; (*): mean significantly different from other groups (* p < 0.001, n=8 per group). Short AMTN peptide containing SSEEL motif did not have any effect on mineralization whereas, short AMTN phosphopeptide containing PS²SEEL motif facilitated mineralization significantly. Buffer containing the full-length AMTN protein had the highest amount of mineral precipitation. Crystal deposits from the phosphorylated peptide samples (a, b) were irregular in micromorphology compared to spherical mineral deposits in rh-AMTN samples (c, d). However, the SAED pattern of these minerals revealed the same hydroxyapatite structure (e). Scale bars = 1 µm (a, c) and 5 µm (b, d).

Myo. The binding of AMTN to the mineral could result in steric hindrance and/or conformational changes to the secondary structure of the protein that would make potential cleavage sites unavailable to the proteases such as KLK4, present in the matrix. This effect has
been shown for some of the saliva proteins that are strong binders to hydroxyapatite and remain mostly intact when bound to the mineral (Helmerhorst et al., 2010; McDonald et al., 2011). Next, we investigated whether the moderate affinity of rh-AMTN to HA would have an effect on in vitro mineralization. Our system involved a modified SBF buffer (Oyane et al., 2003) that has a similar degree of supersaturation with respect to HA to that of enamel fluid during amelogenesis (Buerlein et al., 2007) and is stable for up to three weeks at 37°C. Rh-AMTN as low as 4 µg/ml in solution accelerated the spontaneous HA precipitation from the buffer. We employed light scattering using a monochromatic plate reader as a simple yet definitive technique to characterize the concentration-dependent effect of AMTN on the kinetics and extent of HA mineralization and precipitate formation. To further validate the in vitro system, we compared the mineralizing effect of Myo, a protein not associated with mineralized tissues, and OPN, a protein that is known to bind to HA with relatively high affinity and to inhibit mineralization (Boskey et al., 2012; Goldberg et al., 2001), both of which did not cause any precipitation from the buffer (Figure 2.4). Other more advanced and labour-intensive methods such as measurement of titrant volume/pH versus time in a constant composition system (Yang et al., 2010b) or dynamic light scattering of forming calcium phosphate nuclei (de Bruyn et al., 2013) have been employed to study the kinetics of mineralization influenced by biomolecules while monitoring the onset of crystal formation at the molecular level. Capturing the earliest mineral nucleation events in the presence of AMTN was beyond the scope of this study and requires future investigation. The incorporation of AMTN molecules within HA crystals in our immunogold labelling experiments suggests a possible role for the protein to assemble calcium and phosphate ions together and integrate with them to form CaP clusters and later stable crystal nuclei, as suggested for some other mineral regulatory proteins (Fang et al., 2011; He et al., 2005a). We next queried whether overexpression of AMTN in a collagen-based mineralization
system, mouse calvaria-derived MC3T3-E1 osteoblast cells, would have an effect on the formation of mineral precipitates and indeed found an increased deposition of alizarin red-positive calcium deposits. This increased mineralization tendency is apparently not due to accelerated differentiation of the cells, since the expression level of osteoblast marker gene alkaline phosphatase (ALP) was unaffected by AMTN overexpression. This finding suggests that AMTN can exert its mineralization-promoting effect outside of the enamel environment, and may open interesting perspectives toward tissue-to-mineral attachment in a non-dental setting. We lastly characterized the early secretory-stage forming enamel matrix in tg57 mice and consistent with our in vitro findings, observed an accelerated mineral formation phenomena in AMTN-overexpressed matrix. In agreement with previous observations of secretory enamel matrix in WT mice (Beniash et al., 2009), these mineral deposits were still amorphous. As the expression of major secretory stage enamel proteins including AMEL remains at a normal level in tg57 (Lacruz et al., 2012a), the extensive mineralization that occurs in an uncontrolled manner is due to the presence of elevated levels of AMTN in the matrix. Progression of enamel rods from retracting ameloblasts (Nanci and Ten Cate 2008) normally creates a picket-fence appearance at the secretory end of the cells known as the Tomes’ process (Smith 1998; Warshawsky et al., 1981). It is not surprising that this ordered structure is disrupted by the rapid mineralization front induced by AMTN in tg57 and the final enamel is densely mineralized but significantly thinner than normal enamel (Lacruz et al., 2012a).

Our results provide solid evidence that AMTN is a unique enamel matrix protein with remarkable capability to promote calcium phosphate mineralization. Some studies have reported a similar function for rh-AMEL in vitro (Tarasevich et al., 2007; Wang et al., 2008; Yang et al., 2010b). Unlike AMTN however, AMEL-knock out enamel is not hypomineralized but is
hypoplastic and disorganized (Gibson et al., 2001; Wright et al., 2008) suggesting a critical role in regulating the mineral organization and morphology (Du et al., 2005; Fang et al., 2011) rather than a promoting function which has been confirmed in numerous in vitro studies (Reviewed by (Moradian-Oldak 2012)). In search of mineral promoting sites in the AMTN sequence we focused on an SSEEL motif defined in evolutionary analysis reports as a highly conserved region and possibly phosphorylated (Gasse, Silvent, and Sire 2012; Sire et al., 2007). The SXE motif has been shown as a common sequence in the secretory calcium binding phosphoprotein (SCPP) family and many small integrin-binding ligand N-linked glycoproteins (SIBLING) involved in the mineralization of bone and teeth (Kawasaki and Weiss 2003). It has also been identified as a critical motif for HA mineralization in the product of the C4orf26 gene, which is mutated in various forms of amelogenesis imperfecta (Parry et al., 2012). Mutations in the conserved nucleotides including the first serine in SSEEL motif has been detected in silico for AMTN pseudogene in enamel-less species denoting the importance of this site for a functional enamel-specific AMTN (Gasse, Silvent, and Sire 2012). Here we showed that the two serine residues in this sequence are indeed critical for the HA mineralizing activity of the AMTN protein. Complete deletion of SSEEL or replacing both serine residues by alanine reduced the in vitro mineral precipitation to less than 25%. Functional sites containing the serine/threonine residues in many mineralization regulatory proteins of calcified tissues undergo phosphorylation in vivo (George and Veis 2008). This post-translational modification could substantially alter the role of the biomolecule in control of biomineralization; for instance transforming a mineral nucleator to an inhibitor or vice versa (He et al., 2005b; Kwak et al., 2009; Milan et al., 2006). AMTN produced by mammalian cells is significantly larger than predicted from the primary amino acid sequence (Moffatt et al., 2006a; Somogyi-Ganss et al., 2012), and this increase in molecular weight may be due to post-translational modifications that include serine phosphorylation.
However, the nature and extent of such modifications in native AMTN has not been investigated. Interestingly, the short AMTN peptide containing phosphorylated serine residues promoted HA precipitation from the solution, albeit to a lesser extent than the rh-AMTN protein, while the non-phosphorylated peptide had no effect on mineralization. These results have two possible implications: 1) the phosphorylated full-length protein has a much higher potential to precipitate apatite than the recombinant protein and 2) Other mineral interacting sites exist on the sequence that are involved in HA mineralization and required for a fully functional protein.

2.5. Conclusions

Our findings in vitro, in cell culture, and in vivo indicate that the enamel protein AMTN is a potent promoter of calcium phosphate mineralization. We propose that AMTN is likely a key player in the establishment of the compact, aprismatic surface enamel layer since its expression coincides with the formation of this layer. As the most superficial layer of enamel is the direct interface with the oral cavity, surface enamel is colonized by microbial biofilms and is the initial site for the formation of dental plaque, and highly prevalent caries and erosion. This finding may have direct implications for the development of preventive and/or restorative strategies for dental enamel.

2.6. Disclosures

All authors state that they have no conflicts of interest.

2.7. Acknowledgments

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(Aarhus University, Denmark) for providing the milk osteopontin protein and Douglas Holmyard (Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto) for his expert assistance with the TEM imaging.

**Authors’ roles:** NA, SSM, JH, KI, and BG designed the experiments; NA, SSM, JH, and KI conducted the experiments; NA and KI analyzed the data; NA and BG interpreted the data and drafted the manuscript. NA and BG accept responsibility for the integrity of the data analysis.

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**Figure 2.10 Supplemental** (A, B) HPLC results, and (C, D) mass spectrometry results of non-phosphorylated and phosphorylated AMTN peptides, respectively.
Chapter 3

3. The Enamel Protein Amelotin Promotes Mineralization in Collagen-based Systems *In vitro*

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Except for Figure 3.3 A, all the experiments were conducted by me (NA). For the collagen gel mineralization experiments, collagen gels were designed and produced by YI. I prepared the mineralization buffers and performed the SEM imaging. JH contributed to the production of recombinant AMTN protein. The TEM imaging was performed by Douglas Holmyard at the Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital.
Abstract

Amelotin (AMTN) is an ameloblast-secreted protein found mainly on the surface of maturing dental enamel before tooth eruption and in the junctional epithelium covering the tooth surface at the dentinogingival junction. AMTN knockout mice have hypomineralized enamel and reduced hardness especially on the enamel surface whereas mice overexpressing AMTN develop hypoplastic enamel with a compact and disorganized structure. We have recently reported that AMTN is a promoter of hydroxyapatite mineralization and proposed that it is likely a key player in the establishment of the compact surface enamel layer. Here we investigated whether AMTN is also able to promote mineralization in in vitro systems that mimic the natural environment of collagen-based calcified tissues. Introducing recombinant human (rh) AMTN to the culture media of MC3T3-E1 osteoblast cell line resulted in significant reduction in the expression of bone markers bone sialoprotein, osteocalcin, and alkaline phosphatase; yet accelerated mineralization in these cultures. The incorporation of rh-AMTN with collagen type I gels resulted in the formation of hydroxyapatite deposits onto and within the gel matrix when incubated in metastable mineralization buffer at 37°C within 5 hours. These findings demonstrate that AMTN is able to accelerate mineralization in collagen-based systems in vitro.
3.1. Introduction

Amelotin (AMTN) is an enamel-specific protein (Gasse, Silvent, and Sire 2012; Iwasaki et al., 2005) that is secreted by the enamel forming cells, known as ameloblasts, during the maturation stage of enamel formation where the already formed enamel crystals grow in width and thickness and the entire enamel layer fully matures into an approximately 96% by weight mineralized tissue. During this stage, AMTN molecules are localized on the enamel surface at the cell-mineral interface (Moffatt et al., 2006a; Somogyi-Ganss et al., 2012). Recent investigations suggest that AMTN protein maybe directly involved in enamel mineralization during the maturation phase. Transgenic mice that overexpress AMTN develop a hypoplastic yet compact and disorganized enamel (Lacruz et al., 2012a). Further analysis of the early secretory enamel matrix in these animals revealed a rapid and uncontrolled mineralization front in the matrix (Abbarin et al., 2015). The enamel layer in AMTN knockout mice on the other hand, is hypomineralized (Nakayama, Holcroft, and Ganss 2015). AMTN is also expressed by the junctional epithelial cells that cover the mature enamel surface of erupted teeth (Moffatt et al., 2006a; Nishio 2010). In calcifying cystic odontogenic tumors AMTN molecules co-localize with calcium phosphate deposits (Stolf et al., 2013). We have recently shown that AMTN promotes calcium phosphate mineralization in vitro (Abbarin et al., 2015). Bacterially-expressed recombinant human (rh) AMTN accelerated hydroxyapatite (HA) mineralization in a dose-dependent manner when dissolved in a modified simulated body fluid (SBF) buffer with ion concentrations similar to those of enamel fluid. Immunogold labeling of AMTN in the precipitated crystals in solution showed the protein molecules associated with HA crystals. We also tested the effect of AMTN on mineralization in mouse calvarial MC3T3-E1 cultures and found that overexpression of AMTN in this cell line resulted in increased formation of Alizarin-
red stained mineralized nodules. This established osteoblast-mediated cell culture system allowed us to not only evaluate the function of the protein expressed in a mammalian system as opposed to bacterially-produced, but also revealed that AMTN may be able to accelerate mineralization in both non-collagen based and collagen based systems. The aim of this study is to investigate the mineralizing effect of rh-AMTN in two different collagen based systems: first when the protein is added to the MC3T3-E1 culture medium and second when it is incorporated with collagen gels and incubated in the mineralization buffer in vitro.

3.2. Materials and Methods

3.2.1. Recombinant AMTN protein production
Rh-AMTN protein tagged with six histidine molecules on the N-terminus was expressed in E. coli (pET-15b expression system- Novagen, Merck KGaA, Darmstadt, Germany) and affinity purified on a Ni-NTA Agarose column (Qiagen, Valencia, CA, USA) to near homogeneity. The protein elute was then dialysed against ultrapure water, filtered using a 0.22 µm filter, dried, and sterilized by gamma irradiation at a dose of 4.0 MRad. Samples were then stored at -80°C.

3.2.2. Cell culture experiments
Mouse osteogenic cell line MC3T3-E1 subclone 14 (ATCC # CRL-2594) was grown in αMEM (without ascorbic acid- Gibco A1049001) containing 10% fetal bovine serum (FBS- Sigma F1051), 250µg/ml Penicillin (Sigma P3032), 60 µg/ml gentamycin (Wisent 450-135-XL), and 0.25µg/ml Fungizone® (Invitrogen 15290). At 80% confluence, cells were seeded at 50,000 cells/cm² in a 24-well plate (Falcon™ 087721H). The next day (set at day 0) confluent cells were supplemented with regular αMEM containing 50µg/ml ascorbic acid (Gibco 12571-063), as well as 3mM sodium dihydrogen phosphate (BioShop SPM306), and 100µg/ml rh-AMTN. Inorganic phosphate was also added to control cells. The cell media was changed every
three days. Experiments were performed in triplicate. On day 10 cells were washed with D-PBS (Wisent Inc. 311-425-CL), fixed with 10% v/v formaldehyde (Sigma F8775) at room temperature for 10 minutes, and stained with 2% Alizarin red S (Sigma A-5533), as described (Gregory et al., 2004).

3.2.3. RNA isolation and real-time PCR analysis

Total RNA was extracted from osteoblast cells containing 100µg/ml rh-AMTN in their media or control cells without any protein additions on days 3 and 10 and purified using the RNeasy® Plus Mini kit (Qiagen 74134) and according to the manufacturer’s instructions. For quantitative real-time PCR, 0.6 µl of RNA was prepared with an iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad 172-5150) and reverse transcribed and amplified with mouse gene-specific primers as listed in Table 1. The RT-PCR conditions were 50°C for 30 minutes (cDNA synthesis), 95°C for 5 minutes (reverse transcriptase inactivation), and alternating incubations at 95°C for 15 seconds and 60° for 1 minute, for 30 cycles (PCR reaction). The reaction was performed and analyzed for expression quantity in a CFX96™ thermal cycler (Biorad). Data were normalized relative to the control gene GAPDH. Alkaline phosphatase (ALP) expression was analyzed semi-quantitatively (Hoac et al., 2013) using primers reported previously (Addison et al., 2008). The reactions were performed in three triplicate measurements. The PCR products were then run on a 2% agarose gel stained with ethidium bromide and visualized under UV light.
Table 1 Mouse gene-specific primers used for RT-PCR reactions

<table>
<thead>
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<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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<tr>
<td>Collagen typeIa1</td>
<td>GAGCGGAGAGTACTGGATCG</td>
<td>TACTCGAACGGGAATCCATC</td>
</tr>
<tr>
<td>BSP</td>
<td>GGGAGGCGTGTACTGTCTCTCTCCCAT</td>
<td>TCGTCGCTTTACCTGCTTTCTCCA</td>
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<td>Osteocalcin</td>
<td>GCGCTCTGTCTCTCTGACCT</td>
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<tr>
<td>Osterix</td>
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<td>TGCAGGAGAGAGGGGTCACTG</td>
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<tr>
<td>GAPDH</td>
<td>AACTTTGGCATTTGAGAAG</td>
<td>ACACATTGGGGGTAGGAACA</td>
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3.2.4. Preparation of collagen gels

6mg/ml Bovine collagen solution type I (Nutragen® Catalog #5010), 0.1M NaOH, 20mM Genipin cross linker (Sigma G4796), and PBS were mixed to a final collagen gel concentration of 3.4mg/ml. In gels embedded with rh-AMTN, the protein was dissolved in PBS buffer to a final concentration of 580µg/ml in the final gel product (Figure 3.3 A). The gels were then incubated in the mineralization SBF buffer (detailed components described previously (Abbarin et al., 2015)) at 37°C for several days. Gels were then removed from the buffer, fixed in 2% glutaraldehyde in 0.1M sodium cacodylate, dried and gold coated, and imaged with scanning (SEM, FEI XL30 ESEM, Hillsboro, OR, USA) and transmission electron microscopy (TEM, FEI Tecnai™) analyses. TEM Images were taken at 200kV using bright field and selected area electron diffraction (SAED) modes.

3.3. Results

3.3.1. Rh-AMTN promotes mineralization in osteoblast cultures

On day 10, numerous Alizarin-red stained mineralized nodules were seen in cultures supplemented with 100µg/ml rh-AMTN whereas control cultures remained mostly clear (Figure 3.1).
Figure 3.1 Alizarin red stained cells on day 10. Mineralized nodules were observed in cultures supplemented with 100µg/ml rh-AMTN. At this time point, no mineralization was detected in control cultures.

QPCR analysis revealed that introducing rh-AMTN to the differentiating osteoblast cultures significantly down-regulates the expression of bone markers osteocalcin (OSC), alkaline phosphatase (ALP), and bone sialoprotein (BSP) at the earlier time point day 3 (Figure 3.2 A). Maturing osteoblasts containing rh-AMTN on day 10 also showed marked reduction in expression of OSC and ALP genes (Figure 3.2 B). Collagen type I (Col1a1) and osterix (OSX) expression levels were similar in control and rh-AMTN cultures at both time points.

3.3.2. Mineralization of AMTN-embedded collagen gels

After 5 hours of incubation in the mineralization buffer, calcium phosphate precipitates were observed on the surface of the AMTN-embedded collagen gel. The minerals could also be observed within several micrometers deep into the gel and embedded within collagen fibers.
(Figure 3.3 B-a). TEM and SAED results revealed that these minerals are needle-like crystallites of hydroxyapatite (Figure 3.3 B-c, d). No mineralization was observed in control collagen samples (Figure 3.3 B-b). The buffers containing the collagen samples remained clear of any mineral deposits indicating that the observed minerals on the AMTN containing gels are not the result of any precipitation from the buffer.

3.4. Discussion

In the natural environment of bone, dentin, and cementum, collagen mineralization is regulated by various non-collagenous proteins that are members of the SIBLING (small integrin-binding ligand N-linked glycoproteins) family. These proteins interact with collagen, cells, and calcium and phosphate ions in the extracellular matrix to mediate mineralization (George and Veis 2008). In this study we show that the enamel matrix protein amelotin accelerates mineralization in an osteogenic cell culture based system. Interestingly, the presence of AMTN molecules in the media affects the expression of bone markers OSC, BSP, and ALP. BSP is an early marker of bone formation and differentiation (Chen, Shapiro, and Sodek 1992) and its absence results in significant reduction in osteoblast-mediated mineralization (Gordon et al., 2007). OCN is a late-stage bone marker and a regulator of bone remodeling and mineralization. As expected, the expression levels of OCN rise from day 3 to day 10 in control MC3T3 cells lacking rh-AMTN in their media. Notably, addition of AMTN significantly down-regulates the expression of OSC in these cultures (Figure 3.2). ALP, the critical enzyme responsible for hydrolyzing pyrophosphates into inorganic phosphate required
A) Day 3

Gene expression relative to GAPDH

B) Day 10

Gene expression relative to GAPDH
**Figure 3.2** RT-PCR results of bone markers in MC3T3-E1 mineralizing cultures with or without 100µg/ml rh-AMTN in their media. Data is presented for day 3 (A) and day 10 (B). The 2% agarose gels showing the PCR products are shown on the left side. The quantitative RT-PCR data is shown on the right. Data is presented as mean ± standard deviation; (*) mean significantly different between the groups (* p < 0.05 for day 3 and p < 0.1 for day 10, n=3 experiments).

**Figure 3.3** A) Incorporation of rh-AMTN with collagen solution to yield homogenous gels embedded with AMTN molecules; B) SEM (a and b, picture taken at a 45° angle to the gel surface) and TEM (c and d) images of collagen samples incubated in the mineralization SBF buffer for 5 hours at 37°C. Calcium phosphate precipitates are seen on the surface and deeper into and embedded within the collagen gel (a). TEM and SAED analyses revealed that these precipitates have a hydroxyapatite crystalline structure (c and d). Control gels with no AMTN incorporation contained no minerals (b).
for mineral formation (Addison et al., 2007; Orimo 2010), also appears to be at lower levels of gene expression in MC3T3 cultures supplemented with rh-AMTN. Strikingly, the presence of AMTN molecules in the media reverses the inhibitory effect on osteoblast mineralization that would be expected from a decrease in genes ALP and BSP. These results provide more evidence in addition to our recent report (Abbarin et al., 2015) that AMTN is a promoter of mineralization. Hypothetically, AMTN may be involved in a feedback mechanism whereby the osteoblast cells “sense” the induction of mineralization by AMTN and hence reduce the expression of endogenous “mineral-inducing” genes. Further investigations are required to elucidate the presence of such mechanism. Additionally, it will be interesting to learn whether or not the accelerated mineralization is associated with the collagen structure.

It is especially intriguing that collagen mineralization is observed only within a few hours of incubation in SBF buffer when the AMTN molecules are embedded within the collagen gel matrix. Many SIBLING proteins contain conserved potential serine phosphorylation, mineralization-regulating SXE motifs (Kawasaki and Weiss 2003). In vivo, many SIBLING proteins are bound to collagen (George and Veis 2008) and some, such as dentine phosphophoryn (DPP) and dentin matrix protein 1(DMP1) promote collagen mineralization when immobilized on its surface in vitro (He et al., 2005a; Milan et al., 2006). Similarly, AMTN protein has a conserved SSEEL motif, which is involved in the mineralization promoting effect of the full-length molecule (Abbarin et al., 2015). Here, AMTN trapped in the collagen matrix may locally concentrate calcium and phosphate ions to precipitate onto and within the collagen meshwork. Collagen material has gained increasing interest for hard-to-soft tissue attachment applications due to its biocompatibility, ease-of-use, and biodegradability, and is now widely tested alone or in combination with osteoinductive biologics such as bone morphogenetic protein.
(BMP) in tissue engineering of bone and cartilage and periodontal regeneration (Geiger, Li, and Friess 2003; Stoecklin-Wasmer et al., 2013). The promoting effect of AMTN on collagen mineralization may open up interesting perspectives towards optimal design of calcified tissue regenerative collagen-based implants.

In conclusion, our findings in osteoblast-mediated cell culture and in vitro mineralization systems suggest that enamel matrix protein amelotin is able to exert its mineralizing capacity outside the enamel matrix in bone-like environments. The ability to accelerate mineralization in both non-collagen based and collagen based systems encourages more in-depth investigations into the molecular structure of the molecule and decipher its unique mineral and collagen interacting properties.

3.5. Acknowledgments

This project was supported by the National Sciences and Engineering Research Council of Canada (NSERC, Operating grant #490975) and the Canadian Institutes of Health Research (CIHR, Operating Grant MOP-492418) to BG. We would like to thank Douglas Holmyard (Advanced Bioimaging Centre, Mount Sinai Hospital, Toronto) for his expert assistance with the TEM imaging.
Chapter 4

4. Other Studies Related to the Mineralization Capabilities of AMTN
4.1. HA-Binding Affinity and effect on *In vitro* Mineralization of Various Proteins

4.1.1. Introduction

The aim of this study was to assess the effect of some other enamel matrix proteins on calcium phosphate mineralization in comparison with AMTN using the *in vitro* assay detailed in chapter 2. As the major enamel protein and the key biomolecule in regulating the formation of organized HA rods in bulk enamel, AMEL was the first candidate protein to be tested in this system. It was also worthwhile to investigate other components of the maturation-stage basal-lamina layer that are co-expressed with and located in close proximity to AMTN molecules. ODAM and the novel BL protein SCPQQP1 (Moffatt *et al.*, 2014) were therefore, also generated in the lab in *E.Coli* to be tested in the *in vitro* mineralization system.

Besides AMTN, Myo, and milk OPN, the Langmuir adsorption isotherms of ODAM, AMTN mutants, and a few proteins not associated with the mineralized tissues were also obtained to assess possible correlations between their mineralization regulatory behaviors and affinities to HA mineral. It should be noted that Langmuir theory assumes that the solid (here HA) has an energetically homogeneous surface with the same adsorption energy for every adsorption site; each site may be occupied by only one adsorbate molecule (a monolayer coverage); and no interaction occurs between the adsorbed species. A correlation coefficient (R² value) greater than 0.9 obtained from the best-fit line of Ceq/Q versus Q (chapter 2, section 2.2.3, Equation 1) indicates whether Langmuir is a suitable model to define the affinity of the molecule of interest to HA.
4.1.2. Materials and Methods

As described in chapter 2 section 2.2.1, similar to rh-AMTN and its mutants, the N-terminally 6×His tagged recombinant human amelogenin (rh-AMEL) and SCPPPQ1 (rh-SCPPPQ1), and a truncated version of recombinant human ODAM (rh-ODAM) (Figure 4.1) were expressed in E.Coli (pET-15b expression system) and affinity purified using a Ni-NTA column. Their 6×His tag was then cleaved from the protein N-terminus using a thrombin cleavage kit (CleanCleave™, Sigma-Aldrich).

Figure 4.1 Human ODAM sequence without the signal peptide. The bold letters indicate the well-conserved amino acids by alignment of 10 complete mammalian ODAM sequences and a putative ancestral sequence (Sire et al., 2007). The sequence in blue is the ODAM peptide expressed in E.Coli and affinity purified in this project.

The in vitro mineralization experiments were performed by dissolving rh-AMTN or equimolar concentrations of rh-ODAM peptide, rh-AMEL, or rh-SCPPP-Q1 in the mineralization buffer and incubated in the humidified chamber at 37°C (The methodology is detailed in chapter 2 section 2.2.4). Light scattering data were obtained at 540 nm at different time points during the mineralization experiments.

The HA binding affinity of rh-ODAM peptide, AMTN mutants (rh-AMTN<sub>S→A</sub> and rh-AMTN<sub>DEL</sub>, Figure 2.1 B), and non-mineral associated proteins BSA (bovine serum albumin
fraction V- Roche) and ovalbumin (Albumin from chicken egg white; Sigma A5503) were also obtained, as described in chapter 2 section 2.2.3.

4.1.3. Results

Figure 4.2 shows the affinity purified 6-HIS tag cleaved rh-AMTN (A), rh-ODAM (B), rh-AMEL (C), and rh-SCPPP-Q1 (D). The short rh-ODAM containing the C-terminus (Figure 4.1) is about 16kDa. The rh-AMEL monomer, expected to be about 25kDa in size, is shown by white arrow (Figure 4.2 C). However, the intense band with the molecular weight between 58 and 80 kDa indicates that most of the protein has formed oligomers of about three times larger in size. The rh-SCPPP-Q1 is expected to be around 10kDa (white arrow). Interestingly, this protein has also formed aggregates of about 5 times larger than the monomeric form, as can be seen in Figure 4.2 D.
Figure 4.2 Coomassie-blue-stained 15% SDS/polyacrylamide gel of rh-AMTN (A), rh-ODAM peptide (B), rh-AMEL (C), and rh-SCPPP-Q1 (D) expressed in E.Coli bacteria and affinity purified. The 6-HIS tags have been removed. The AMEL and SCPPP-Q1 in monomer form are shown by white arrows.

About 145 µg/ml rh-ODAM peptide in SBF buffer gradually precipitated calcium phosphate minerals in much lower extent than the amounts precipitated from 100µg/ml rh-AMTN within a period of 10 days. The two proteins added together to the SBF buffer in equimolar concentrations appeared to initially result in higher levels of HA precipitation from the solution (Figure 4.3). Control buffer without any proteins did not precipitate any minerals (Figure 4.3).

The microstructure of calcium phosphate deposits from ODAM- or AMTN + ODAM-containing buffers was similar to those of AMTN-containing buffer (Figure 4.4 A, B and C). TEM and
SAED analysis confirmed that the precipitates are needle-like crystallites of HA (Figure 4.4 D and E).

**Figure 4.3** Light scattering plots of mineralization buffers containing 100μg/ml AMTN, 145 μg/ml ODAM, or 100μg/ml AMTN+145 μg/ml ODAM up to 10 days. Some minerals precipitated from ODAM containing buffer after a week. The mineralization promoting effect of AMTN appeared to be intensified when combined with ODAM. Data is presented as mean ± standard error of the mean; (*) mean significantly different from other groups at that time point (* p < 0.05, sample size (n) = 8 per group).
Figure 4.4 Mineral precipitates from the mineralization buffers containing rh-AMTN (A) rh-ODAM peptide (B) or ODAM+AMTN (C, D) after 14 days of incubation at 37°C. (A), (B), and (C) SEM images; bar scales = 5µm (D) TEM imaging of the minerals precipitated from AMTN+ODAM containing buffer consisting of needle-like crystallites; bar scale = 100nm. The crystalline structure of these precipitates was hydroxyapatite (SAED pattern in E).

Figure 4.5 shows light scattering results of SBF buffers containing equimolar concentrations of rh-AMEL or rh-SCPPP-Q1 to rh-AMTN. Neither AMEL nor SCPPP-Q1 caused any precipitation from the SBF buffer even after 2 weeks.
Figure 4.5 Light scattering plots of mineralization buffers containing AMTN, AMEL, or SCPPP-Q1 in equimolar concentrations, up to 15 days. AMEL or SCPPP-Q1 did not cause any precipitation from the SBF buffer. Data is presented as mean ± standard error of the mean; (*): mean significantly different from other groups at that time point (* p < 0.1, sample size (n) = 7 per group).

Table 2 summarizes the HA binding affinity of various proteins obtained during the course of this PhD project. K and N values of the same proteins reported in the literature are also listed for comparison. A similar N value but a K value of an order of magnitude lower than rh-AMTN was obtained for rh-ODAM peptide. The affinities of AMTN mutants to HA were not different from the full-length molecule.
Table 2 HA adsorption isotherm results of various proteins obtained compared to values reported in the literature. K and N represent the affinity of the protein molecules for HA adsorption sites and the maximum number of adsorption sites per unit of HA surface area, respectively. $R^2$ is the correlation coefficient obtained for linear adsorption isotherms.

<table>
<thead>
<tr>
<th>Protein</th>
<th>N (mol/m²)</th>
<th>K (M⁻¹)</th>
<th>$R^2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk OPN (bovine)</td>
<td>$1.97 \times 10^{-8}$</td>
<td>$8.3 \times 10^6$</td>
<td>0.9998</td>
<td>This work</td>
</tr>
<tr>
<td>Bone OPN (porcine)</td>
<td>$1.53 \times 10^{-8}$</td>
<td>$3 \times 10^6$</td>
<td>0.915</td>
<td>(Goldberg et al., 2001)</td>
</tr>
<tr>
<td>rm-AMEL (mouse)</td>
<td>$6.09 \times 10^{-7}$</td>
<td>$19.7 \times 10^5$</td>
<td>0.99</td>
<td>(Bouropoulos and Moradian-Oldak 2003)</td>
</tr>
<tr>
<td>rp-AMEL (porcine)</td>
<td>$8.24 \times 10^{-7}$</td>
<td>$6.38 \times 10^5$</td>
<td>0.9916</td>
<td>(Sun et al., 2008)</td>
</tr>
<tr>
<td><strong>rh-AMTN</strong></td>
<td>$5.98 \times 10^{-8}$</td>
<td>$3.86 \times 10^5$</td>
<td><strong>0.9736</strong></td>
<td>This work</td>
</tr>
<tr>
<td>rh-AMTN₅→₆</td>
<td>$5.83 \times 10^{-8}$</td>
<td>$3.51 \times 10^5$</td>
<td>0.9504</td>
<td>This work</td>
</tr>
<tr>
<td>rh-AMTN₆ΔDEL</td>
<td>$6.96 \times 10^{-8}$</td>
<td>$3.75 \times 10^5$</td>
<td>0.9805</td>
<td>This work</td>
</tr>
<tr>
<td>rh-ODAM peptide</td>
<td>$5.50 \times 10^{-8}$</td>
<td>$7.11 \times 10^4$</td>
<td>0.8828</td>
<td>This work</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>$2.10 \times 10^{-8}$</td>
<td>$1.4 \times 10^5$</td>
<td>0.9697</td>
<td>This work</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>$4.41 \times 10^{-8}$</td>
<td>$0.7 \times 10^5$</td>
<td>0.993</td>
<td>(Iafisco et al., 2008)</td>
</tr>
<tr>
<td>BSA</td>
<td>$(23.4 – 42.1) \times 10^{-8}$</td>
<td>$(0.3 – 160) \times 10^5$</td>
<td>NA</td>
<td>(Ouizat et al., 1999)</td>
</tr>
<tr>
<td>BSA</td>
<td>$(8.7 – 26.1) \times 10^{-9}$</td>
<td>$(0.1 – 1160) \times 10^5$</td>
<td>NA</td>
<td>(Wassell, Hall, and Embery 1995)</td>
</tr>
<tr>
<td>BSA</td>
<td>$1.8 \times 10^{-8}$</td>
<td>$124.8 \times 10^5$</td>
<td>0.9938</td>
<td>This work</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>$2.8 \times 10^{-8}$</td>
<td>$8 \times 10^5$</td>
<td>0.9945</td>
<td>This work</td>
</tr>
</tbody>
</table>
4.2. Cell Culture Observations

4.2.1. Introduction

The goal of this study was to investigate the role of AMTN in biomineralization in a natural extracellular matrix (ECM) produced by ameloblasts. This system will allow for assessing the function of AMTN in an environment that mimics the enamel matrix during amelogenesis in vitro. In addition, the protein expressed from mammalian cells will be post-translationally modified and therefore, will structurally and functionally be in the most similar state to its natural in vivo condition. As the attempts to culture ameloblasts have been unsuccessful (Den Besten et al., 1998), a number of ameloblast-like cell lines have been established. Ameloblast lineage cells (ALC) (Nakata et al., 2003), cultured form mouse molar tooth germ, reportedly have the ability to differentiate into ameloblasts, express enamel genes, and mineralize in vitro (Huang et al., 2008; Nakata et al., 2003; Yan et al., 2006). ALCs have been reported to express AMEL, AMBN, ENAM, MMP20, KLK4, and ODAM (Gao et al., 2009; Lee et al., 2010; Nakata et al., 2003; Yan et al., 2006), but not AMTN. Therefore, this cell line was considered suitable for transfection with a mammalian expression vector containing the AMTN cDNA insert to investigate the effect of exogenously-overexpressed AMTN on mineralization in vitro.

Additionally, to assess whether AMTN can exert its mineralization-inducing effect outside of the enamel environment, a bone-mimicking cell line was also investigated. For this purpose, three different subclones of mouse calvaria osteoblast precursor cell line MC3T3-E1 (subclones 4, 14, and 30) were initially compared. The aim was to study whether introducing AMTN to the system accelerates mineralization in the already mineralizing matrix of subclones 4 and 14 or promotes mineralization in the ECM of subclone 30 which does not normally mineralize (Wang et al., 1999).
4.2.2. Materials and Methods

ALCs used in this study were kindly supplied by the laboratories of Dr. M. Paine (University of Southern California, Los Angeles, USA) and Dr. J-C Park (Seoul National University, Seoul, Korea), who currently work with this cell line. The passage number of these cells was unknown. Cells were either grown in Dulbecco’s Modified Eagle medium (DMEM- Gibco 21068-028), 10% FBS, and antibiotics according to the culture procedure of Dr. Paine’s group (Sarkar et al., 2014) ; or they were grown in minimum essential medium (MEM), 5% FBS, 10 ng/ml human epithelial growth factor (EGF- Gibco PHG0311), and antibiotics according to the culture procedure of Dr. Park’s group (Lee et al., 2012a) and were incubated in 5% CO₂ humidified chamber at 37°C. In addition to ALCs, another enamel-like cell line designated LS8 was also cultured using DMEM, 10% FBS, and antibiotics as culture media. LS8 has been derived from mouse enamel organ epithelia (Chen et al., 1992) and is reported to express some of the enamel genes including amelogenin, but does not mineralize (Sarkar et al., 2014). Figure 4.6 shows ALC and LS8 morphologies. To induce mineralization, confluent cells were supplemented with 5mM β-glycerolphosphate. Cell medium was changed every 3 days. To visualize mineral nodules cells were fixed with 10% v/v buffered formaldehyde and stained with 2% Alizarin red S (Gregory et al., 2004). Alternatively, after fixing they were treated with Oli red O to stain for lipid droplets (Sigma O0625).

RNA was isolated from the cells on different culture days using the RNeasy® Plus Mini kit (Qiagen). Gene transcription analysis was performed using iScript™ One-Step RT-PCR Kit (Bio-Rad) and gene-specific primers to assess whether these cells express AMEL as the main enamel matrix gene, and are therefore considered as enamel-mimicking cell lines. The AMEL forward and reverse primer sequences are 5’-TTTTGCTATGCCCTACCAC-3’ and 5’-
GTGATGAGGCTGAAGGGTGT-3’, respectively. GAPDH with forward primer of 5’-
AACTTTGGCATTGTGGAAGG-3’ and reverse primer of 5’-
ACACATTGGGGGTAGGAACA-3’ was used as the control gene.

**Figure 4.6** ALC and LS8 cell lines grown to about 70% confluence and imaged by phase contrast microscopy.

MC3T3-E1 subclones were purchased from ATCC (subclone 4: ATCC#2593, passage 17; subclone 14: ATCC # CRL-2594, passage 16; subclone 30: ATCC#2596, passage 12) and passaged according to the manufacturer’s protocol. There is no clear consensus regarding the optimal concentration of different components of “osteogenic media” in the literature (Boskey and Roy 2008). These also include the use of organic (β-glycerophosphate) or inorganic phosphate source in osteoblast mineralization assays (Hoemann, El-Gabalawy, and McKee 2009). In order to obtain optimum conditions, all three MC3T3-E1 subclones were grown in αMEM (without ascorbic acid (AA)), 10% FBS, and antibiotics. At 80% confluence, they were seeded at 50,000 cells/cm² as recommended in literature (Addison *et al.*, 2007; Addison *et al.*, 2015; Petryk *et al.*, 2005) in a 24-well plate. Similar passage numbers were chosen for this
experiment (subclone 4: p22, subclone 14: p19, and subclone 30: p20). The next day (set at day 0) the media was changed to αMEM that contains 50µg/ml ascorbic acid to initiate collagen production and cell differentiation. On day 4 the media was supplemented with either 3mM sodium dihydrogen phosphate or 10mM β-glycerophosphate. After 2 weeks, cells were fixed in 4% buffered paraformaldehyde solution and stained with 2% alizarin red to visualize the mineralization capacity of different subclones of MC3T3-E1 cell line.

4.2.3. Results

Confluent ALC cultures supplemented with 5mM β-glycerol phosphate for up to 14 days showed weak Alizarin red staining (Figure 4.7). No mineral nodules were observed in these cultures. Once they reached 100% confluence, LS8 cells started to peel off the plastic substrate and eventually completely detached from the well within a week. Therefore, it was not possible to culture them for longer than a few days.

![Figure 4.7](image)

**Figure 4.7** ALC cultures in a 6-well plate up to 14 days and stained with Alizarin red. Experiment was performed in triplicate.

During the initial attempts to culture ALCs, several small nodules appeared within or on top of the confluent cells after about 10 days and increased in number in time. These nodules were stained intense red with Oil red O and were thus, regarded as “lipid droplets” (Figure 4.8). Interestingly, RT-PCR failed to detect amelogenin expression in any of these cell lines. Figure
4.9 shows the 2.5% agarose gel of the RT-PCR results for different samples after 25 cycles and using mouse amelogenin and GAPDH (control) primers. Amelogenin expression was only found in the positive control (enamel matrix from the incisor of freshly-dissected mice-lane7). We thus concluded that none of these cell lines were suitable ameloblast cells and decided not to pursue further experiments with these cell lines.

**Figure 4.8** ALCs after 21 days of culture in DMEM, 10% FBS, and antibiotics. Cells have either been stained by Oil Red O or Alizarin Red to observe the formation of oil droplets (white arrows) or mineralized nodules.
Figure 4.9 2.5% agarose gel of the RT-PCR results for different RNA samples. Top gels: Lanes 1 and 3: total RNA collected from the ALC cells 4 days after they were confluent (day4). Lanes 2 and 4: total RNA of ALC cells collected before confluence (day0). Lanes 5 and 6: total RNA of LS8 cells on days 2 and 0. Lane 7: RNA collected from the mouse incisor enamel; and lane 8: mouse fibroblast cell line NIH3T3 as a negative control. Bottom gel: Same samples with no reverse transcriptase (RT) to confirm the absence of contaminating genomic DNA in these samples.

With regards to MC3T3-E1 subclones, unlike the original report (Wang et al., 1999) which had identified subclones 4 and 14 with great mineralizing potentials and subclone 30 with no mineralizing potential, in this study, subclones 14 and 30 were both found to mineralize when supplemented with 3mM inorganic phosphate. Subclone 4 however, showed the lowest mineralizing potential (Figure 4.10). None of the subclones mineralized in 2 weeks, when supplemented with organic phosphate source. Due to these inconsistencies observed in comparison with the literature, we decided to use subclone 14 (chapter 3) that has also been used in most recent publications on MC3T3-E1 mineralization.

1. ALC (Paine), day 4
2. ALC (Paine), day 0
3. ALC (Park), day 4
4. ALC (Park), day 0
5. LS8, day 2
6. LS8, day 0
7. Ameloblasts
8. NIH3T3
**Figure 4.10** MC3T3-E1 subclones cultured in αMEM media supplemented with inorganic (left panel) or organic (right panel) phosphate source and stained with alizarin red after 2 weeks. The greatest mineralizing potential was observed in subclones 14 and 30 when provided with inorganic phosphate.

4.3. Mineralization of AMTN-coated collagen membranes *in vitro*

4.3.1. Introduction

Here, I present preliminary experiments of incorporating AMTN molecules within collagen and studying its effect on collagen mineralization when adsorbed onto it. We first started with a commercially available collagen membrane and investigated whether AMTN binds to collagen structure just by simply incubating the membrane in the AMTN solution. Presumably, non-covalent associations such as hydrophobic interactions are possible to occur between collagen and AMTN molecules, rich in non-polar residues like proline, leucine, and glycine. We then examined whether weakly-bound AMTN can facilitate accelerated CaP deposition from the
metastable mineralization buffer on the surrounding collagen molecules *in vitro*. Results from this study further encouraged us to perform the mineralization experiments when AMTN is incorporated within the collagen structure in a more stable fashion (chapter 3).

### 4.3.2. Materials and Methods

Collagen membranes (resorbable type I bovine- Cytoplast RTM, Osteogenics Biomedical, Lubbock, TX, USA) were cut into 4×4 mm pieces and were immersed in 1 mg/ml rh-AMTN solution in distilled water overnight on a rocking platform at room temperature. Control membranes were immersed in water only overnight. The membranes were then removed from the solution and left to dry in the fume hood. Each piece was then immersed in 300 µl mineralization buffer (the composition is detailed in chapter 2 section 2.2.4) and incubated at 37°C for 6 days. After incubation, the membranes were removed from the buffer, washed with distilled water, and dried. Samples were gold coated and imaged using scanning electron microscopy (FEI XL30) with a secondary electron detector and at 20kv accelerating voltage.

### 4.3.3. Results

After 6 days calcium phosphate particles were formed on AMTN-coated membranes while the mineralization buffer remained clear with no signs of mineral precipitation. Figure 4.11 A and B show the AMTN-coated membranes after incubation in mineralization buffer at 37°C for 6 days. Numerous calcium phosphate particles can be seen associated with the membrane fibers. Figure 4.11 C shows the control membrane with no AMTN coating, but incubated in the buffer also for 6 days.
Figure 4.11 Cytoplasm membranes coated with rh-AMTN (A and B) or without any protein coating (C) and after incubation in the mineralization buffer for 6 days. Calcium phosphate precipitates can be seen within the collagen fibers in A and B. Bar scale = 50μm in A and C; bar scale = 2μm in B.

4.4. Discussion

This chapter summarizes other *in vitro* and cell culture findings with regards to the mineralizing capability of AMTN that have not been discussed in chapters 2 and 3. I first described the results of examining the effect of some of the other enamel matrix proteins on *in vitro* mineralization compared to AMTN. The second part of the chapter outlines the results of testing different cell lines to find the most reliable culture system for mineralization studies. These findings led to the selection of osteoblast-mediated MC3T3-E1 subclone 14 for experiments examining the effect of rh-AMTN on *in vitro* mineralization of a collagen-based culture system (described in chapter 3).
Lastly, I presented the results of my initial investigations of *in vitro* AMTN-incorporated collagen mineralization.

The *in vitro* accelerating effect that the C-terminal half of ODAM protein appears to have on the AMTN mineralizing ability is intriguing (Figure 4.3). ODAM and AMTN are both found in the BL layer and post-maturation JE (Dos Santos Neves *et al.*, 2012; Nishio *et al.*, 2013) and have been reported to interact *in vitro* (Holcroft and Ganss 2011). In addition, recombinant ODAM has been shown to promote mineralization in odontoblastic (Yang *et al.*, 2010a) and ameloblastic (Lee *et al.*, 2010) cells. It is not surprising to see that the truncated version of rh-ODAM alone lacking the N-terminus does not have a significant promoting effect on mineralization. The N-terminal domain contains most of the conserved residues including a SASNSxELL motif that is postulated to be serine phosphorylated *in vivo* (Sire *et al.*, 2007). It has also been shown *in vitro* that while overexpressing ODAM in ALCs induces mineralization, this effect is retarded when the cells are transfected with ODAM mutant constructs coding for AxE instead of the SxE or without the SxE motif entirely (Lee *et al.*, 2012a). In agreement with the mineralization data, the HA binding affinity of rh-ODAM peptide is about an order of magnitude lower than that of rh-AMTN (Table 2). It should be noted though, that the $R^2$ value of the linear isotherm for HA-ODAM interaction is less than 0.9 and therefore, the Langmuir theory may not be the best model to calculate the affinity of ODAM peptide for HA.

Figure 4.2 C shows the purified rh-AMEL that is mostly much larger than the 25kDa monomer size on the SDS-PAGE gel. Affinity purification using Ni-NTA columns requires washing and elution steps with increasing concentrations of imidazole at pH8.0. The next 6-HIS tag cleavage step is also performed at pH 8.0 resulting in further aggregation of AMEL molecules to larger oligomer and nanomer assemblies (Fang *et al.*, 2011). Therefore, it is not surprising to see that
most of the purified rh-AMEL protein is not in the monomeric form. As demonstrated by numerous in vitro and in vivo studies (reviewed by (Moradian-Oldak 2012)), the function of AMEL is not primarily to promote mineralization but to regulate and guide the formation of aligned HA crystallites. Our in vitro mineralization system consisting of a metastable SBF buffer is designed to demonstrate mainly any accelerating effects and so as expected, did not show any precipitation from the solution containing AMEL compared with AMTN even after 2 weeks (Figure 4.5). Similar to AMEL, the new BL protein SCPPP-Q1 produced in E.coli and affinity purified the same way also appeared at a much higher molecular weight than its expected monomer size on the SDS-PAGE gel. It will be interesting to conduct pH/temperature dynamic light scattering studies on this molecule as has been done for AMEL (Wiedemann-Bidlack et al., 2011), to elucidate possible self-assembly mechanisms.

It is also worthwhile to discuss the large variation in timing for SBF containing AMTN to initiate HA precipitation (2-7 days) (Figure 2.4, Figure 4.3, and Figure 4.5). Minute changes in various parameters such as temperature, composition and pH of SBF, and concentration of protein may have caused the differences observed. Another possibility is the limitation of the method that was employed to detect the onset of precipitation. As discussed in chapter 2, light scattering assay is a simple yet definitive technique to characterize, with limited sensitivity, the relative accelerating effects on precipitation from SBF and therefore, may have contributed to the variations observed among the mineralization experiments.

Another noteworthy observation is the Langmuir isotherm results of rh-AMTN mutants. While mutating the conserved SSEEL motif in rh-AMTN$_{S\rightarrow A}$ or rh-AMTN$_{DEL}$ variants of the molecule significantly diminished the mineralizing effect of the protein in solution (detailed in chapter 2 section 2.3.5), the affinity of the protein molecules for HA adsorption sites (K) and the maximum
number of adsorption sites per unit of HA surface area (N) remained unaffected (Table 2). Additionally, the SSEEL motif alone was not able to promote mineralization (Figure 2.9) suggesting the presence of other mineralization regulatory site(s) on the AMTN sequence. It is possible that the SSEEL motif is one of the sites that interacts with calcium and phosphate ions in solution to promote mineralization. But it is not involved in interactions with the HA crystals and therefore removing it from the sequence of the molecule does not impact the HA affinity of the protein.

Our findings with regards to the so-called “ameloblast-like” cells were contradictory to the published literature. A recent review on ALC and LS8 (Sarkar et al., 2014) reported higher expression of secretory enamel matrix genes in LS8 and maturation enamel matrix genes except for AMTN in ALC analyzed by quantitative PCR. They were also able to detect AMEL, AMBN, and ODAM proteins in ALC by western blot. All enamel matrix proteins were absent in LS8 cells and they were not able to mineralize whereas ALC formed calcified nodules starting from day 3 post inoculation. Following the same cell culture procedures as reported in the literature, we did not detect significant mineralization in ALC except for low intensity Alizarin red staining that was considered as background staining (Figure 4.7). Later observations of oil droplets forming in these cells and the absence of AMEL expression in either ALC or LS8 suggested to us that these cells may have undergone phenotypic drift with increasing passage numbers such that they can no longer be considered appropriate secretory or maturation ameloblast, enamel-matrix-producing cell lines. We also observed different results from the original report (Wang et al., 1999) with regards to the osteogenic capacity of different subclones of pre-osteoblast MC3T3-E1 cell line. It has been shown for subclone 4 that osteogenicity and the expression of bone markers such as ALP and OPN are decreased with increase in passage number (Yan et al.,
2014). It is therefore not surprising to see altered mineralizing effects in these subclones with passage numbers above 15 in comparison with the first few passages tested in the original study.

Our initial results on coating the Cytoplast membranes with AMTN were encouraging in that merely dipping collagen in rh-AMTN solution seemed to facilitate some weak bindings between the two molecules that result in deposition of calcium phosphate minerals onto the collagen substrate only, and not in the SBF solution. We do not know however, how much of the protein at a concentration of 1mg/ml in solution attaches to the membrane and how much will remain solubilized in the mineralization buffer. SEM imaging of the Cytoplast membranes also revealed a heterogeneous surface topography with some areas having more compact structure and other areas appearing more porous for this material which could affect mineralization studies. We thus developed collagen gels that are more homogenously structured with AMTN molecules stably incorporated within them and followed up with the mineralization experiments detailed in chapter 3.
Chapter 5

5. Summary and Future Directions
5.1. Summary

This PhD project elucidated the role of the maturation stage enamel protein amelotin in mineralization. Using different model systems, I showed for the first time that the enamel protein amelotin is a direct regulator of biomineralization. Below is a summary of conclusions that address the objectives defined for this research thesis:

**Conclusion 1** Based on *in vitro, in vivo*, and cell culture studies the enamel protein amelotin is a promoter of calcium phosphate mineralization.

In chapter 2, I showed that rh-AMTN dissolved in a metastable mineralization buffer with ion concentrations similar to those of enamel fluid promotes hydroxyapatite mineralization in a dose-dependent manner. Using the immunogold labelling technique, we detected AMTN molecules evenly distributed within growing hydroxyapatite crystals. We also generated mutated versions of the full-length rh-AMTN molecule lacking a functional conserved SSEEL motif and showed that the mineralizing ability of mutants is significantly reduced in comparison to the AMTN protein. A synthetic short peptide containing phosphorylated SSEEL motif promoted mineralization to a lesser degree than full-length recombinant molecule suggesting that the phosphorylated full-length AMTN has a stronger mineralizing capacity than the recombinant molecule. The same peptide but non-phosphorylated completely lacked the mineralizing ability indicating that SSEEL motif is not the only mineral interacting site on the AMTN sequence. A detailed characterization of secretory matrix of developing enamel in transgenic mice overexpressing AMTN under the AMEL gene promoter showed a rapid and disorganized mineralization front due to the presence of AMTN molecules in the matrix confirming the *in vitro* results. The overexpression of AMTN in the mouse calvaria MC3T3-E1 cell line similarly
resulted in accelerated formation of calcified nodules. Using the *in vitro* crystallization assay, I also showed in chapter 4 that the mineralizing potential of rh-AMTN may increase when combined with another component of the maturing enamel basal lamina layer, ODAM.

**Conclusion 2** No reliable enamel matrix producing cell culture model is currently available.

In hopes of further investigating the role of AMTN in mineralization in a model system that resembles the forming enamel environment, I tested two ameloblast-like cells ALC and LS8. I was not able to culture LS8 beyond 1 week post-confluence as they tend to detach from their solid substrate. ALC did not show strong mineralizing capacity and formed oil droplets suggesting a phenotypic drift in these cells after several passages. QPCR analysis showed no AMEL expression in either of these cell lines. The results of this part of the work are presented in chapter 4.

**Conclusion 3** AMTN, transfected and overexpressed in cells or introduced in culture media as bacterially-expressed recombinant form, can exert its mineralization-inducing effect outside of the enamel environment, in a collagen-based cell culture system.

In chapters 2 and 3 I presented the results of the effect of AMTN on mineralization in an established cell culture system of osteoblast mediated hydroxyapatite formation, MC3T3-E1 subclone 14. The accelerating effect on mineralization was consistently observed for both transfected AMTN and the recombinant protein added into the culture. Considering the fact that real-time PCR results showed a significant down-regulation of some of the bone formation promoting molecules such as BSP and ALP in cultures supplemented with rh-AMTN, the observed provides further evidence for the ability of AMTN to promote mineralization.
Conclusion 4 Rh-AMTN incorporated with collagen promotes collagen mineralization *in vitro* in an SBF solution.

In a simple experiment, presented in chapter 4, Cytoplast collagen membranes coated with rh-AMTN and tested in the *in vitro* crystallization assay showed calcium phosphate deposition after a few days. These exciting results led us to test the ability of AMTN to mineralize collagen in a modified *in vitro* system when AMTN is mixed with the collagen solution and the resulting gel is incubated in the mineralization buffer in a similar manner to the first experiment. Here again, we observed consistent promotion of collagen mineralization, this time remarkably within only a few hours (chapter 3).

In summary, we propose that AMTN is a key player in the establishment of the compact, aprismatic surface enamel layer. As the most superficial layer of enamel is the direct interface with the oral cavity, surface enamel is colonized by microbial biofilms and is the initial site for the formation of dental plaque and highly prevalent caries and erosion. The outcomes of this work may thus have direct implications for the development of new preventive and/or restorative strategies for dental enamel. In addition, the mineralization-promoting effect of AMTN in collagen-based systems opens interesting perspectives toward regenerative orthopedics and tissue-to-mineral attachment applications in a non-dental setting.
5.2. Future Directions

i. Identification of other mineral interacting site(s) within the sequence of AMTN

The mineralizing ability of AMTN was reduced to 25% but was not completely abolished when its conserved SSEEL motif was mutated (Figure 2.8). The Langmuir HA binding affinity of AMTN mutants was also similar to that of AMTN (Table 2). A synthetic peptide consisting of a short region of AMTN sequence including the SSEEL motif did not have any mineralizing potential either (Figure 2.9). These results collectively point to other mineralization regulatory site(s) on the sequence of AMTN. A potential region to investigate is the acidic TDDD stretch close to the C-terminus (Figure 1.11). Similar to the SSEEL motif, this region can be mutated by replacing the aspartic acid residues with non-polar residues or completely deleting the TDDD sequence. Another interesting experiment will be to perform site-directed mutagenesis of TDDD combined with SSEEL and investigate whether the mineralizing ability of the resulting molecule will be fully suppressed in vitro.

ii. Is native AMTN phosphorylated?

In vitro crystallization experiments using a short AMTN peptide containing the SSEEL motif phosphorylated on the serine residues, suggested that the full-length molecule is even a stronger promoter of mineralization when phosphorylated. Therefore, an important question is whether the molecule is phosphorylated in vivo and how that affects its function. The isolation and purification of AMTN from wild-type animals, however, is very challenging as the protein is expressed in low amounts in a short period during tooth formation. One way to address this question is to isolate AMTN from transgenic mice that overexpress the protein and purify it using the anti-FLAG antibody, as the expressed protein in transgenic animals contains a
3xFLAG tag at the N-terminus. Mass spectroscopy and western blotting can then be employed using anti-phosphoserine and anti-phosphothreonine antibodies to detect any phosphorylated sites. The native murine AMTN is 34KDa (Somogyi-Ganss et al., 2012), about 14KDa heavier than the recombinant protein which could be due to phosphorylation as well as glycosylation as predicated by evolutionary analysis studies. Alternatively, the protein maybe overexpressed in mammalian cells, purified and analyzed for post-translational modifications. Figure 5.1 shows a silver-stained 15% SDS-PAGE gel of purified human and mouse AMTN proteins from the ALC cultures on day 4 after transfection. Cells were transfected with pCMV plasmids containing mouse or human AMTN DNA inserts with a FLAG tag on the C-terminal end. After collecting the cells, AMTN protein was pulled down using anti-FLAG coated magnetic beads. However, the attempt to characterize the proteins using mass spectroscopy was not successful due to insufficient protein amounts for the analysis. It should be noted that post-translational modifications apparently depend on the cell line used to express the gene of interest. In this case, expression of AMTN in two different mammalian cell lines not related to ameloblasts gave two different molecular weights of about 30kDa (Iwasaki et al., 2005) and 35kDa (Moffatt et al., 2006b). As discussed earlier, no reliable ameloblast cell lines are available either. It is therefore questionable whether this approach will yield any information about the true modification state of the native protein.
iii. Protein-protein interactions and their effect on mineralization

Most of this PhD work has been accomplished using unmodified recombinant AMTN protein alone. *In vivo* however, the protein maybe modified and/or interacting with other proteins especially components of the BL layer in the maturation stage. Yeast two-hybrid studies have shown that AMTN is very specific in interacting with other proteins and only has affinity to bind to itself, possibly forming multimers, and to ODAM (Holcroft and Ganss 2011). Despite containing only the C-terminal half of the full-length molecule, rh-ODAM peptide mixed with rh-AMTN in the SBF buffer already showed an accelerating effect on the mineralizing ability of AMTN *in vitro* (Figure 4.3). It will be very interesting to see how the full-length ODAM molecule containing the N-terminal conserved SASNS motif affects mineralization alone or together with AMTN. ODAM also binds to AMBN (Holcroft and Ganss 2011) that maybe attached to the maturation-stage ameloblasts. Interacting with other molecules such as AMBN may alter the conformation of ODAM and in turn change its binding affinity to AMTN possibly influencing the mineralization behavior. Therefore, it will be also worthwhile to investigate how these molecules combined affect mineralization in the SBF solution.

**Figure 5.1** Silver-stained 15% SDS-PAGE showing FLAG-tagged mouse and human AMTN proteins expressed in ALC.
References


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